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In the loving memory of my mother
Konstantina Koukou-Kotsampasakou
(2 February 1955 - 2 July 2011)
In my heart you live forever!

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Preface

The current work was performed between September 2013 and July 2016 at the Pharmacoinformatics Research Group, Department of Pharmaceutical Chemistry of the University of Vienna, under the supervision of Professor Dr Gerhard F. Ecker.

The 1st chapter describes the motivation and aim of this PhD Thesis and briefly reports the contributions that are further described in the following chapters.

The 2nd chapter presents the biological background of this work in terms of the transporters located in the liver and their association with hepatotoxicity. A book chapter describing the role of organic anion transporting polypeptides (OATPs) as drug targets is also included.

The 3rd chapter describes the two-class classification models developed for OATP1B1 and OATP1B3 inhibition and the biological assay followed to further evaluate the model performance.

Chapters 4, 5 and 6 report two-class classification approaches which were developed in order to predict drug-induced liver injury (DILI), hyperbilirubinemia and cholestasis, respectively. For all three cases the potential association of hepatic transporters inhibition in the hepatotoxic endpoints was investigated.

In chapter 7 two case studies that concern the application of machine learning techniques in order to predict hepatotoxicity endpoints are reported. The first case study reports the development of models for 7 hepatotoxicity-endpoints for animal data and their use for developing a 7-model ensemble approach for predicting global hepatotoxicity. The development of a single global hepatotoxicity model is also described and its performance is compared with the 7-endpoint ensemble model approach. The second case study evaluates the performance of several meta-classifiers for the prediction of imbalanced datasets for transporters and hepatotoxicity endpoints.

Finally, chapter 8 contains the concluding discussion of the Thesis. The major contributions of each chapter are discussed as well as the main outcome and take-home-message of these studies.

In vitro assays of OATP1B1 and OATP1B3 inhibition were performed by Stefan Brenner under the supervision of Professor Walter Jäger in Department of Pharmaceutical Chemistry, University of Vienna. Toxicological studies for the hyperbilirubinemia case study were performed by Dr Sylvia Escher in Fraunhofer Institute of Toxicology and Experimental Medicine (ITEM), Hannover, Germany. Toxicological studies for chapter 7.1, concerning the modeling of animal data, as well as useful advice were provided by Dr Alexander Amberg, Dr Manuela Stolte and Dr Lennart Anger from Sanofi-Aventis Deutschland GmbH, Frankfurt am Main, Germany.

Chapter 1

Introduction

1.1 Motivation and Aim of the Thesis

Drug induced liver injury (DILI) is currently a great issue worldwide for patients, clinicians and health providers.^{1, 2} Furthermore, it is a major challenge for drug development in pharmaceutical industry: it is one of the main causes for attrition during clinical and pre-clinical studies and the primary reason for drug withdrawal from the market or labeling with a black box warning.³⁻⁵ Subsequently, there is great need to be able to recognize or foresee potential hepatotoxicity issues as early as possible. Of course, some efficient *in vitro* assays or animal models for several toxicity endpoints are available, but usually they are time-consuming and expensive.^{6, 7} Unfortunately, for the case of DILI animal models are not always the best choice, due to low concordance (<50%) between human and animal hepatotoxicity.^{5, 8, 9} The important role of DILI gets more and more acknowledged from the scientific community, judged also from the continuously rising number of publications on the topic in PubMed.¹⁰ Proportionally, a constantly increasing effort is being invested towards elucidating the toxicological processes and mechanisms that result in manifestations of DILI.¹¹ Among other parameters, influential factors are the liver basolateral and canalicular transporters. It is widely known that, together with metabolizing enzymes, liver transporters are playing an important role for maintaining the integrity and proper function of the liver and they highly influence the ADMET (absorption, distribution, metabolism, excretion and toxicity) profile of drugs.^{12, 13} Actually, there are several recent publications suggesting that inhibition of liver transporters might result in manifestations of DILI. In particular for cholestasis there is strong evidence towards bile salt export pump (BSEP)^{11, 14-19}, while there is also suspicion for multidrug resistance-associated protein 2 (MRP2)^{18, 20}, breast cancer resistance protein (BCRP)^{18, 20}, P-glycoprotein^{18, 20} (P-gp) and multidrug resistance-associated protein 3 and 4 (MRP3 and MRP4)^{16, 18, 20}. For hyperbilirubinemia, another possible manifestation of hepatotoxicity, the implication of organic anion transporting polypeptide 1B1 and 1B3 (OATP1B1 and OATP1B3)^{21, 22}, MRP2²² and, at a smaller extent, BCRP²² is suggested.

Aim of this thesis is developing *in silico* classification models for the inhibition of hepatic transporters, (OATP1B1 and 1B3), believed to be implicated in manifestations of DILI. These new models, together

with models already available in-house (BSEP, BCRP, P-gp) are further used, together with physicochemical descriptors and molecular fingerprints, in order to predict DILI in general, as well as particular manifestations of hepatotoxicity, like hyperbilirubinemia and cholestasis. Then, the contribution of transporter inhibition will be evaluated in order to investigate potential relationships between hepatic transporters' inhibition and hepatotoxicity endpoints.

These models have been developed within the framework of eTOX project, which is an IMI European project aiming to predict toxicity. We hope that the developed *in silico* models for transporter inhibition, DILI, hyperbilirubinemia and cholestasis will be of use during the drug development process for our eTOX EFPIA partners, as well as for the rest of the community. Furthermore, we anticipate that our study will shed some more light in the role of hepatic transporters in the development of hepatotoxicity.

1.2 Contributions of the Thesis

The particular thesis focuses initially on the *in silico* modeling of OATP1B1 and OATP1B3 inhibition, two basolateral uptake hepatic transporters suspected to be implicated in hyperbilirubinemia.^{21, 22} Other hepatic transporters with potential role in hepatotoxicity, such as BSEP, BCRP and P-gp, have already been studied in the group and in-house *in silico* classification models for these transporters' inhibition with satisfactory statistical performance are available. The predictions of transporter classification models are then used as descriptors, together with physicochemical descriptors and molecular fingerprints for the further development of 2-class classification models for drug-induced liver injury, hyperbilirubinemia and cholestasis. In particular, during this thesis the following models have been developed:

- Six two-class classification models for OATP1B1 and six two-class classification models for OATP1B3, respectively, that work separately or together to provide a consensus prediction. The models have been validated via 5- and 10-fold cross validation and on an external test set. The predictions on an unknown dataset (DrugBank) have further been validated with biological experiments.
- A 2-class classification model for DILI in humans, which explores the contribution of the inhibition of BSEP, BCRP, P-gp, OATP1B1 and 1B3. The model is validated via 10-fold cross validation and with an external test set.
- A 2-class classification model for hyperbilirubinemia in humans, which explores the contribution of the inhibition of OATP1B1 and 1B3 and a respective one for animals. Both models are validated via 10-fold cross validation.

- A 2-class classification model for cholestasis in humans, which explores the contribution of the inhibition of BSEP, BCRP, P-gp, OATP1B1 and 1B3. The model is validated via 10-fold cross validation and with an external test set.
- Two-class classification models for each one of the 7 hepatotoxicity endpoints for animal data: 1) necrosis, 2) steatosis, 3) bile duct abnormalities, 4) preneoplastic effect, 5) inflammation as a 2nd effect, 6) glycogen decrease and 7) hypertrophy.
- A consensus modeling approach for hepatotoxicity based on the individual endpoint models.
- A single global hepatotoxicity model for animals and comparison of it with the consensus 7-model approach.
- A study evaluating the performance of 7 known meta-classifiers for handling imbalanced datasets: Bagging, under-sampled stratified bagging, cost-sensitive classifier, MetaCost, threshold selection, SMOTE and ClassBalancer.

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Chapter 2

Biological Background

2.1 Hepatic Transporters

In general, transmembrane transporters are often expressed in tissues with barrier functions (e.g. blood-brain barrier, kidney, liver, enterocytes, etc) regulating the uptake and efflux of several important endobiotics, as well as of xenobiotics, such as drugs and toxins.¹⁻⁴ Consequently, they are involved in intestinal absorption, tissue distribution, hepatic metabolism, as well as biliary and urinary excretion of exogenous substances. Thus, distinct transporters are inherently linked to the ADME profile of many drugs, influencing the efficacy, as well as toxicity of most of the drugs and drug candidates.^{1, 3-10} Within this interplay of transporters' function, hepatic transporters are playing an extremely crucial role, since liver is the organ of metabolism and detoxification. Thus its integrity and proper function is of vital importance.^{11, 12}

There are two main categories of hepatic transporters, depending on their function:

- i. The uptake transporters, which mediate the transport of endobiotics and xenobiotics from the blood to the interior of the hepatocyte. Those are residing on the basolateral membrane of the hepatocyte.^{11, 13}
- ii. The efflux transporters, which remove the endobiotics and xenobiotics out of the hepatic cell, a) either by forwarding them into bile, when residing on the canalicular membrane, or b) by pumping them back into sinusoidal blood, when residing on the basolateral membrane.^{11, 13}

Among the hepatic transporters, there are basically representatives from 2 main superfamilies: the solute carrier (SLC) and the ATP-binding cassette (ABC) transporters. The SLC transporters discussed here are majorly uptake transporters, even though there are examples of bi-directional transport, and reside on the basolateral membrane of the hepatocyte. ABC transporters are in principle efflux transporters and they reside both on the basolateral and the canalicular membrane of the hepatocytes.¹³

In Figure 1 the main hepatic transporters and their respective location in the hepatocyte are depicted.

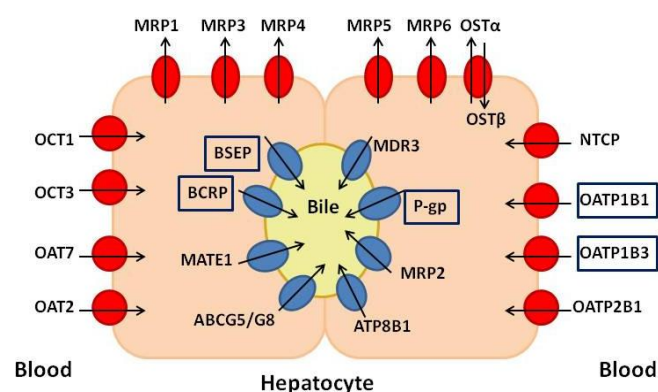


Figure 1: Transporters located on the hepatocyte. Blue symbols represent mainly the canalicular transporters and red ones the basolateral transporters. The arrows define the direction of transport. The transporters more important for the thesis that will be further examined in the next chapters are presented within rectangular frames. The arrows show the direction of transport. MRP1-6: multidrug resistance-associated protein 1-6, OST α /OST β : organic solute transporter, BSEP: bile salt export pump, BCRP: breast cancer resistance protein, MATE1: multidrug and toxin extrusion transporter, 1, ABCG5/G8: ATP-binding cassette sub-family G member 5/8, MDR3: multi-drug resistance protein 3, P-gp: P-glycoprotein, ATP8B1: ATPase-aminophospholipid transporter, OATP: organic anion transporting polypeptide, NTCP: sodium (Na^+) taurocolate co-transporting polypeptide, OCT: organic cation transporter 1, OAT: organic anion transporter.

Adjusted from Pauli-Magnus et al.¹⁴ with addition of more transporters (OCT3,¹⁵ OAT7,¹⁶ OST α /OST β ,¹⁷ ATP8B1¹³, MATE1¹⁸) from other sources.

Discussing the physiological role of all hepatic transporters is not within the scope of the current thesis. However, it is rather necessary to refer to the key players implicated in several pathological liver conditions, either genetically inherited or drug-induced. The transporters that are of greater interest for this thesis are depicted inside a rectangular frame in Figure 1. Among the pathological liver conditions that we will further try to predict with the help of transporters' profiles are drug-induced liver injury, cholestasis and hyperbilirubinemia.

2.2 The Role of Transporters in Hepatotoxicity

*Note: The transporters that have been modeled in our group and their inhibition predictions which are further going to be used for the prediction of DILI, hyperbilirubinemia and cholestasis are marked with *. They are also depicted within a rectangular frame in Figure1.*

2.2.1 Basolateral Uptake transporters

2.2.1.1 Sodium (Na^+) taurocolate co-transporting polypeptide (NTCP)

Sodium taurocholate co-transporter (NTCP) is a hepatic uptake transporter and member of the SLC superfamily, encoded by the SLC10A gene.^{13, 19} It mediates the transport of bile salts from sinusoidal blood to the hepatocyte. NTCP is the main transporter for bile acids and it is able to transport both unconjugated and conjugated bile acids, with higher affinity for the later ones. Apart from bile salts, it also transfers some organic anions, such as estrone-3-sulphate and bromosulfophthalein.^{11, 14, 19-21} In general, it has restricted capability to transport drugs.¹³ The nature of the transport is Na^+ -dependent, electrogenic and of high affinity with bile acids. In hepatocytes it is driven by an inwardly Na^+ gradient, maintained by the activity of Na^+/K^+ ATPase.²¹

Based on the fact that NTCP is the major route for bile salt uptake, this transporter plays an important role in the enterohepatic circulation of bile salts, bile flow and therefore contributes to liver homeostasis and general health. NTCP's levels have been found to adapt in some specific physiological conditions, like pregnancy, or due to some pathological conditions, respectively.²¹ Among the pathological conditions are inflammation-induced icteric cholestasis (e.g. cholestatic alcoholic hepatitis)^{21, 22}, advanced stage of primary biliary cirrhosis^{21, 22}, progressive familial intrahepatic cholestasis type 2 and 3, primary sclerosing cholangitis and extrahepatic biliary atresia.²² In case of cholestasis, NTCP is down-regulated, in order to prevent hepatocytes from the accumulation of toxic bile salts.^{14, 23} Interestingly, in hepatocellular carcinoma NTCP is down-regulated in comparison to adjacent healthy tissue, while in HepG2 cells (perpetual liver cancer cell line) NTCP is not expressed at all. This is considered an adaptive protective down-regulation from the potentially cytotoxic high concentration of bile salts.²⁴

Moreover, it has been proposed that the mechanistic basis of some hepatotoxic –and in particular cholestatic drugs- includes the inhibition of NTCP. In particular, it has been found by Mita et al. that

cholestatic agents like rifampicin, rifamycin SV, glibenclamide and cyclosporine inhibit the transport of taurocholate in NTCP- (and also BSEP-) overexpressing cells.¹⁹

Finally, recently Vaz and colleagues (2014) have identified a genetic disease, conjugated hypercholanemia that is characterized by deficiency in NTCP, which resulted in high plasma levels of unconjugated bile acids, without any clear clinical symptoms. The phenotype of the disease, which is not clear, includes some hypotonia and delay in the first motor milestones of the patient (5-year old female on 2014 when the study was conducted) that afterwards improved, as well as delay in speech development and learning difficulties. Nevertheless, the patient showed good progression in cognitive development, as well as normal social behavior. The detection of this NTCP deficiency proves that NTCP is the main transporter for the uptake of conjugated bile salts in the liver. However, the mild phenotype suggests that the rest of the uptake transporters are partially able to compensate the deficiency and maintain the enterohepatic cycle in the liver.²⁰

2.2.1.2 Organic anion transporting polypeptides (OATPs)*

From the OATP family, OATP1B1, OATP1B3, OATP2B1 belong to the SLCO superfamily (genes SLCO1B1, SLCO1B3 and SLCO2B1 respectively) and they are expressed in the liver; in particular OATP1B1 and 1B3 are selectively expressed in the liver under normal conditions.^{6, 25, 26} They have a wide range of substrates and inhibitors, including many endobiotics, such as bilirubin, bile salts, estradiol-17 β -glucuronide and thyroxine, which constitutes them important regulators of bile acids, bilirubin and cholesterol homeostasis.^{21, 27}

More information regarding the physiological role of OATPs can be found in Chapter 3 of the thesis, as well the book chapter entitled: “Organic Anion Transporting Polypeptides as Drug Targets”, by Eleni Kotsampasakou and Gerhard F. Ecker, for the book “Transporters as Drug Targets”. The book chapter has been written within the framework of this thesis and it discusses the general function of the whole OATP family and the liver OATPs in particular. Additionally, it describes the altered expression of OATPs in pathological conditions, such as genetic diseases and cancer, as well as their potential emerging role as drug targets and diagnostic biomarkers. However, its length is quite extensive and it concerns not only the hepatic OATPs, but the whole family. Thus, for the sake of reading flow, it was considered preferential to be provided in the end of the thesis, in the Appendix.

Here it is also noteworthy to mention the suspected correlation of OATP1B1 and 1B3 with hyperbilirubinemia, a pathological accumulation of conjugated or unconjugated bilirubin in sinusoidal

blood.^{28, 29} Excessive bilirubin in blood can be toxic, since it is associated with neural and non neural damage²⁸, and it is usually an indication of hepatotoxicity.^{30, 31} Bilirubin –the byproduct of heme catabolism- is taken up by OATP1B1 and OATP1B3 into the hepatocyte. There, it is metabolized by UDP-glucuronosyltransferase 1A1 (UGT1A1) into mono- and bi-glucuronidated products that are exported into bile primarily by MRP2 and in a smaller extent by BCRP. A portion of the glucuronidated or unglucuronidated bilirubin is not transported into bile, but instead it is effluxed into sinusoidal blood by MRP4. This can be further taken up from OATPs and the cycle is repeated. Thus, inhibition of any of these stages of the cycle of bilirubin is suspected to result in conjugated or unconjugated hyperbilirubinemia.^{28, 29, 32-34} Genetic diseases having hyperbilirubinemia as a phenotype, such as Rotor syndrome, which is accompanied by total deficiency for OATP1B1 and 1B3 suggest the potentially important role of OATP1B1 and 1B3 for the case of hyperbilirubinemia.^{29, 32, 33, 35-38}

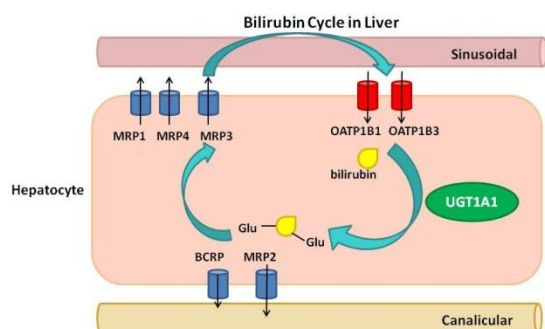


Figure 2. The cycle of bilirubin in the liver. Bilirubin is taken up from sinusoidal blood by OATP1B1 and OATP1B3. It is metabolized by UGT1A1 into mono- and bi-glucuronidated products that are exported into bile primarily by MRP2 and in smaller extent (smaller arrow) by BCRP. A portion of the glucuronidated or unglucuronidated bilirubin is effluxed into sinusoidal blood by MRP4 and the cycle is repeated. Adjusted from Sticova et. al.³²

Moreover, similarly to NTCP, a decrease in the expression of OATP1B1 in cases of cholestasis has been reported, as a compensatory mechanism.^{11, 14} In particular, the levels of OATP1B1 are decreased in cases of inflammation-induced icteric cholestasis²², in primary sclerosing cholangitis^{21, 22, 25}, in late stage of primary biliary cholangitis^{21, 22}, progressive familial intrahepatic cholestasis type 2 and 3 and extrahepatic biliary atresia²². OATP1B3 is also down-regulated in cases of progressive familial intrahepatic cholestasis type 1 and 2.²² This is believed to be a compensatory mechanism in cases of cholestasis, in order to maintain the hepatic extraction of endobiotics and xenobiotics from sinusoidal blood.^{14, 21, 25} This fact

would raise concern in case of drug-induced OATP1B3 inhibition during cholestatic conditions or in case of co-administration of some cholestatic drug with some OATP1B3 inhibitor.

2.2.1.3 Organic anion transporters (OATs)

Organic anion transporters (OATs) are encoded by the SLC22A family gene. Among the family members 1-10, only OAT1-4 and OAT7 have been found in humans and only OAT2 and OAT7 are expressed in the liver. Both of them are expressed on the basolateral membrane of the hepatocyte and they are primarily transporting organic anionic compounds – as their name suggests.^{16, 26, 39-41} The uptake of an anion into the cell requires energy, due to the negative potential of the interior of the membrane. OATs obtain this energy by exchanging extracellular with intracellular organic anions. The intracellular exchange ion can be monovalent (e.g. short chain fatty acid anions for OAT7) or bivalent (e.g. succinate for OAT2).¹⁶ However, apart from organic anions, OATs also show multispecificity, interacting with a wide range of chemical compounds, including several endobiotics and xenobiotics.^{16, 26, 39}

OAT2 (gene SLC22A7), in particular, transports diuretics (chlorothiazide, furosemide), statins, antibiotics (erythromycin, tetracycline, cephalosporins), antivirals (zidovudine, gancyclovir), NSAIDs (naproxen, diclofenac, salicylate) and H2 antagonists (cimetidine, ranitidine).^{16, 39} NSAIDs have also been found to inhibit OAT2. Currently, there are 3 known variants of OAT2, however, no functional tests have been performed to elucidate their effect in a human phenotype.¹⁶

OAT7 (gene SLC22A9) is selectively expressed in the liver and presents a narrower range of substrates, in comparison to the other OAT members. OAT7 is believed to play an important role in the transport of sulfated steroid hormone metabolites, like estrone sulfate from hepatocytes in exchange for circulating short-chain fatty acids like butyrate, which are not used as energy source for colonocytes. So far, there are no drugs known to be substrates or inhibitors of OAT7.^{16, 39}

The polyspecificity of OATs and their ability to transport drugs and toxins has been associated with toxicity, but only in kidneys.^{26, 41} So far, up to our knowledge, there are no reports associating OATs with any form of hepatotoxicity.

2.2.1.4 Organic cation transporters (OCTs)

Apart from OATs, also the organic cation transporters OCT1, and OCT3 -encoded by the genes SLC22A1, SLC22A3 respectively and belonging to the SLC22A family- are expressed in the liver in humans.^{15, 26}

OCT transporters transport mainly organic cations down their electrochemical gradient independently from the pH or sodium. Transport is feasible in both directions –depending on the transmembrane concentration gradient or the membrane potential- and the transport of charged substrates is electrogenic.^{11, 26} Among their physiological functions, OCTs contribute in the reabsorption and excretion of endogenous compounds, such as choline, dopamine and guanidine.⁴²

Regarding their substrates and inhibitors, there is high overlap between the OCTs, nevertheless there are some distinct differences in affinity and maximum transport rate between subtypes and species.¹⁵

Among the OCTs substrates belong to various chemical structures of organic cations, both endobiotics and xenobiotics, including drugs.²⁶ Non-charged compounds and even anions can also be transported.¹⁵

Some classes of drugs that interact with OCTs are antiviral drugs (acyclovir, ganciclovir etc.), ion channel blockers (verapamil, quinidine), the antimalarial drug quinine, some adrenergic receptor agonists and antagonists, antidepressants and some antidiabetic drugs (metformin, phenformin).^{15, 42}

There are no known human polymorphisms for OCT1 and OCT3 associated with pathologic phenotypes.²⁶ However, polymorphisms and mutations in human OCT1, leading to decreased transport activity of OCT1 in the liver, can obstruct the biliary excretion of hydrophobic cationic drugs.¹⁵ Moreover, it has been identified that cholestasis and genetic variants can influence OCT1 and OCT3 expression levels, which can affect the hepatic elimination of OCT substrates, like metformin.²⁶

2.2.2 Basolateral Efflux Transporters

2.2.2.1 Multidrug resistance-associated proteins (MRPs)

Multidrug resistance-associated proteins belong to the family of adenosine triphosphate (ATP) – dependent efflux pumps. There are totally 6 multidrug resistance proteins expressed in the liver. MRP1 (ABCC1 gene), MRP3 (ABCC3 gene), MRP4 (ABCC4 gene), MRP5 (ABCC5 gene) and MRP6 (ABCC6 gene) are located on the basolateral membrane of the hepatocyte, while MRP2 (ABCC2) is the only multidrug resistance-associated protein located on the canalicular membrane.^{14, 21, 43, 44} Nevertheless, the existence

of MRP1 and MRP5 in the basolateral membrane of hepatocytes seems controversial, since according to other authors only MRP3 and MRP4 are expressed in hepatocytes.^{45, 46}

Regarding basolateral MRPs, they are transporting:

- MRP1: drug-glutathione, -glucuronide and -sulfate conjugates^{14, 43}
- MRP3: bile salts¹⁴, drug-glucuronide conjugates^{31, 43}
- MRP4: sulfated drugs and bile acids,³¹ nucleotide analog drugs (e.g. zidovudine, stavudine, lamivudine)^{14, 43}
- MRP5: glutathione conjugate, nucleotide analogs^{14, 43}
- MRP6: unknown

MRP1, under normal conditions, according to some studies, is expressed in low levels in the liver. However, its expression levels are increased in cases of liver regeneration and during endotoxin- and bile duct ligation-induced cholestasis in rats.^{11, 47, 48} Moreover, an increase in protein levels of **MRP1**, as well as **MRP3**, **MRP4** and **MRP5** in cases of advanced stages of primary biliary cirrhosis has been reported.²²

Regarding **MRP3**, even before its physiological role had been definitely established, an up-regulation of the transporter was noticed in cholestatic rats and humans.^{11, 43} This can allow the efflux of organic anions from liver into blood when there is an obstruction of the bile flow.⁴³ Apart from cholestasis, it is also up-regulated during genetic diseases, such as Dubin-Johnson syndrome, which is characterized by MRP2 deficiency.¹¹ Thus, **MRP3**, as well as **MRP1**, may act as a compensatory mechanism to alleviate from potential toxic effects of high bile acids' concentration inside liver, when the canalicular efflux transporters such as BSEP and MRP2 are blocked.^{11, 49} Similar to **MRP3**, **MRP4** is also up-regulated in cholestatic liver.¹³ It has additionally been found that the protein levels of **MRP3** and **MRP4** are increased during progressive familial intrahepatic cholestasis type 3.²² Similarly to other transporters, adaptive alterations of MRPs expression also take place.

2.2.2.2 Organic solute transporter alpha-beta (OST α -OST β)

Organic solute transporter alpha-beta (OST α -OST β) is one of the newest members of the solute carrier superfamily. OST α is encoded by gene SLC51A and OST β by gene SLC51B, which are located in different chromosomes. Both proteins have no sequence identity, however they need to dimerize in order to stabilize and form a functional heterodimer transporter.⁵⁰ This dimeric transporter is ubiquitous

expressed on the basolateral membrane of several tissues, including the liver, even though its primary localization is in the ileum.^{17, 50, 51} The transport of substrates can be bidirectional, depending on the electrochemical gradient.⁵⁰ OST α -OST β plays a pivotal role in bile acid and steroid homeostasis and is a key player in enterohepatic circulation.^{17, 50, 51}

OST α -OST β dimer is a multispecific transporter. It can transport a wide range of taurine- and glycine-conjugated bile salts, as well as estrone-3-sulfate, digoxin and prostaglandin E₂. It also transports the neurosteroids dehydroepiandrosterone sulfate (DHEAS) and pregnenolone sulfate (PREGS), which suggests a possible function of the dimer in the brain. Bile salts –mainly conjugated- as well as sulfate- and glucuronic acid-conjugates of steroids can inhibit OST α -OST β .⁵⁰

In cases of cholestasis and cholestatic conditions such as primary biliary cirrhosis and biliary atresia, OST α -OST β is up-regulated, acting this way as a protective mechanism. Together with other basolateral membrane efflux proteins it prevents the accumulation of toxic bile salts in the hepatocyte.⁵¹

2.2.3 Canalicular Efflux Transporters

Hepatic bile circulation, which is tightly connected to liver health, is mediated by a series of canalicular ATP-binding cassette transporters.^{14, 52, 53} The usual “transport unit” of ABC transporters consists of two intracellular nucleotide binding domains (NBDs) and two transmembrane domains (TMDs). The nucleotide binding domains, which are usually well conserved across subfamilies, bind and hydrolyze ATP, the energy source for transport function. The transmembrane domains create the translocation chamber across which the substrates are transported. These regions are usually little conserved and are responsible for the substrate specificity of the different transporters. Based on their structural organization, ABC transporters can be classified into full- and half transporters. While full ABC transporters consist of 2 NBDs and 2 TMDs, half ABC transporters contain only one NBD and one TMD and have to form heterodimers or homodimers for functionality.^{45, 54} Among them are the bile salt export pump (BSEP), breast cancer resistance protein (BCRP), multidrug resistance associated protein 2 (MRP2) and multidrug resistance proteins 1 and 3 (MDR1/P-gp and MDR3).^{14, 52, 53}

2.2.3.1 Bile salt export pump (BSEP)*

The transport of bile salts from the hepatocyte into bile, against a high concentration gradient needs an ATP-dependent process; this function is accomplished mainly by the bile salt export pump (BSEP).^{23, 31, 55,}

⁵⁶ BSEP is an ABC transporter, encoded by the ABCB11 gene.^{22, 56} BSEP basically transports monovalent bile acids and salts^{21, 22, 57}, with order of preference taurochenodeoxycholate > taurocholate = tauroursodeoxycholate > glyocholate = cholate.^{55, 56} Actually, BSEP is the major driving force of the enterohepatic circulation of bile salts.²⁴ It can also transport some drugs, like pravastatin.^{31, 58}

Due to its pivotal role in bile salt circulation, BSEP is associated with cholestatic conditions, both genetically- and drug-induced. The hypothesis is that in case of BSEP deficiency, there is accumulation of bile salts inside the hepatocyte. The bile acids in high concentrations are toxic: they can induce mitochondrial toxicity, solubilize membrane components that may result in apoptosis and in general cause morphological lesions.⁵⁹ Moreover, bile acids are also suspected to be implicated in liver carcinogenesis.^{60, 61}

In particular, there are genetic diseases caused due to mutations on the ABCB11 gene that encodes BSEP.⁶² Among them are progressive familial intrahepatic cholestasis type 2^{21, 52, 56, 57}, a severe disease, with fatal outcome, unless a liver transplantation occurs, as well as a milder form termed benign recurrent intrahepatic cholestasis type 2.⁵⁷

Moreover, there is evidence in literature that drugs inducing cholestatic or mixed type of DILI (hepatocellular and cholestatic)⁵² –both in humans and in rodents- are actually inhibiting BSEP and its rodent analog Bsep *in vitro*.^{52, 57, 59, 63, 64} Interestingly, this was not the case for compounds exhibiting the hepatocellular type of DILI.⁶⁴ In general, during the stages of drug development, screening for BSEP inhibition is highly recommended for early prevention of side-effect such as DILI and cholestasis.^{65, 66} Finally, genetic variants in BSEP may also increase the susceptibility to drug-induced cholestasis.³¹

2.2.3.2 Multidrug resistance-associated protein 2 (MRP2)

Multidrug resistance-associated protein 2 (MRP2) –also termed canalicular multispecific organic anion transporter (cMOAT) or canalicular multidrug resistance-associated protein (cMRP)- is the only canalicular efflux representative of the MRPs in the liver. It is also an ABC transporter encoded by the ABCC2 gene. It transports a wide range of organic anions, including glucuronide and glutathione conjugates of endogenous and exogenous substances. Among the endogenous substances are bivalent bile salts, mono- and bi-glucuronidated bilirubin.^{14, 21, 22, 53, 67} It seems that MRP2 and the basolateral

MRP3 are counter-regulated; when there is down-regulation of MRP2, protein levels of MRP3 tend to increase as a compensatory mechanism for the removal of potentially toxic bile salts analogs or metabolites out of the hepatic cell.⁴⁹

There are genetic diseases as a result of deficiency of MRP2. Dubin-Johnson syndrome is a characteristic example that results from mutations on the ABCC2 gene. It is a relatively rare, benign condition and it is accompanied by a chronic conjugated hyperbilirubinemia phenotype and elevated levels of serum γ -glutamyl-transpeptidase.^{13, 21, 67-69} Moreover, in cases of cholestatic diseases, MRP2 has been found to be down-regulated.^{14, 67} However, this was not a catholic phenomenon; there were contradictory results among studies.^{58, 14}

Due to its important role in transporting endogenous substances, like bilirubin and bile salts, MRP2 is suggested to be correlated with drug induced hyperbilirubinemia^{32, 70} and cholestasis^{31, 67, 71}. However, a study conducted by Chang and co-workers²⁸ showed that the contribution of MRP2 inhibition towards hyperbilirubinemia would be minor. Nevertheless, their study concerned a small number of drugs (7 drugs in total), thus this might not be totally representative. In terms of cholestasis the landscape is quite similar. There are studies supporting the hypothesis that MRP2 plays a crucial role to the cholestasis induced by estradiol-17 β -glucuronide (E₂17G)⁷¹, while others suggest that it is not the major cause.⁷² Another study by Saab et al. showed association of MRP2 inhibition and inflammation-associated drug-induced hepatotoxicity.⁷³ Moreover, studies have shown that the risk for drug-induced DILI/cholestasis is greater, if apart from BSEP, more efflux transporters are inhibited, like MRP2⁷⁴, MRP3 and MRP4^{64, 74}. In any case, MRP2 functional testing is also required from US Food and Drug Administration (FDA) –together with BSEP- during drug development.⁵⁸

2.2.3.3 Breast cancer resistance protein (BCRP)*

Breast cancer resistance protein (BCRP) is also a member of the ABC superfamily, but is considered a half transporter. It is encoded by the ABCG2 gene.^{13, 75} BCRP transports compounds similar to MRP2: organic anions (biliary or drug metabolites) conjugated with glucuronate, glutathione, or sulfate.⁵⁶ Moreover, it presents overlapping substrates and inhibitors with P-gp.^{76, 77} Several drugs are BCRP substrates, including many anti-cancer agents, statins, antibiotics, as well as environmental toxins.⁷⁸ BCRP, together with MRP2 and P-gp are transporters associated with multidrug resistance.⁷⁹ The role of

BCRP in cancer resistance and tumor progression/development is well known^{18, 75, 78}, but it is out of our scope.

All in all, to our knowledge, genetic diseases associated with hepatotoxicity phenotypes have not been described yet. The role of BCRP variants in drug induced cholestasis needs to be further investigated since genetic studies regarding the role of ABCG2 gene showed conflicting results.⁵⁸

BCRP is believed to have a possible contribution in the efflux of bilirubin conjugates into bile,³² which could constitute potential implication in hyperbilirubinemia. Similarly to the other efflux transporters, deficiency of BCRP is believed to result in accumulation of toxic bile salts in the liver, which induce toxicity issues in the liver.⁸⁰ However, to our knowledge, there are not yet studies available proving hepatotoxic dynamic (hyperbilirubinemia or cholestasis) for BCRP.

2.2.3.4 Multidrug resistance proteins (MDRs)

There are two multidrug resistance proteins (MDRs) located in the liver, both on the canalicular membrane of the hepatocytes: **MDR1** or more widely known as **P-glycoprotein (P-gp)**, and **MDR3**. They are both members of the ABC superfamily of transporters, encoded by the genes ABCB1 and ABCB4 respectively.

2.2.3.4.1 MDR1 (P-glycoprotein/P-gp)*

MDR1/P-gp has a wide range of substrates among endobiotics and xenobiotics and its role in drug resistance during cancer therapy is very well described.^{75, 78} As mentioned above, P-gp presents significant overlap regarding substrates and inhibitors with BCRP.^{76, 77} The exact role of P-gp in bile formation has not yet been fully established, but it definitely contributes in the efflux of endobiotics and xenobiotics into bile.⁵³ The ABCB1 gene that encodes P-gp is highly polymorphic, however, the role of all these variants has not been elucidated yet.⁷⁸ In principle, genetic diseases caused due to a mutation on P-gp in humans have not been described up to now.³¹ Moreover, no P-gp genetic variants have been proven to be implicated in drug-induced cholestasis⁵⁸ or hepatotoxicity, as shown by a recent study in a Spanish cohort⁸¹.

The study by Saab et al. showed association of P-gp inhibition –apart from MRP2- and inflammation-associated drug-induced hepatotoxicity.⁷³ Nevertheless, in most of the cases, the implication of P-gp in drug-induced hepatotoxicity or cholestasis is attributed to its localization in several organ membranes

and its great number of its substrates. These two factors, in combination with its implication in drug-drug interactions, constitute P-gp responsible for the clearance and the toxicity of several drugs.^{14, 58}

2.2.3.4.2 MDR3

Even though MDR3 is not a drug transporter³¹, it significantly contributes in liver health by maintaining the integrity of the membrane and conducting the phospholipid flow across the canalicular membrane of the hepatocyte.²³ It is also known as phosphatidylcholine translocase or flippase^{14, 23, 53}, since it translocates from the hepatocyte into bile across the canalicular membrane.^{13, 14, 23, 31, 53, 58, 80} This way canalicular phospholipids are solubilized by bile salts, forming mixed micelles, thus protecting cholangiocytes from the detergent properties of bile salts.^{13, 14, 80}

Progressive familial intrahepatic cholestasis type 3 results from mutations in ABCB4 gene that encodes MDR3.^{11, 13, 23, 31, 53, 80, 82, 83} Additionally, variants of ABCB4 have been associated with intrahepatic cholestasis of pregnancy^{11, 13, 53, 83} and gallstones^{13, 83}, while impaired expression of MDR3 can lead in cholangiolytic cholestasis and vanishing bile duct syndrome³¹. MDR3 inhibition is also suspected to be a risk factor for drug-induced cholestasis^{13, 23, 31, 80}. A recent study by He and colleagues in 2015 identified new potential MDR3 inhibitors that are drugs associated with DILI and cholestasis.⁸⁴ In accordance to this, a more recent study by Mahdi et al. in 2016 showed inhibition of MDR3 by antifungal azoles: posaconazole, itraconazole and ketoconazole (itraconazole and ketoconazole have also been identified as MDR3 inhibitors by He et al.). Additionally, their data indicated potential increase of drugs' cholestatic effect in case of simultaneous inhibition of BSEP and MDR3.⁸⁵

2.2.3.5 ATP-Binding Cassette Subfamily G Members 5 and 8 (ABCG5/G8)

ATP-Binding Cassette Subfamily G Members 5 and 8 (ABCG5 and ABCG8), located both on the canalicular membrane of the hepatocyte, dimerize and function as an obligate heterodimer, translocating cholesterol and other plant sterols from the canalicular membrane into bile.^{13, 56, 58} Normally, the metabolism of cholesterol into bile salts transforms the hydrophobic cholesterol into amphipathic bile salts. Bile salts form micelles with phospholipids and solubilize cholesterol effluxed by ABCG5/G8, contributing in the homeostasis of cholesterol.⁵⁶

Mutations in the genes encoding either ABCG5 or ABCG8 disrupt the heterodimers' transporting capacity. This results in sitosterolemia, an autosomal recessive disorder, characterized by impaired

sterol clearance, xanthomas and atherosclerosis.^{13, 56, 80, 86, 87} Currently, up to our knowledge, there are no drugs known to inhibit either ABCG5 or ABCG8. Thus, the result of the drug-induced inhibition of these transporters is unknown.

2.2.3.6 ATPase Class I Type 8B Member 1 (ATP8B1, FIC1)

ATPase Class I Type 8B Member 1, also known as ATPase-aminophospholipid transporter, ATP8B1 or FIC1, belongs to the type 4 subfamily of P-type ATPases that are termed flippases.^{13, 56} ATP8B1 is the second transporter –together with MDR3- coordinating the maintenance of the cell membrane integrity and the flow of phospholipids across the canalicular membrane.²³ Due to ATP8B1 activity the proportion of sphingomyelin and cholesterol in the outer part of canalicular membrane is increased, which is believed to contribute to its resistance against the detergent properties of bile acids.¹³ Maturation, transport and function of ATP8B1 requires the presence of the chaperone protein CDC50A. In the case it is not heterodimerized with CDC50A, it remains in the endoplasmic reticulum and degrades prematurely. In presence of CDC50A, it translocates from the endoplasmic reticulum to the canalicular membrane where after heterodimerization with CDC50A it acts as a flippase.⁵⁶ In particular, ATP8B1 transports phosphatidylserine from the outer to the inner leaflet of the membrane, which keeps the membrane in the liquid-ordered state that allows the secretion of bile salts into bile.^{13, 56}

Mutations in the gene encoding ATP8B1 result in progressive familial intrahepatic cholestasis type 1, also known as Byler disease or Greenland familial cholestasis^{13, 56, 88} and benign recurrent intrahepatic cholestasis^{56, 88}.

To date, up to our knowledge, there are no drugs known to inhibit ATP8B1. Thus, it is not known what would be the effect of drug-induced inhibition of ATP8B1.

2.2.3.7 Multidrug and toxin extrusion transporter 1 (MATE1)

Multidrug and toxin extrusion transporter 1 (MATE1) belongs to the solute carrier superfamily and is encoded by the gene SLC47A1. It is the only one of the two human MATE transporters that is expressed also in the liver, although its predominant expression has been reported in the kidney.^{18, 89, 90} MATE1 functions on the basis of H⁺/organic cation antiport to transport cationic compounds from the hepatocyte into bile.^{18, 89-91} MATE1 in principle transports cationic, large, lipophilic molecules, nevertheless, it has been also reported to transport some anionic compounds.^{18, 89-91} The MATE

transporters transport metabolic products such as N-methylnicotinamide, choline, guanidine and serotonin.⁹¹ Moreover, several endobiotics and xenobiotics are substrates of MATE1, such as flavonoids⁹², metformin, cimetidine, cis-platin, tetraethylammonium, paraquat and others.^{89, 90} There are also several drugs identified to be MATE1 inhibitors such as cimetidine, metformin, imatinib, pazopanib and many more.^{89, 90, 93}

In humans there is a mutation on the SLC47A1 gene that encodes MATE1, but concerns only heterozygous carriers; no homozygous carriers have been reported. This could be explained by low allelic frequency, but it could also be possible that this sort of mutation is lethal in humans.⁸⁹ In respect to MATE1 drug-induced inhibition, the resulted effect is tightly associated with the functionality of the rest of the hepatic transporters, thus it is not easy to estimate.⁹⁰ In the liver, to our knowledge, no cases of toxicity has been reported. However, in the kidneys MATE transporters have been associated with nephrotoxicity and drug-drug interactions.^{89-91, 93}

2.2.4 Miscellaneous Transporters

The following transporters, even though they cannot be classified in the conventional classes of basolateral/canicular or uptake/efflux transporters, are important for liver homeostasis, while their malfunction is associated with several liver conditions. Thus, for the importance of their pathophysiological role, it was considered appropriate to make a short mention on them.

2.2.4.1 Cystic fibrosis transmembrane conductance regulator (CFTR)

Cystic fibrosis transmembrane conductance regulator (CFTR), encoded by the gene ABCC7, is a chlorine and an atypical ABC transporter.⁹⁴⁻⁹⁷ Human CFTR mRNA is expressed in higher levels in the gastrointestinal tract and in lower levels in kidney and lungs. During development, CFTR is expressed in less differentiated endodermal cells. High levels can be traced in several tissues, liver among others.⁹⁴ CFTR acts as a hydrolyzable-ligand-gated channel, where the two processes of the ATP ligand binding and the channel gating are allosterically coupled.⁹⁶ The major function of CFTR is the maintenance of the epithelia homeostasis by facilitating the epithelial fluid secretion.^{94, 98}

Mutations that lead in loss of function of CFTR cause cystic fibrosis, a genetic disease that is accompanied by progressive loss of lung function, pancreas abnormalities, infertility and other organ malfunctions.^{94, 98} Liver disease has been associated with cystic fibrosis and it has actually been recognized as the main cause of death during cystic fibrosis. In the liver, CFTR is localized in biliary

epithelial cells and over- or under-expression of CFTR may determine the development of cystic fibrosis liver disease (CFLD). Moreover, it has been found that hepatic CFTR levels are decreased in cases of familial intrahepatic cholestasis. Furthermore, CFTR expression is decreased in the human biliary epithelial cell line Mz-ChA-2, which is associated with deficiency in ATP8B1 by siRNA.⁹⁴ Finally, native CFTR plays an important role for two human diseases: enterotoxin-mediated secretory diarrheas and autosomal dominant polycystic kidney disease (PKD). It is believed that inhibitors of CFTR can have a therapeutic effect for these medical conditions.⁹⁸

2.2.4.2 Copper-transporting P-type ATP-ase (ATP7B)

Copper-transporting P-type ATP-ase (ATP7B) –and its highly homologous ATP7A– belong to the highly conserved family of P_{1B}-ATPases, which use the energy of ATP hydrolysis to transport copper across the membranes.⁹⁹⁻¹⁰² ATP7B is primarily expressed in the liver and kidney.¹⁰¹ Under normal copper levels, ATP7B resides in the trans-Golgi-network (TGN) of the hepatocytes, and loads Cu on newly synthesized ceruloplasmin, the major protein that carries copper in the blood. When intracellular levels of copper increase, ATP7B exits TGN and moves towards the canalicular membrane inside distinct vehicles that are associated with level of copper into bile.⁹⁹⁻¹⁰¹

Mutations in ATP7B are associated with Wilson's disease, a monogenic autosomal-recessive disorder that is characterized by copper accumulation⁹⁹⁻¹⁰³ and it is accompanied by chronic liver and/or neurological disease and sometimes kidney damage^{101, 103}.

2.2.4.3 SLC30A10- Manganese transporter

SLC30A10 belongs to the solute carrier superfamily of transporters and is a member of a big family of transporters that transport bivalent ions.¹⁰⁴⁻¹⁰⁷ In particular, it is a cell-surface localized efflux transporter.¹⁰⁶ Initially, it was believed to transport zinc, like other members of the SLC30A1-10 family, but it was finally discovered that it transports manganese.^{104, 107} SLC30A10 is highly expressed in the liver and brain.¹⁰⁵

Mutations and loss of function of SLC30A10 result in liver diseases and neurological disorder, due to Mn²⁺ accumulation in blood (hypermanganesemia) and tissues, which is toxic in high concentrations.¹⁰³⁻¹⁰⁸ In particularly chronic liver disease^{105, 108}, hepatic cirrhosis^{103, 104, 108}, polycythemia^{104, 105, 108} (abnormally increased concentration of hemoglobin in the blood), Parkinsonism and dystonia¹⁰³⁻¹⁰⁸ have been reported.

2.2.4.4 Sugar-phosphate/phosphate exchangers/antiporters

The SLC37 family is consisted of four sugar-phosphate exchangers: SLC37A1 (SPX1), SLC37A2 (SPX2), SLC37A3 (SPX3) and SLC37A4 (SPX4, G6PT) that are located in the endoplasmic reticulum (ER) membrane.^{109, 110} They are all ubiquitous, nevertheless only SLC37A2 (SPX2) and SLC37A4, also known as glucose-6-phosphate (G6P) transporter, are expressed in high levels in the liver.¹¹⁰ SLC37A1, SLC37A2 and SLC37A4 function as phosphate (P_i)- linked G6P antiporters catalyzing G6P: P_i and P_i : P_i exchanges. The function of SLC37A3 is unknown. Even though there have not been reported many structure-function studies for SLC37A1-3, SLC37A4 is well characterized. The main function of G6PT is to translocate G6P from the cytoplasm into the endoplasmic reticulum, where it is hydrolyzed by glucose-6-phosphatase (G6Pase) into glucose and P_i . The transport activity is dependent on the ability of G6PT to form a functional complex with G6Pase. In absence of G6Pase, the transport capacity of G6PT is minimal. There are two enzymatically active forms of G6Pase: G6Pase- α (or G6PC) is mainly expressed in the liver, while G6Pase- β is ubiquitous.^{109, 110}

There have been described several mutations in the G6Pase and SLC37A4 gene, leading to G6PT deficiency that results in the genetic autosomal recessive disorder called glycogen storage disease (GSD) type I (GSD-I). It represents the 90% of all cases and mainly affects the liver and kidneys.¹⁰⁹⁻¹¹¹ GSD has two subtypes GSD-I α and GSD-I β . Mutations in the G6Pase gene cause GSD-I α and in the SLC37A4 gene cause GSD-I β . They present almost the same phenotype, which includes hypoglycemia, hepatomegaly, hyperuricemia, lactic acidemia and hyperlipidemia, while GSD-I β also includes neutropenia and myeloid dysfunction, while the individual are susceptible to recurrent bacterial infections and inflammatory bowel disease.^{109, 111} Chlorogenic acid is known to be a reversible competitive inhibitor of G6PT, used in mechanistic studies.¹¹⁰

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Chapter 3

***In Silico* Classification Modeling of OATP1B1 and OATP1B3 Inhibition**

Identification of Novel Inhibitors of Organic Anion Transporting Polypeptides 1B1 and 1B3 (OATP1B1 and OATP1B3) Using a Consensus Vote of Six Classification Models, Eleni Kotsampasakou, Stefan Brenner, Walter Jäger, and Gerhard F. Ecker*, *Mol Pharm* **2015**, 12, (12), 4395-404

In the following paper the generation of 6 classification models for OATP1B1 and 6 respective ones for OATP1B3 is reported. The training set for the models was the one published in 2013 by De Bruyn and co-workers with over 1700 compounds after curation. The models were built in WEKA using 2 sets of 6 physicochemical descriptors and 11 physicochemical and topological descriptors from PaDEL and 1 set of 6 physicochemical descriptors from MOE. On the three sets of descriptors two sets of base-classifiers were applied: Random Forest and Support Vector Machines resulting into six models (2 sets of classifiers * 3 sets of descriptors). For all six models, apart from the base-classifier, the cost-sensitive meta-classifier MetaCost was applied, in order to artificially balance the dataset.

The models were validated via 5-fold and 10-fold cross validation, as well as with an external test set of over 200 compounds published by Kalgren et al. (2012) with satisfactory results. Using the consensus predictions out of the six models, we further screened DrugBank and selected 10 compounds, 9 as dual inhibitors for OATP1B1 and 1 as selective OATP1B3 inhibitor. The compounds were biologically tested for independent validation yielding an accuracy of 90% for OATP1B1 and 80% for OATP1B3.

E. Kotsampasakou performed the *in silico* study: gathered and curated the datasets, generated and validated the models, selected and bought the compounds for testing, and wrote the manuscript, apart from the Methods part: "Inhibition Assay for OATP1B1 and OATP1B3". S. Brenner performed the inhibition assay for OATP1B1 and 1B3 inhibition. W. Jäger supervised the conduction of the inhibition

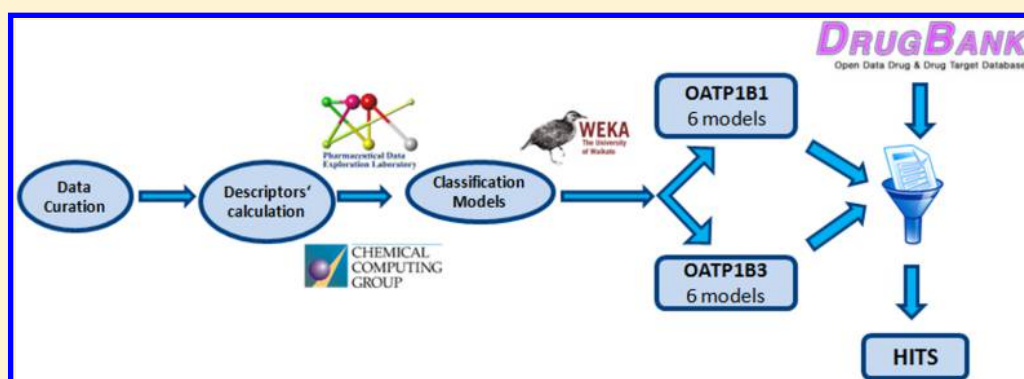
assay, wrote the methods part “Inhibition Assay for OATP1B1 and OATP1B3” and reviewed the manuscript. G. F. Ecker designed and supervised the *in silico* study and critically reviewed the manuscript.

Identification of Novel Inhibitors of Organic Anion Transporting Polypeptides 1B1 and 1B3 (OATP1B1 and OATP1B3) Using a Consensus Vote of Six Classification Models

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S Supporting Information



ABSTRACT: Organic anion transporting polypeptides 1B1 and 1B3 are transporters selectively expressed on the basolateral membrane of the hepatocyte. Several studies reveal that they are involved in drug–drug interactions, cancer, and hyperbilirubinemia. In this study, we developed a set of classification models for OATP1B1 and 1B3 inhibition based on more than 1700 carefully curated compounds from literature, which were validated via cross-validation and by use of an external test set. After combining several sets of descriptors and classifiers, the 6 best models were selected according to their statistical performance and were used for virtual screening of DrugBank. Consensus scoring of the screened compounds resulted in the selection and purchase of nine compounds as potential dual inhibitors and of one compound as potential selective OATP1B3 inhibitor. Biological testing of the compounds confirmed the validity of the models, yielding an accuracy of 90% for OATP1B1 and 80% for OATP1B3, respectively. Moreover, at least half of the new identified inhibitors are associated with hyperbilirubinemia or hepatotoxicity, implying a relationship between OATP inhibition and these severe side effects.

KEYWORDS: organic anion transporting polypeptide B1, organic anion transporting polypeptide B3, OATP1B1, OATP1B3, liver, hepatocyte, transporters, inhibitors, classification, Random Forest, Support Vector Machines, DrugBank, virtual screening

INTRODUCTION

Detoxification mainly takes place in the hepatocyte and is accomplished by a diverse series of transferase-mediated conjugation reactions with charged moieties such as glutathione, glucuronide, and sulfate, resulting in negatively charged, amphiphilic compounds that are efficiently secreted into bile or urine. The hepatocyte is an epithelial cell which comprises two membrane domains, the basolateral (sinusoidal) and the apical (canalicular) membrane.^{1,2} Together with metabolizing enzymes, transmembrane transporters are important determinants regarding drug metabolism and drug clearance by the liver. Their significant role has been increasingly recognized in terms of drug and metabolite pharmacokinetics.^{2,3} Transport proteins in the basolateral membrane of the liver cause drugs to enter the hepatocyte, where metabolism takes place, while in the apical membrane of the hepatocyte the residing ATP-dependent efflux pumps transfer drugs and metabolites from the hepatocyte to bile. Among the transporters residing on the

basolateral (sinusoidal) membrane of human hepatocytes are organic anion transporting polypeptides (OATP1B1, 1B3, and 2B1), NTCP, OAT2, and OCT1. Among the canalicular transporters are MRPs (1, 2, 3, and 6), MDRs (1 and 3), BSEP (ABCB11), and BCRP (ABCG2).^{2,4,5}

OATPs are encoded by the genes of the *SLCO/Slco* (*SLCO* for humans/*Slco* for rodents) superfamily.^{3,6–9} The particular superfamily was originally named *SLC21A*. However, the nomenclature of its members was updated and standardized in 2004 on the basis of phylogenetic relationships, resulting in its being renamed *SLCO*, the solute carrier family of OATPs.^{3,6,7,9} 11 human OATPs have been identified, which are organized in 6 distinct families: OATP1, OATP2, OATP3,

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OATP4, OATP5, and OATP6. These might be split further into subfamilies (OATP1A, OATP1B, and OATP1C).^{7,10–13} OATP1B1 (encoded by the *SLCO1B1* gene) and OATP1B3 (encoded by the *SLCO1B3* gene) are transporters exclusively expressed on the basolateral membrane of the hepatocyte.⁶ They have a wide and overlapping range of substrates and inhibitors, including various endobiotics, such as bilirubin, estradiol-17 β -glucuronide, thyroxine (T4), cholate, and taurocholate. In the liver, OATPs take up bile acids, thus helping in preservation of a circulating pool of bile acids, an important factor for bile flow. This way they contribute to the bile acids and cholesterol homeostasis.^{14,15} Furthermore, OATPs are among the transmembrane transporters that regulate the uptake of thyroid hormones into their target cells throughout the body, as well as from the mother to the fetus.^{14,16–19} Apart from endogenous compounds, OATPs can transport many marketed drugs, such as erythromycin, levofloxacin, imatinib, pitavastatin, and enalapril (substrates) and cyclosporine, atorvastatin, telmisartan, and diazepam (inhibitors). Due to their wide range of substrates and inhibitors, they are implicated in various drug–drug interactions.^{20–24}

Additionally, they are closely associated with cancer, as many anticancer agents are OATP1B1 and 1B3 substrates or/and inhibitors. Therefore, they affect the intracellular concentration of these drugs and alter their effectiveness.^{25–27} The association between OATPs and cancer is also based on the fact that the localization and the expression level of these transporters alters in cancer tissues, which further influences the uptake and exposure of drugs.^{25,28–30} Moreover, since these influx transporters are working together with efflux transporters and metabolizing enzymes, they are suspected to play an important role in chemoresistance during chemotherapy.^{25,31,32}

Last, but not least, OATPs are correlated to hyperbilirubinemia, a condition of accumulation of bilirubin in the body. Hyperbilirubinemia has been extensively studied in terms of neurotoxicity, where it appeared that bilirubin may change synaptic potentials and functions of neurotransmitters. It can also interfere with oxidative phosphorylation, enhance DNA instability, interrupt protein synthesis, and block the activity of mitochondrial enzymes. Therefore, apart from neurotoxicity, bilirubin may lead to non-neural organ dysfunctions. Moreover, hyperbilirubinemia can be considered as an early warning of possible adverse effects such as hepatotoxicity, since hepatotoxicity is often accompanied by elevated levels of bilirubin.^{33–35}

Bilirubin is taken up to the hepatocyte by OATP1B1 and 1B3 and is subsequently metabolized into mono- and diglucuronide conjugates by UGT1A1 (UDP-glucuronosyl-transferase 1A1). These conjugated bilirubin-glucuronides are excreted into bile by the hepatobiliary ABC-transporter MRP2 (multidrug resistance protein 2), as well as, to a smaller extent, by BCRP.^{5,36} In the case of impaired biliary excretion, as a compensatory pathway, the glucuronidated bilirubin may also be secreted back to the sinusoidal blood by MRP3.^{5,33,36,37} Thus, since bilirubin is imported by OATP1B1 and 1B3, a potential inhibition of those transporters can lead to the increase of unconjugated bilirubin in the blood and eventually cause hyperbilirubinemia.

Considering the multifactorial role of OATP1B1 and OATP1B3 for drug uptake, efficacy, and metabolism, they also have been included in the table of “Selected Transporter-Mediated Clinical Significant Drug–Drug Interactions (7/28/2011)” of the FDA.³⁸ Therefore, predictive models allowing the assessment of risk for a compound to interact with OATP1B1

and OATP1B3 would be useful tools at the early stage of drug development. Classification models for OATP1B1 and B3 inhibition are already available in the literature.^{39–41} Karlgren et al.⁴⁰ generated a computational model for OATP1B1, based on 146 compounds (98 in the training set and 48 in the test set) using orthogonal partial least-squares projection to latent structures discriminant analysis (OPLS-DA) based on a set of molecular descriptors. As a follow-up,⁴¹ they also published a model for OATP1B1 and OATP1B3 inhibition, based on 225 compounds (two-thirds randomly assigned as a training set and one-third as a test set), using multivariate partial least-squares (PLS) regression and physicochemical descriptors. De Bruyn et al.³⁹ followed a proteochemometric modeling approach, using almost 2000 compounds for their training set and 54 compounds as an external test set, combining protein-based and ligand-based molecular descriptors and using Random Forest as a classifier. After careful manual curation and removal of compounds that showed contradictory class labels, we used these data sets to develop a set of *in silico* classification models suitable for virtual screening of compound libraries. This was followed by virtual screening of DrugBank and subsequent biological evaluation of the top ranked compounds, in order to identify existent inhibitors among drugs that are currently on the market or in the stage of clinical trials.

■ EXPERIMENTAL SECTION

In Silico Modeling for the Prediction of OATP1B1 and OATP1B3 Inhibition. Selection and Curation of Data Sets.

High quality data sets are key for statistical modeling.^{42–45} For our study we used two recently published large data sets for the inhibition of OATP1B1 and 1B3, one containing 2000 compounds³⁹ and one consisting of 225 compounds.⁴¹ The first data set was used as a training set and the second data set as an external test set. The external test set was downloaded from ChEMBL,⁴⁶ and the training set was kindly provided by Gerard J. P. van Westen. Subsequently, both data sets were curated according to a set of protocols, which have been developed in house:⁴⁷

- Inorganic compounds, salt parts as well as compounds containing metals and rare or special atoms were removed (MOE 2013.0801).⁴⁸
- The chemotypes were standardized using an in-house Pipeline Pilot (version 9.1.0.13)⁴⁹ workflow.
- Duplicates and permanently charged compounds were removed.
- 3D structures were generated using CORINA (version 3.4),⁵⁰ and their energy was minimized with MOE 2013.0801, using default settings with an extra setting of preserving the existing chirality and changing the gradient to 0.05 RMS kcal/mol/Å².

Finally, the training and the test set were checked for duplicates. In total, 68 and 70 overlapping compounds were identified for OATP1B1 and OATP1B3, respectively. In most cases, the overlapping compounds were of the same class (using 50% ($\pm 10\%$) inhibition as threshold, as defined by the initial authors). For these cases, since the overlapping compounds were mostly noninhibitors, we decided to remove them from the training set and keep them in the test instead. Those compounds showing contradictory class labels (10 compounds for OATP1B1 and 2 compounds for OATP1B3) were removed from both data sets.

This procedure finally led to a training set of 1708 compounds (190 inhibitors and 1518 noninhibitors) for OATP1B1 and of 1725 compounds (124 inhibitors and 1601 noninhibitors) for OATP1B3, respectively. The external test set contained 201 compounds for OATP1B1 (64 inhibitors and 137 noninhibitors) and 209 compounds for OATP1B3 (40 inhibitors and 169 noninhibitors).

Generation of Statistical Models. Algorithms Used. The open-source software WEKA (version 3-7-10)⁵¹ served as the basis for generating classification models. The following classifiers were explored: Naive Bayes, *k* Nearest Neighbors (*k* = 5), Decision Tree (J48 in WEKA), Random Forest, and Support Vector Machines (SMO in WEKA). Furthermore, because of the highly imbalanced training set, the meta-classifiers MetaCost and CostSensitive Classifier, as implemented in WEKA, were used. They are both cost-sensitive meta-classifiers that artificially balance the training set. In each case, the cost matrix was set according to the ratio of noninhibitors vs inhibitors. In the case of OATP1B1 the ratio noninhibitors/inhibitors was equal to 8, thus the matrix used during the application of cost was [0.0, 1.0; 8.0, 0.0]. For OATP1B3 the respective ratio was equal to 13, thus the respective cost matrix was [0.0, 1.0; 13.0, 0.0].

The best results were obtained using MetaCost⁵² as meta-classifier and Random Forest (RF) and Support Vector Machines (SMO) as base-classifiers.

Molecular Descriptors. Using MOE 2013.0801,⁴⁸ all the available 2D and selected 3D molecular descriptors (like the whole series of Volsurf descriptors) were calculated. Additionally, in order to generate models with open-source descriptors, an analogous set of descriptors was calculated with PaDEL-Descriptor (version 2.18).⁵³ Additionally, several fingerprints such as MACCS-keys using PaDEL and ECFPs using RDkit were also calculated.

In a first run, a set of basic physicochemical descriptors were used for model generation. This should allow us to derive basic physicochemical properties driving OATP1B inhibition. For MOE, these comprised a_acc (number of H-bond acceptors), a_don (number of H-bond donors), logP (o/w) (lipophilicity), mr (molecular refractivity), TPSA (topological polar surface area), and weight (molecular weight, MW). The analogous descriptors calculated with PaDEL included nHBAcc_Lipinski, nHBDOn_Lipinski, CrippenLogP, CrippenMR, TopoPSA, and MW. The absolute values were not fully identical to those calculated with MOE, as slightly different algorithms are used by the two software packages. In order to further enrich the original set of the six descriptors, a few topological descriptors were additionally calculated, thus leading to a third set comprising 11 molecular descriptors: nHBAcc_Lipinski, nHBDOn_Lipinski (number of H-bond donors and acceptors according to Lipinski), CrippenLogP, CrippenMR (Wildman–Crippen logP and mr), TopoPSA, MW, nRotB (number of rotatable bonds), topoRadius (topological radius), topoDiameter (topological diameter), topoShape (topological shape), and globalTopoChargeIndex (global topological charge index).

Finally, combining the three sets of descriptors with the two base-classifier methods selected, six models were generated for each transporter. A detailed description of the model settings is given in the [Supporting Information](#).

Model Validation. The statistical models were validated using 5-fold and 10-fold cross-validation, as well as with the external test set. The parameters used comprised Accuracy, Sensitivity (True Positive Rate), Specificity, Mathews Correla-

tion Coefficient (MCC), and Receiver Operating Characteristic (ROC) Area.⁵⁴ A detailed description of all parameters is provided in the [Supporting Information](#). The cost for the MetaCost meta-classifier was applied based on a standard confusion matrix.

The performance of all models was relatively equivalent with total accuracy values and ROC areas for the test set in the range of 0.81–0.86 and of 0.81–0.92, respectively. Generally, the OATP1B3 models performed slightly better than the ones for OATP1B1. In order to retain as much information as possible, all models were subsequently used for the virtual screening of DrugBank, implementing a consensus scoring approach. Therefore, the prediction score of each classification model for every compound was summed up, giving a float score prediction number between 0 and 6.

In Silico Screening of DrugBank. In order to perform a prospective assessment of the predictivity of our models, DrugBank (Version 4.1)⁵⁵ (<http://www.drugbank.ca/>), which contains 7740 drug entries including 1584 FDA-approved small molecule drugs, 157 FDA-approved biotech (protein/peptide) drugs, 89 nutraceuticals, and over 6000 experimental drugs, was virtually screened, and the top ranked compounds were purchased and experimentally tested. The in silico screen was restricted to the small molecules (either approved or experimental), since this is the chemical space upon which the models were generated. Before the screening, the compounds underwent the same curation process as the compounds from the training and test sets. This resulted in a screening set of 6279 compounds in total. For each screened compound we obtained two scores for each model: (i) a binary score, 0 if the compound was predicted as noninhibitor and 1 if the compound was predicted as inhibitor; and (ii) a float-number score between 0 and 1, [0, 0.5] if the compound is predicted as noninhibitor and [0.5, 1] if the compound is predicted as inhibitor. The individual binary and the float-number scores for each model were added up and gave a consensus class prediction (integer consensus score) and a predictive score (float consensus score) for each compound, which were afterward ranked from inhibitors to noninhibitors according to these additive scores. In general, a compound was considered as being an inhibitor if it was predicted as inhibitor by at least 3 out of the 6 models for each transporter, while the float-number score was also taken into consideration.

Selection of Compounds for Biological Testing. For the selection and purchase of potential inhibitors, those compounds having an integer consensus score of 6 were taken and ranked according to their float consensus score. Subsequently, a similarity search based on MACCS fingerprints and the Tanimoto coefficient was performed with MOE, comparing the selected screening hits from DrugBank with the compounds included in the training and in the external test set. Thus, any high ranked compound in DrugBank showing a Tanimoto similarity higher than 0.85 to inhibitors from the training set or the test set was excluded from the shopping list. Furthermore, compounds that are known OATP1B1 and/or OATP1B3 inhibitors were also excluded. Last but not least, the final selection of compounds for purchase was influenced by their commercial availability and the respective costs. The ten compounds that were finally selected were purchased from Glentham Life Sciences, U.K. (<http://www.glenthamls.com/>, 6 compounds) and from Sigma-Aldrich (<https://www.sigmaaldrich.com>, 4 compounds). The purity of all compounds was ≥95%. Out of the ten compounds, nine were predicted as

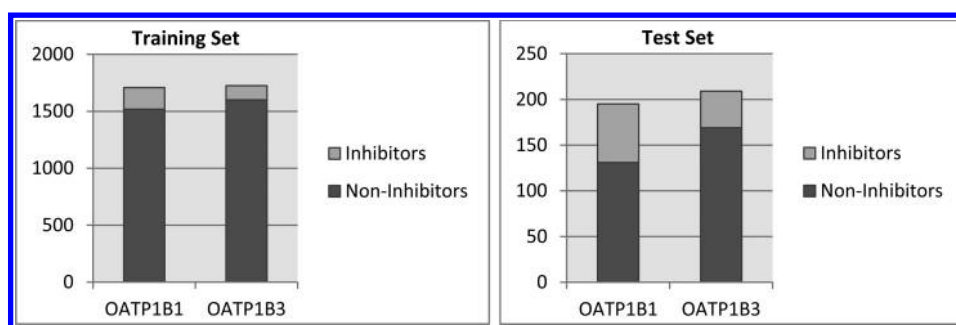


Figure 1. Graphical representation of the numeric gap between inhibitors and noninhibitors of OATP1B1 and OATP1B3 for both the training and the test set.

Table 1. Detailed Statistical Results of OATP1B1 Inhibition Models

model	validation	accuracy	sensitivity	specificity	precision	MCC	ROC area
B1_6MOE_RF	test set	0.846	0.719	0.905	0.780	0.638	0.815
	10-fold CV	0.843	0.611	0.872	0.374	0.394	0.795
	5-fold CV	0.858	0.621	0.888	0.410	0.428	0.790
B1_6MOE_SMO	test set	0.841	0.672	0.920	0.796	0.622	0.869
	10-fold CV	0.862	0.489	0.909	0.403	0.366	0.808
	5-fold CV	0.862	0.495	0.908	0.402	0.368	0.791
B1_6PaD_SMO	test set	0.811	0.719	0.854	0.697	0.568	0.814
	10-fold CV	0.841	0.626	0.868	0.373	0.399	0.790
	5-fold CV	0.843	0.605	0.872	0.372	0.390	0.787
B1_6PaD_SMO	test set	0.821	0.609	0.920	0.780	0.570	0.851
	10-fold CV	0.867	0.453	0.919	0.411	0.357	0.806
	5-fold CV	0.864	0.453	0.915	0.400	0.348	0.799
B1_11PaD_RF	test set	0.831	0.719	0.833	0.742	0.607	0.845
	10-fold CV	0.854	0.579	0.889	0.394	0.398	0.797
	5-fold CV	0.853	0.595	0.885	0.394	0.404	0.801
B1_11PaD_SMO	test set	0.821	0.547	0.949	0.833	0.568	0.868
	10-fold CV	0.861	0.505	0.905	0.400	0.371	0.801
	5-fold CV	0.861	0.500	0.906	0.401	0.370	0.790
consensus model							0.859

inhibitors for both OATP1B1 and OATP1B3 and one was predicted as selective OATP1B3 inhibitor with a binary score of 6 for OATP1B3 and of 1 for OATP1B1.

Inhibition Assay for OATP1B1 and OATP1B3. Chinese hamster ovary (CHO) cells that were stably transfected with OATP1B1 or OATP1B3 and wild-type CHO cells were provided by the University of Zurich, Switzerland, and have been extensively characterized previously.^{24,56,57} Cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% FCS, 50 $\mu\text{g}/\text{mL}$ L-proline, 100 U/mL penicillin, and 100 $\mu\text{g}/\text{mL}$ streptomycin. The culture media of the transfected CHO cells additionally contained 500 $\mu\text{g}/\text{mL}$ Geneticin sulfate (G418) (Sigma-Aldrich, Munich, Germany). Media and supplements were obtained from Invitrogen (Karlsruhe, Germany). Cells were incubated at 5% CO_2 and 37 $^{\circ}\text{C}$. For uptake experiments, CHO cells were seeded in 24-well plates (BD Biosciences, Heidelberg, Germany) at a density of 25,000 cells/well. Uptake assays were generally performed on day 3 after seeding, when the cells had grown to confluence. 24 h before starting the transport experiments, cells were additionally treated with 5 mM sodium butyrate (Sigma-Aldrich, Munich, Germany) to induce gene expression. Prior to the uptake experiments, cells were rinsed twice with 2 mL of prewarmed (37 $^{\circ}\text{C}$) uptake buffer (116.4 mM NaCl, 5.3 mM KCl, 1 mM NaH_2PO_4 , 0.8 mM MgSO_4 , 5.5 mM D-glucose, and 20 mM Hepes, pH adjusted to 7.4). Uptake was initiated

by adding 0.5 mL of uptake buffer containing 5 μM of the fluorescent OATP1B1/1B3 substrate FMTX⁵⁸ in the presence or absence of inhibitors. After 10 min culture at 37 $^{\circ}\text{C}$, uptake was stopped by removing the uptake solution and washing the cells 3 times with ice-cold uptake buffer. The cells were then lysed with 0.5 mL of 0.5% Triton X-100 solution dissolved in PBS and placed on a plate shaker for 30 min. Fluorescence was measured in an Enspire Multimode plate reader (PerkinElmer, Waltham, MA) at an excitation wavelength of 485 and an emission wavelength of 528 nm.

IC_{50} values were determined by plotting the log inhibitor concentration against the net uptake rate and nonlinear regression of the data set using the equation

$$y = \frac{a}{1 + [I/(\text{IC}_{50})]^s + b}$$

in which y is the net uptake rate (pmol/ μg of protein/min), I is the inhibitor concentration (μM), s is the slope at the point of inversion, and a and b are the maximum and minimum values for cellular uptake (GraphPad Software, San Diego, CA, USA). Net uptake was calculated for each inhibitor concentration as the difference in the uptake rates of the transporter-expressing and wild-type cell lines. Unless otherwise indicated, values are expressed as mean $\pm$ SD of three individual experiments. Significant differences from control values were determined using a Student's paired t test at a significance level of $p < 0.05$.

Table 2. Detailed Statistical Results of OATP1B3 Inhibition Models

model	validation	accuracy	sensitivity	specificity	precision	MCC	ROC area
B3_6MOE_RF	test set	0.847	0.775	0.864	0.574	0.574	0.847
	10-fold CV	0.876	0.677	0.891	0.326	0.412	0.842
	5-fold CV	0.871	0.661	0.887	0.312	0.394	0.821
B3_6MOE_SMO	test set	0.852	0.825	0.858	0.579	0.603	0.919
	10-fold CV	0.900	0.597	0.923	0.376	0.422	0.866
	5-fold CV	0.893	0.589	0.916	0.353	0.401	0.852
B3_6PaD_RF	test set	0.828	0.825	0.828	0.532	0.563	0.877
	10-fold CV	0.870	0.677	0.884	0.312	0.400	0.844
	5-fold CV	0.863	0.669	0.878	0.299	0.385	0.814
B3_6PaD_SMO	test set	0.847	0.725	0.876	0.580	0.554	0.925
	10-fold CV	0.894	0.516	0.923	0.342	0.365	0.853
	5-fold CV	0.896	0.508	0.926	0.348	0.366	0.840
B3_11PaD_RF	test set	0.842	0.775	0.858	0.564	0.565	0.886
	10-fold CV	0.866	0.629	0.884	0.295	0.368	0.832
	5-fold CV	0.863	0.645	0.880	0.294	0.372	0.825
B3_11PaD_SMO	test set	0.866	0.750	0.893	0.625	0.602	0.924
	10-fold CV	0.881	0.581	0.904	0.320	0.372	0.849
	5-fold CV	0.883	0.613	0.904	0.332	0.394	0.844
consensus model							0.917

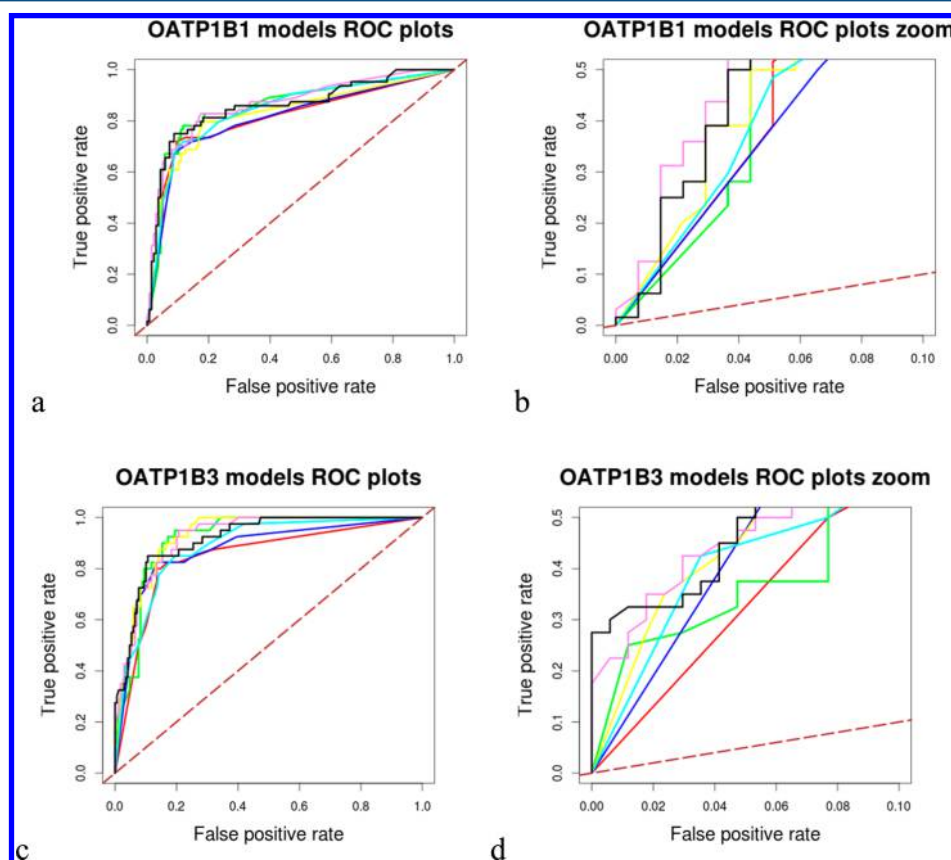


Figure 2. Comparative ROC plots of individual and consensus models for each transporter: (a) total OATP1B1 models ROC plot, (b) OATP1B1 models zoom ROC plot (TP rate [0.0, 0.5] and FP rate [0.0, 0.1]), (c) total OATP1B3 models ROC plot, and (d) OATP1B3 models zoom ROC plot (TP rate [0.0, 0.5] and FP rate [0.0, 0.1]). Black continuous line represents the performance of the consensus model, with red 6MOE_RF, with green 6MOE_SMO, with dark blue 6PaD_RF, with yellow 6PaD_SMO, with cyan 11PaD_RF, with violet 11PaD_SMO, and with dashed brown line a random performance of 50%.

RESULTS AND DISCUSSION

The Problem of Imbalanced Data Sets. One of the major challenges when dealing with real life scenarios is the imbalance of data sets. While most classification studies published in the literature show an equal number of actives

and inactives, our data sets comprised a ratio of 8/1 for noninhibitors/inhibitors for OATP1B1 and of 13/1 for OATP1B3, respectively (Figure 1). This resulted in a very poor performance when applying base classifiers directly on the

Table 3. Inhibition of OATP1B1 and OATP1B3

N	Compound	IC ₅₀ (μM) OATP1B1	IC ₅₀ (μM) OATP1B3	Compounds predicted as dual inhibitors	Compounds predicted as selective OATP1B3 inhibitors	Correctness of prediction	Purity of compound %
1	Carfilzomib	0.38 ± 0.12	0.90 ± 0.17	+		+++ , +++	≥98
2	Dronedaron hydrochlorid e	0.32 ± 0.07	1.12 ± 0.20	+		+++ , ++	≥98
3	Flavin adenine dinucleotide	6.62 ± 1.15	10.02 ± 0.78		+	x , +	≥95
4	Fosinopril sodium	6.28 ± 1.13	5.19 ± 0.98	+		+, +	≥98
5	Gliquidone	7.45 ± 1.64	1.17 ± 0.31	+		+, ++	≥98
6	Lapatinib	0.37 ± 0.19	33.78 ± 2.45(s.i.)	+		+++ , -+	≥99
7	N,O- Didansyl-L- Tyrosine	6.07 ± 0.56	2.94 ± 0.75	+		+, ++	≥95
8	Rapamycin (Sirolimus)	0.62 ± 0.19	0.40 ± 0.12		+	+++ , +++	≥98
9	Trametinib	0.34 ± 0.09	30.82 ± 4.01(s.i.)	+		+++ , -+	≥98
10	Zafirlukast	7.84 ± 0.49	4.96 ± 0.92	+		+, ++	≥98.5

s.i.: slight inhibition; results are means ± SD of three independent experiments; the – symbol and the red color are missing from table

+++ : strong inhibitor (IC₅₀ values less than 1 μM)

++ : moderately strong inhibitor (IC₅₀ values between 1 and 5 μM)

+: moderate inhibitors (IC₅₀ values between 5 and 10 μM)

-+: slight inhibitors (IC₅₀ values above 10 μM)

-: non-inhibitor

x: wrong class prediction

❖ green: totally correct prediction = correct prediction for both transporters

❖ yellow: almost correct prediction = correct prediction for one transporter and slight mistakes for the other (for the case of the slight inhibitors; it is not a mistaken prediction, but also not totally correct)

❖ orange: correct prediction for one transporter but incorrect for the other

training set, with sensitivity values lower than 0.2 (data not shown).

There are several methods for dealing with imbalanced data when using machine learning techniques.^{59–61} Indicatively, they comprise undersampling, oversampling, bagging, boosting, and application of costs. In our case, the application of a cost for misclassification of the minority class, using the meta-classifier MetaCost in WEKA, yielded the best results.

Classification Models for OATP1B1 and OATP1B3. Combining several sets of descriptors with various base- and meta-classifiers resulted in a cluster of models, based on Random Forest and Support Vector Machines (SMO) in combination with MetaCost as a cost-sensitive meta-classifier. All models present in the final cluster were validated via 5- and 10-fold cross-validation, as well as with the use of an external

test set, composed of 201 and 209 compounds for OATP1B1 and OATP1B3, respectively.⁴¹ Although the latter data set has been measured under different assay conditions than the respective one used in the training set, a comparison of the overlapping compounds showed high consistency. The statistical results of all models were quite similar and are presented in Tables 1 and 2.

As can be seen in Tables 1 and 2, all six models for each transporter showed approximately the same performance. Thus, we decided to implement a consensus scoring approach to allow input of all models when screening DrugBank, since it has been often suggested in the literature that consensus modeling outperforms single modeling approaches.^{62–66} This would also increase our confidence regarding the selection of potential OATP1B1 and 1B3 inhibitors for experimental testing,

especially in the case of contradictory results among different models. For getting the consensus score, the prediction scores of all models were summed up in order to get a final prediction. The validity of this approach was partially confirmed by calculating the ROC area of the consensus models based on the results of the external test set, as well as by plotting the respective ROC curves, using R^{67} (Figure 2). Although for both transporters the consensus models did not exhibit the highest AUC, the consensus model for OATP1B3 had the steepest ROC curve vs all the individual ones and was thus selected as the best solution for the subsequent in silico screen of DrugBank. In the case of OATP1B1, the ROC curve was steeper than the curves of five of the individual models, while there was one model, the SMO_11 PaD_B1, which had a slightly steeper curve. However, also for this case the consensus model was used for screening, since the difference was almost insignificant and we were in favor of using a majority vote for screening and compound selection rather than relying on a single model.

In Silico Screening of DrugBank. In order to prospectively validate the in silico models, DrugBank was virtually screened using all of the six classification models for each transporter, and the compounds were ranked according to the probability score of being an inhibitor. For OATP1B1, 5371/6279 compounds of DrugBank were predicted as noninhibitors by the consensus vote of the 6 models (85.5%), while 908/6279 were predicted as inhibitors. From the predicted inhibitors, 271 compounds were given an integer score of 6, i.e., they were predicted as inhibitors by all 6 classification models (4.36% of whole DrugBank). For OATP1B3, the overall figures were quite identical (905/6279 compounds were predicted as inhibitors, with 407 compounds showing a consensus score of 6/6). Integer and float consensus scores of all compounds are provided in the [Supporting Information](#).

Besides validation of our models by identification of new, hitherto unknown inhibitors of OATP1B1 and OATP1B3 from DrugBank, we also aimed at identifying subtype selective inhibitors. Unfortunately, the development of a 4-class classification model gave poor statistical results (data not shown). Thus, for each compound we compared the predictive scores for both transporters. However, this was quite challenging, since most of the compounds either were presenting the same inhibition profile for both transporters or they were already known OATP1B1 or 1B3 selective inhibitors. Finally, with an integer consensus score of 1 and a float consensus score of 2.062 for OATP1B1 vs 6 (integer score) and 4.430 (float score) for OATP1B3, flavin adenine dinucleotide was proposed as potential selective OATP1B3 inhibitor. As we could not identify a suitable OATP1B1 selective inhibitor, the remaining nine compounds that were selected for biological testing were predicted to inhibit both transporters. All of the selected OATP1B1/1B3 inhibitors, as well as their assay results, are presented in [Table 3](#).

Results of the Inhibition Assay. Since the model's threshold for inhibitors was 10 μM , compounds with IC_{50} values less than 1 μM were considered as strong inhibitors (+++), compounds with IC_{50} values between 1 and 5 μM as moderately strong inhibitors (++), compounds with IC_{50} values between 5 and 10 μM as moderate inhibitors (+), and compounds having IC_{50} values above 10 μM as slight inhibitors as long as an IC_{50} value could be obtained. In cases in which it

was impossible to obtain an IC_{50} value, the compound was considered as noninhibitor.

Considering that the classification models were generated on a threshold of 10 μM , the obtained results are very encouraging regarding the predictive capabilities of the models. The consensus model for OATP1B1 was correct for 9/9 inhibitors, while it was mistaken for the case of the selective OATP1B3 inhibitor. Flavin adenine dinucleotide was also an OATP1B1 inhibitor, which renders it a false negative. For OATP1B3, the respective consensus model was able to predict correctly 8/10 compounds. The two remaining compounds (lapatinib and trametinib) that were predicted as inhibitors had IC_{50} values above the threshold of the model.

Searching in the literature for any association between these newly identified OATP inhibitors and hepatotoxicity manifestations, such as hyperbilirubinemia, revealed the following findings: Carfilzomib was specifically reported as nonhepatotoxic,⁶⁸ and we could not find any association to hepatotoxicity for flavin adenine dinucleotide, gliquidone, and *N,O*-didansyl-L-tyrosine. Flavin adenine dinucleotide is a redox factor, important for the function of many flavoenzymes,⁶⁹ thus it could not be particularly toxic, while gliquidone is considered a safe antidiabetic drug and has actually been found to improve liver injury in diabetic patients.⁷⁰ *N,O*-Didansyl-L-tyrosine is an antibacterial agent, still in the experimental stage, so it is quite unlikely to have already reports regarding its toxicity. For trametinib, no reports for hyperbilirubinemia were found. However, it is known for elevating hepatic serum enzymes.⁷¹ Finally, dronedarone, fosinopril, lapatinib, rapamycin, and zafirlukast are reported for causing hyperbilirubinemia, when checking in online sources⁷² and in the literature,⁷³ while there are also some literature reports for hepatotoxicity of these compounds.^{68,73–78}

During the preparation of this manuscript, an additional OATP1B1 classification model was published by van de Steeg et al.⁷⁹ Their Bayesian model was based on a training set of 437 compounds (37 inhibitors and 400 noninhibitors) and an internal set of 155 compounds for validation (12 inhibitors and 143 noninhibitors), resulting from the screening of a commercial library of 640 FDA-approved drugs. Among the 20 strongest OATP1B1 and OATP1B3 inhibitors are rapamycin and fosinopril, which were also in our hit list. For the rest of the compounds we tested, to the best of our knowledge, they are reported for the first time in our study as OATP1B1 and/or 1B3 inhibitors. Moreover, the analysis of the top 20 compounds from van de Steeg et al. further confirmed the validity and high predictivity of our models. For OATP1B1, 5 compounds were not virtually screened by us, either because they did not exist in DrugBank or because they were removed in some stage of the data set curation. Another 7 compounds (cyclosporin A, atazanavir, dipyrindamiole, telmisartan, nifedipine, estradiol, spironolactone) were already included either in our training set or in the test set (6/7 predicted correctly as inhibitors). For the remaining 6 compounds, 5/6 are predicted correctly as inhibitors by our consensus model (bromocriptine mesylate, pranlukast, suramin, troglitazone and docetaxel), while sulfasalazine is predicted as noninhibitor. However, for sulfasalazine we must note that it was initially part of both the De Bruyn³⁹ data set and the Karlgren⁴¹ data set. As De Bruyn et al. annotated it as noninhibitor, and Karlgren et al. evaluated it as inhibitor, it was removed from both data sets. Nevertheless, we must emphasize that De Bruyn et al. and Karlgren et al. use different assays. The assay we used is similar

to the one from De Bruyn et al. (the source of our training set), while van de Steeg et al. use an assay similar to the one by Karlgren et al. (the source of our test set). This implies that the particular compound might give different results for different assays, and that this is a probable reason for its misclassification by our model.

For OATP1B3, an analogous picture occurs. Six compounds were not virtually screened because of their absence in DrugBank, 7 compounds (cyclosporin A, atazanavir, dipyridamole, telmisartan, mifepristone, fluvastatin, clarithromycin) were included either in our training set or our test set, and for the remaining 5 compounds we had an accuracy of 100% by our consensus model (suramin, docetaxel, clobetasol propionate, bromocriptine mesylate, and losartan).

CONCLUSIONS

The transportome of the liver is most probably the most complex one in the human body. It comprises numerous uptake and efflux transporters that regulate the concentrations of metabolites and endogenous substrates, such as bile acids and bilirubin. Thus, perturbation of this system by drugs might lead to symptoms such as cholestasis or hyperbilirubinemia. With this manuscript we introduce a set of in silico models which aid in the potential early detection of hepatotoxicity manifestations, such as hyperbilirubinemia, by predicting the probability of a compound to block OATP1B1 and OATP1B3 mediated transport of bilirubin. The models have been derived on the basis of a large, manually curated data set, and have been extensively validated by statistical methods, as well as by in silico screening of DrugBank followed by experimental testing of top ranked hits. Among the 9/10 hits confirmed as OATP inhibitors, five are reported for causing hyperbilirubinemia. These results strongly support the use of validated in silico models for prioritizing compounds in the hit triaging process.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.molpharmaceut.5b00583.

Settings for classification model generation (PDF)

Training and test set for OATP1B1 and OATP1B3 (SMILES format); scores for DrugBank compounds (XLSX)

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS USED

OATP1B1, organic anion transporting polypeptide 1B1; OATP1B3, organic anion transporting polypeptide 1B3; IC₅₀, half maximal inhibitory concentration; OATP2B1, organic anion transporting polypeptide 2B1; NTCP, Na⁺-taurocholate cotransporting polypeptide; OAT2, organic anion transporter 2; OCT1, organic cation transporter 1; MRPs, multidrug resistance associated proteins; MDRs, multidrug resistance proteins; ABC transporters, ATP-binding cassette transporters; BSEP, bile salt export pump; BCRP, breast cancer resistance protein; UGT1A1, UDP-glucuronosyltransferase 1A1; E1S, estrone sulfate; DHEAS, dehydroepiandrosterone sulfate; RF, Random Forest; SVM, Support Vector Machines; SMO, sequential minimal optimization; FMTX, fluoro-methotrexate; CHO cells, Chinese hamster ovary cells

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Chapter 4

Classification of Drug-Induced Liver Injury (DILI)

A simple 2-class classification model for drug-induced liver injury (DILI) – the importance of data curation

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In this paper we report the development of an *in silico* classification model for drug-induced liver injury (DILI). For the development of the model, a big dataset of 1770 compounds has been compiled as a training set from public sources and has been gradually reduced to 986 compounds of higher confidence regarding the class label. We tried to use hepatic transporters' interaction profiles (BSEP, BCRP, P-gp, OATP1B1 and OATP1B3), in combination with physicochemical descriptors, in order to generate the classification model. Unfortunately, liver transporters' inhibition predictions didn't seem to contribute significantly to the prediction of DILI; we suspect several reasons for this outcome, which are analyzed in the manuscript. The obtained model is based on a simple Random Forest of 100 trees and, despite the complexity of the endpoint, it performs satisfactory, both for 10-fold cross validation and on 2 external test sets. We emphasize on the importance of the carefully curated data, both in terms of chemotypes and class labels- especially when it comes to toxicity endpoints- that can give satisfactory results even with a rather simple classification scheme.

E. Kotsampasakou has compiled and curated the training and test set, calculated the transporters' inhibition predictions, generated the models, made the statistical analysis and wrote the manuscript. G.F. Ecker supervised the conducted work, reviewed the manuscript and contributed to writing.

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A simple 2-class classification model for drug-induced liver injury (DILI) – the importance of data curation

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A simple 2-class classification model for drug-induced liver injury (DILI) – the importance of data curation

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KEYWORDS

DILI, drug-induced liver injury, Random Forest, machine learning, 2-class classification, liver transporters, BSEP, BCRP, P-glycoprotein, OATP1B1, OATP1B3, data curation, toxicity reports

ABSTRACT

Drug-induced liver injury (DILI) is a major issue for both patients and pharmaceutical industry due to insufficient means of prevention/prediction. In the current work we present a 2-class classification model for DILI, generated with Random Forest and 2D descriptors on a dataset of 968 compounds. Priority was given to careful compilation and curation of literature data for

DILI, rather than the application of complex and computationally demanding algorithms. The initially compiled dataset of 1777 compounds was reduced via a 2-step approach to 968 compounds, resulting in a significant increase in the model's performance. The models have been validated via 10-fold cross-validation and against two external test sets of 910 and 1586 compounds, respectively. The final model showed an accuracy of 64% (AUC 69%) for 10-fold cross-validation (average of 50 iterations) and comparable values for two test sets (AUC 78% and 64%, respectively). Interestingly, including the predictions of our in-house transporter inhibition models for BSEP, BCRP, PgP and OATP1B1 and 1B3, did not improve the DILI model.

Introduction

Drug-induced liver injury (DILI) is the term used for liver damage that is caused by drugs, herbal agents or nutritional supplements.^{1,2} DILI has gained increasing attention in recent years,³ as it is one of the main causes for attrition during clinical and pre-clinical studies and the main reason for drug withdrawal from the market or labeling with a black box warning.⁴⁻⁷ Thus, great effort has been invested towards elucidating the toxicological processes and mechanisms that result in manifestations of DILI.⁸ It is widely accepted that, together with metabolizing enzymes, liver transporters play an important role for maintaining the integrity and proper function of the liver, and also influence the ADME (absorption, distribution, metabolism, excretion and toxicity) profile of drugs.^{9, 10} Actually, there are several recent publications suggesting that inhibition of liver transporters might result in manifestations of DILI. In particular for cholestasis strong evidence towards the role of the bile salt export pump (BSEP)^{8, 11-16} has been posed. There is also evidence for the multidrug resistance-associated protein 2 (MRP2)^{15, 17}, breast cancer

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3 resistance protein (BCRP)^{15, 17}, P-glycoprotein^{15, 17} and multidrug resistance-associated protein 3
4 and 4 (MRP3 and MRP4)^{13, 15, 17} to be involved. For hyperbilirubinemia, another possible
5 manifestation of hepatotoxicity, involvement of organic anion transporting polypeptides 1B1 and
6 1B3 (OATP1B1 and OATP1B3)^{18, 19}, MRP2¹⁹ and to a smaller extent BCRP¹⁹ is discussed.
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10 Although *in vitro* predictive methods are efficient for many toxic endpoints, they are time-
11 consuming and expensive.^{20, 21} In addition, for assessing hepatotoxicity, experimental methods
12 such as *in vitro* tests and animal models, have been shown to share low concordance (<50%)
13 with human hepatotoxicity.^{6, 22, 23}
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22 This led to the development of predictive computational methods, which are summarized in
23 two recent reviews by Chen et al.²⁴ and Ekins.²⁵ In particular, Chen divides these methods into:
24 i) knowledge-based models, which are based on structural alerts, ii) QSAR based models, which
25 utilize physicochemical 2D/3D descriptors and/or molecular fingerprints in combination with
26 statistical modeling or machine learning, iii) *in vitro* assay-based models that utilize multiple
27 toxicological assays and iv) toxicogenomics models taking advantage of microarray-based
28 technology.⁶
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39 Additional recent work, that is not reported in the reviews above, is a study by Liu J. et al.
40 (2014), where they used ToxCast bioactivity data and chemical structure information
41 (physicochemical descriptors and fingerprints) to predict hepatotoxicity in animals.²⁶ In a study
42 of 2015, Liu R. and co-workers are able to identify 12 structural alerts that are quite common
43 among drugs, but also implicated in drug-induced liver injury.²⁷ In the same year, Muller et al.²⁸
44 use molecular and biological descriptors to predict DILI on basis of particular hepatotoxicity
45 endpoints using various machine learning methods. Also in 2015, Xu et al. apply a deep neural
46 network approach to predict DILI.²⁹ Mulliner et al. (2016) present support vector machine
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(SVM) classification models to predict human and animal hepatotoxicity on multiple levels - from specific hepatotoxic endpoints to global hepatotoxicity.³⁰ Although all these models generally perform quite well, they sometimes suffer from low statistical performance, imbalanced sensitivity vs specificity, or small data sets (Table 1). Zhang and co-workers (2016) developed classification models with MACCS and FP4 fingerprints, based on a training set of 1317 compounds.³¹ Finally, in a very recent paper, Chen et al. analyzed the association of daily dose, lipophilicity (logP) and formation of reactive metabolites (RM), and developed an algorithm that allows quantitative assessment of risk of clinical DILI, by assigning a DILI score on each compound.³² Although all these models generally perform quite well, they sometimes suffer from low statistical performance, imbalanced sensitivity vs specificity, or small data sets (Table 1).

Table 1: Classification models for DILI reported in literature. Acc stands for accuracy, Sen for sensitivity, Spec for specificity, BA for balanced accuracy

Reference	Descriptors	Classification algorithm	Data used	Reported performance
Cheng & Dixon (2003) ³³	2D molecular descriptor	Ensemble recursive partitioning	382 drugs for CV 54 drugs for EV	CV: 76% Acc; 76% Sen; and 75% Spec EV: 81% Acc; 70% Sen; and 90% Spec
Cruz-Monteagudo et al. (2008) ³⁴	Radial distribution function molecular descriptors	Linear discriminant analysis	74 drugs for CV 13 drugs for EV	CV: 84% Acc; 78% Sen; and 90% Spec EV: 82% Acc
Matthews et al. (2009) ³⁵	Molecular descriptors	4 commercial QSAR programs	~1600 drugs for CV 18 drugs for EV	CV: 39% Sen; and 87% Spec EV: 89% Sen

Rodgers et al. (2010) ³⁶	MolConnZ and Dragon molecular descriptors	k-nearest neighbor	37 drugs for EV	84% Acc; 74% Sen; and 94% Spec
Fourches et al. (2010) ³⁷	2D fragments and Dragon molecular descriptors	Support vector machine	531 drugs for CV 18 compounds for EV	CV: 62–68% Accs EV: 78% Acc
Ekins et al. (2010) ³⁸	ECFC_6 molecular descriptors	Linear discriminant analysis	295 compound for CV 237 compounds for EV	CV: 59% ACC; 53% Sen; and 65% Spec EV: 60% Acc; 56% Sen; and 67% Spec
Liew et al. (2011) ³⁹	PaDEL molecular descriptor	Ensemble of mixed learning	1087 compounds for CV 120 compounds for EV	CV: 68% Accs; 67% Sen; and 70% Spec EV: 75% Acc; 82% Sen; 65% Spec
Liu et al. (2011) ²²	ECFC_6 molecular descriptors	Bayesian models	888 drugs for training 3 data sets with 40–148 drugs for EV	EV: 60–70% Accs
Chen et al. (2013) ⁴⁰	Mold2 chemical descriptor	Decision Forest	197 drugs for CV Three data sets with 190–348 drugs for EV	CV: 70% Acc EV: 62–69% Accs
Liu et al. (2014) ²⁶	physicochemical descriptors and fingerprints	Ensemble classifier	677 compounds for CV	81% BA; 66% Sen; and 95% Spec
Muller et al. ²⁸	ISIDA fragment descriptors	SVM	424 drugs for CV	66% BA
Xu et al.	Encoding layers based on SMILES, PaDEL descriptors	Deep Learning Neural Networks	190, 475 & 1065 compounds for CV 185,320, 236,198 & 119 compounds	CV: 70-88% Accs; 70-90% Sens; 70-87% Specs EV: 62-87% Accs; 62-83% Sens; 62-93% Specs

			for EV	
Mulliner et al. ³⁰	2D and 3D physicochemical descriptors	SVM with a genetic algorithm	3712 compounds for training	IV: 75% Acc; and 73% AUC
			221 compounds for IV	
			269 compounds for EV	
Zhang et al. ³¹	FP4 fingerprints	SVM	1317 compounds for training	Training set: 66% Acc; 85% Sen; and 34% Spec; 55% AUC
			88 compounds for EV	EV: 75% Acc; 93% Sen; 38% Spec; 61% AUC

In this we generate *in silico* 2-class classification models for DILI by compiling multiple and diverse datasets from literature. We carefully curated these data regarding the chemotypes, as well as the accuracy of the class label. In addition, we are exploring the importance of hepatic transporter inhibition on DILI. For this, we use the predictions of our in-house *in silico* classification models for the inhibition of the basolateral transporters OATP1B1 and 1B3⁴¹ and the canalicular transporters P-glycoprotein⁴², breast cancer resistance protein (BCRP)⁴² and BSEP⁴³ as additional descriptors for the DILI model.

Methods

Data Compilation

Training Set

For compiling the DILI training dataset we searched in PubMed⁴⁴, Google⁴⁵ and Scopus⁴⁶ using the search terms: “drug-induced liver injury”, “DILI”, “drug-induced hepatotoxicity”. Subsequently, the retrieved publications were manually investigated for data, i.e. compounds that

are positive or negative for drug-induced liver injury. This allowed to identify 9 unique datasets as follows (all numbers are after curation of the data):

1. O'Brien et al.⁴ developed an *in vitro* cell-based model for assessing drug –induced liver injury in humans. We consider drugs “severely” and “moderately” hepatotoxic as DILI positives and the non-toxic drugs as DILI negatives, which leads to 132 compounds (100 positives/32 negatives).
2. Rodgers et al.⁴⁷ developed a QSAR model for adverse effects of drugs (AEDs) in the liver, based on a dataset from the FDA spontaneous reporting database of human liver AEDs (i.e. elevations in activity of serum liver enzymes). After curation, the dataset consisted of 473 compounds, but only 382 of them (75 positives/307 negatives) had a class for general hepatotoxicity.
3. Fourches et al.⁴⁸ compiled datasets for human, rodent and non-rodent (animal) hepatotoxicity for further QSAR analysis by applying text mining techniques on MEDLINE. For human hepatotoxicity we collected 902 compounds (620 positives/282 negatives).
4. Greene and coworkers⁴⁹ developed an SAR study for hepatotoxicity based on a big collection of drugs and chemicals, which they implemented as structure alerts using Derek for Windows. For general hepatotoxicity 385 compounds (252 positives/133 negatives) were compiled.
5. Ekins et al.³⁸ developed a Bayesian ligand-based model with extended connectivity fingerprints dividing his data into training set (295 compounds) and test set (237 compounds). This lead to 499 compounds (294 positives/205 negatives).

6. Chen and coworkers⁶ proposed an FDA-based labeling system for DILI compounds. The initial system consisted of 3 classes with the following order of severity: i) most DILI concern, ii) less DILI concern and iii) no DILI concern. By transforming the data into a binary system, the “most DILI concern” and “less DILI concern” were considered as positives and the “no DILI concern” as negatives, which provided 279 compounds (279 positives/61 negatives) were collected.
7. Liu Z. et al.²² developed a DILI prediction system based upon 13 hepatotoxic side-effects with data compiled by the SIDER 2^{50, 51} database and validated it using the data sets from O’Brien et al.⁴, Greene et al.⁴⁹ and Chen et al.⁶ The authors demonstrated that for a compound to be considered as DILI positive, it should be positive for at least 3 out of the 13 hepatotoxic endpoints. Following this concept yielded 835 drugs in total (188 DILI positives and 647 DILI negatives).
8. Zhu and Kruhlak⁵² set up a human hepatotoxicity database using post-market safety data and recommend this database for further QSAR studies. Using only the data of highest certainty regarding the class (according to authors), we retrieved 1948 compounds (651 DILI positives and 1297 DILI negatives).
9. In their 2015 study, Liu and coworkers²⁷ identified 12 structural alerts for DILI based on LiverTox data. Once more, the data were initially divided into 3 classes: “hepatotoxic”, “possible hepatotoxic” and “nonhepatotoxic”. In order to transform them into our binary system, we considered the “hepatotoxic” and “possible hepatotoxic” as DILI positives and the “nonhepatotoxic” as DILI negatives, giving 583 compounds (409 positives and 174 negatives).

For visualizing the data and for converting the names into structures Marvin from ChemAxon⁵³ was used.

Chemical curation

- For each dataset we applied the following chemotype curation:
- All inorganic compounds are removed using MOE 2014.09.⁵⁴
- Using the Standardiser tool⁵⁵ created by Francis Atkinson, all salt parts and any compounds containing metals and rare or special atoms are removed from the dataset and the structures are standardized.
- Duplicates and permanently charged compounds are removed using MOE 2014.09.⁵⁴
- 3D structures are generated using CORINA (version 3.4)⁵⁶ and their energy is minimized with MOE 2014.09⁵⁴, using default settings, but changing the gradient to 0.05 RMS kcal/mol/Å². Existing chirality is preserved.

Class-label curation

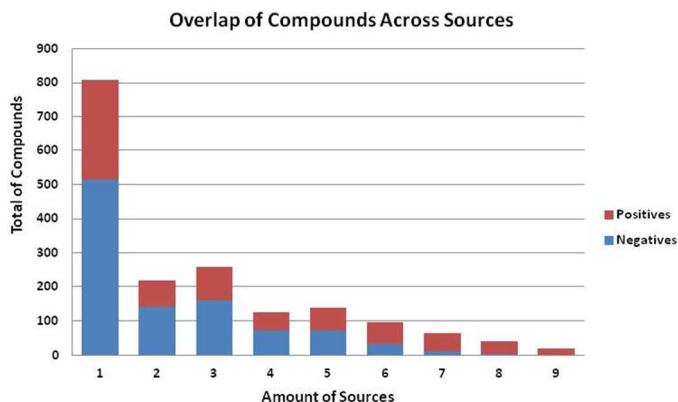
Apart from the chemical curation of the data, we apply careful curation regarding the class label of the compounds. In particular, after merging all the single datasets in one database using MOE 2014.09⁵⁴, the majority of the compounds are overlapping for at least two datasets. Thus, we keep track of the sources for each particular compound and the class that was assigned by each source. In total, we might have up to 9 sources for an individual compound (in case it is included in all 9 datasets). In case of conflicting class labels, we assigned the majority label to the compound. In case the class labels (positive or negative) are equally distributed, the compound is considered as “ambiguous” and it is removed from the dataset. This leads to 1777

compounds, 796 positives and 981 negatives. In Table 2 and Chart 1 the overlap of compounds (positives and negatives) across the different amount of sources is depicted.

Table 2: Overlap of DILI positives and negatives across the different amount of sources.

Total Compounds	Positives	Negatives	Amount of Sources
809	296	513	1
217	77	140	2
260	101	159	3
124	52	72	4
137	65	72	5
97	66	31	6
65	53	12	7
39	37	2	8
20	20	0	9

Chart 1: Overlap of DILI positives and negatives across the different amount of sources.



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6 However, despite the careful curation, the first modeling attempt of the dataset gave moderate
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8 results. Therefore, in order to improve the dataset quality, we decided to remove all compounds
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10 that were coming solely from the Fourches dataset (227 compounds). As mentioned above, the
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12 Fourches dataset was compiled via a text mining approach⁴⁸. The method was very sophisticated
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14 and resulted in a pretty good quality level, regarding the statistics obtained. However text-mining
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16 approaches are error-prone and definitely less reliable than manual human curation.^{57, 58} This
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18 reduces the data set to 1550 compounds in the dataset: 699 positives and 851 negatives.
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22 To further improve the quality of the data set, we removed all compounds coming from only a
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24 single source, as they do not allow us to counter check the class level with at least one additional
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26 source. Following this concept leads to the removal of additional 582 compounds, which
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28 provides the final set of 968 compounds: 501 positives and 467 negatives.
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34 External test sets

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36 After compiling the training set and generating the DILI model, we came across one more
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38 dataset that had initially escaped our attention, published by Liew et al.³⁹ in 2011. In their study,
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40 Liew and coworker develop an ensemble modeling approach based on 617 base classifiers.
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42 Considering both training and external test set and after chemical curation (as described above),
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44 1217 compounds remained in the dataset. Removing the overlapping compounds with the
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46 training set leads to 910 compounds (527 positives and 383 negatives), which served as first
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48 external test set.
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As second external test set we used the human hepatotoxicity dataset very recently published by Mulliner et al.³⁰ Removing overlapping compounds and chemical curation leads to 1586 compounds: 980 positives and 606 negatives.

Both the training set and the two external test sets are provided in the supporting information.

Generation of statistical models

Algorithms used

The 2-class classification models are built using the software package WEKA (version 3.7.12)⁵⁹. The training set for all stages of curation (1777 compounds, 1550 compounds, and 968 compounds) is almost balanced, thus there is no need for artificially balancing it via meta-classifiers. We investigated the performance of several base classifiers, such as logistic regression, tree methods (Random Forest and J48), Support Vector Machines (SMO in WEKA with polynomial, RBF and Puk kernels), Naïve Bayes, and k-nearest neighbors. Additionally, several meta-classifiers were explored for attribute selection (AttributeSelectedClassifier), as well as for improving the statistical performance such as Bagging⁶⁰ and Boosting^{61, 62}.

Molecular descriptors

For both datasets, several types of molecular descriptors have been calculated, comprising all 2D MOE descriptors and the 3D Volsurf series of descriptors⁵⁴, PaDEL descriptors⁶³ and ECFP6 fingerprints (extended connectivity fingerprints) using RDKit⁶⁴.

In order to investigate the potential influence of transporter inhibition in DILI manifestation, we predicted the transporter inhibition profile of all compounds and used it as additional descriptors. In particular, for OATP1B1 and 1B3 inhibition we used our previously published

models based on PaDEL descriptors,⁴¹ as implemented in eTOXlab.⁶⁵ The binary output (positive or negative) was transformed to 1 or 0 and the sum of the binary scores for all 4 models per transporter, which results in “sum binary score” values between 0 and 4. For basolateral transporters, we used the float predictions obtained for BSEP⁴³ inhibition from its implementation as a KNIME workflow. Also for P-glycoprotein⁴² and for BCRP⁴² inhibition, the respective float prediction scores were used.

Model validation

For a first assessment, the models were validated via 10-fold cross validation. Subsequently, for the best models obtained, we performed 50 iterations by changing the cross-validation seed (for splitting the data within cross validation) and further performed a two-sample t-test in R⁶⁶ to assess if the models’ performance for the different data sets is indeed significantly different. This was also done to compare whether the inclusion of the predicted transporter interaction profiles significantly improves model performance. The best models were further validated via external validation by using the datasets by Liew et al.³⁹ and Mulliner et al.³⁰

Results and Discussion

Optimizing the training dataset

After compiling the DILI dataset from the 9 data sources and performing the curation of the chemotypes and class labels according to majority vote, we initially ended up with 1777 compounds. However, the first modeling approach gave moderate results (Table 3). As this might be due to low data quality rather than the inability to create a valid model, we removed the 227 single-source compounds from the Fourches dataset. This set has been compiled via text

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3 mining approaches and thus might be more prone to errors.^{52, 58} Two examples are tocopherol
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5 and carnitine, which were reported as hepatotoxic only by the Fourches source. In reality, those
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7 two compounds not only are not hepatotoxic, but actually have hepatoprotective effect against
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9 DILI caused by other drugs.^{67, 68} Analysing the respective publications under the titles:
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11 “Hepatoprotective effect of tocopherol against isoniazid and rifampicin induced hepatotoxicity in
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13 albino rabbits”⁶⁷ and “Effect of L-carnitine treatment for valproate-induced hepatotoxicity”⁶⁸,
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15 allows to understand why the text mining failed. Text mining techniques are searching for words
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17 like “induce”, “cause” etc. next to drugs, thus they mistakenly identified tocopherol and carnitine
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19 as hepatotoxic.
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25 The reduction gives back 1550 compounds and improves the statistical performance of the
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27 resulting models (Table 3). In order to further improve the data quality, we also removed all
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29 compounds that are retrieved from a single source. In this case there is possibility to double-
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31 check the class label and to assign the label of the majority vote. This led to the removal of 582
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33 compounds, providing our final training dataset of 968 compounds (501 positives and 467
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35 negatives). The model trained on the final dataset performed even better than the previous one
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37 (Table 3).
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41 In order to evaluate if the difference between the models generated on the three datasets is
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43 statistically significant, we performed 50 iterations of 10-fold cross validation by changing the
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45 cross-validation seed and then performed a two sample t-test to compare the best model from
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47 each dataset. As the statistical performance parameters for all models generated mostly differ
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49 only slightly, we considered a sensitivity/specificity compromise, the number of descriptors used
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51 (e.g. if a combination of 2D and 3D descriptors gave same results as only 2D descriptors, the
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53 latter option was preferred), the complexity of the classifier and the computational cost (i.e. more
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simple classifiers were preferred when they perform equally well as complex ones). For the datasets of 1777 and 1550 compounds Random Forest with 100 trees with the threshold manually set to 0.45 by using the ThresholdSelector meta-classifier in WEKA, was selected. This was done as for the normal default threshold of 0.5 the obtained sensitivity values were less than 50%. Reducing the threshold for class-assignment, results in an increase of sensitivity and decrease of specificity. For the dataset of 968 compounds this issue was not observed, and Random Forest was used without adjusting the threshold. All three models were generated using all 2D MOE descriptors (192 descriptors in total). Table 3 provides the mean and sd-values of a set of statistical parameters for the three data sets when using 10-fold cross validation with 50 iterations. As can be seen, all parameters generally increase with higher quality of the data sets. To further strengthen this, we performed two sample t-tests in R.⁶⁶ Comparing the 1777 compounds and 1550 compounds datasets, we indeed obtained p-values considerably lower than 0.05, which points towards a significantly improved model performance. Also when comparing the 968 and 1550 compounds datasets, apart from specificity ($p = 0.351$) p -values < 0.05 were obtained. Of course for machine learning techniques larger datasets are preferred⁶⁹ unless there is doubt regarding the data quality.

Table 3. The average performance for 10-fold cross validation out of 50 iterations and the standard deviation for accuracy, sensitivity, specificity, MCC, AUC and precision for the three datasets examined for a Random Forest (100 trees) using all 2D MOE descriptors.

Average of 50 iterations						
	Accuracy	Sensitivity	Specificity	MCC	AUC	Precision
DILI_1777cpds	0.595	0.602	0.589	0.189	0.630	0.543

sd	0.007	0.012	0.008	0.015	0.014	0.007
DILI_1550cpds	0.613	0.624	0.605	0.228	0.656	0.566
sd	0.007	0.010	0.011	0.013	0.005	0.010
DILI_968cpds	0.643	0.677	0.607	0.285	0.692	0.649
sd	0.009	0.012	0.015	0.018	0.008	0.009

DILI 2-class classification models

For the final training dataset of 968 compounds, the best models are obtained using either all 2D MOE descriptors or a subset of 93 selected more interpretable descriptors (for a full list of the descriptors see Supporting information), with and without inclusion of the predicted transporter interaction profiles. The models are trained either with Random Forest (RF) of 100 trees as a stand-alone base classifier or with the meta-classifier RealAdaBoost in combination with the Random Forest base classifier. RealAdaBoost was developed by Friedman et al.⁷⁰ as a modification of the initial boosting methodology^{71, 72} of AdaBoost.⁷³ RealAdaBoost, like conventional AdaBoost, increases the performance of the base classifier, while it simultaneously reduces the computation time.⁷⁰

An overview on the statistical performance for all possible combinations of settings (8 models) is provided in Table S2, supporting information. Also in this case we performed a two-sample t-test for the 10-fold cross-validation runs over 50 iterations. In a first run we pair-wise compared the models using different sets of descriptors, with and without predicted transporter interaction profiles as additional descriptor set. The results indicate that models using all 2D MOE descriptors perform better than the ones with a reduced input matrix. Interestingly, there is no statistically significant improvement in the performance if the transporter interaction profiles are

included in the descriptor matrix. With respect to the classifier used, the combination with RealAdaBoost seems slightly superior to Random Forest alone. However, this trend is less visible when the two external validation sets are analyzed. (Table S3, supporting information). Table 4 shows the statistical performance of the proposed optimal model using Random Forest (100 trees) as a classifier and 93 2D MOE descriptors, with (DILI_93_2D_MOE_dscrs_RF model) or without transporters predictions (DILI_93_2D_MOE_dscrs_transp_pred_RF model). All the obtained statistics metrics are reported for the average of 10-fold cross validation for 50 iterations, as well as for the two external test sets.

Table 4: statistical performance of the proposed optimal model using Random Forest (100 trees) as a classifier and 93 2D MOE descriptors, with (DILI_93_2D_MOE_dscrs_RF model) or without transporters predictions (DILI_93_2D_MOE_dscrs_transp_pred_RF model)

			Accuracy	Sensitivity	Specificity	MCC	AUC	Precision
DILI_93_2D_MOE_dscrs_RF								
Average 10-fold CV for 50 iterations			0.630	0.660	0.599	0.266	0.664	0.637
sd			0.008	0.010	0.017	0.061	0.060	0.008
Liew 910 cpds			0.714	0.700	0.734	0.429	0.783	0.783
Mulliner 1586 cpds								
			0.575	0.584	0.561	0.141	0.592	0.683
DILI_93_2D_MOE_dscrs_transp_pred_RF								
Average 10-fold CV for 50 iterations			0.625	0.657	0.591	0.249	0.671	0.633
sd			0.009	0.013	0.012	0.018	0.008	0.008
Liew 910 cpds			0.712	0.704	0.723	0.422	0.784	0.778

Mulliner	0.575	0.597	0.540	0.133	0.587	0.677
1586 cpds						

Considering the results from both 10-fold cross validation and the external validation shows, that the use of transporters predictions does not really improve the quality of the resulting model. Therefore, there is no reason for including them in the descriptors' set. Comparing the use of Random Forest stand-alone and the combination of RealAdaBoost and Random Forest, the results are not fully in agreement between the 10-fold cross validation and the external test set results. Thus, since the difference is not substantial, we decided to focus on the model built upon Random Forest stand-alone, in order to keep the classification scheme as simple as possible. Over all, the performance of all 8 models is quite satisfactory, especially when considering the complexity of DILI as an endpoint. The reported performance might be close to real life scenarios, since the validation took place via 10-fold cross validation over 50-iterations and we further validated the resulting models with two independent datasets of 910 compounds and 1586 compounds. Considering all duplicates, the external validation is based on in total 1672 unique compounds. However, we have to note that for the 307 compounds the Liew dataset shares with the training set, approximately 22% (67 compounds) are assigned with contradictory classes. For the Mulliner dataset of 1586 compounds, the percentage of contradiction for the shared compounds is even larger: out of 503 compounds shared with the training set, 165 of them (33%) show contradictions regarding their class labels. But also in between the two external datasets, for the 412 common compounds, 99 of them (24%) show contradictory class labels. Even though the shared compounds between external test set- training set have been removed, this might be an indication of the whole picture regarding the class labeling for the datasets. For example, in the Mulliner dataset many of the common compounds were labeled as positives for DILI, while they

were characterized as negatives in the training dataset and the Liew dataset. These differences for class labels further strengthen the importance of careful data curation, especially for this type of complex toxicity endpoints.

Importance of transporter inhibition for DILI

Literature definitely suggests association of the liver transporters we investigated - especially BSEP⁴³, but also BCRP^{15, 17}, P-glycoprotein^{15, 17}, OATP1B1 and 1B3^{18, 19} - with DILI. However, the results obtained from the two sample t-test for cross validation results and the external validation strongly suggest that the predicted transporter interaction profiles are not improving the models' performance. This was further verified via the use of AttributeSelectedClassifier, a WEKA meta-classifier for attribute selection. Here, SignificanceAttributeEval and CorrelationAttributeEval are selected as evaluators and Ranker as a search method, in order to assess the descriptors' importance. SignificanceAttributeEval⁷⁴ with Ranker evaluate the worth of an attribute by computing the Probabilistic Significance in a two-way function: attribute-classes and classes-attribute association. Then, Ranker gives back the list of the descriptors with a decimal coefficient number (usually between 1 and 0) in descending order, according to their importance. The higher the coefficient number, the more important is the descriptor. CorrelationAttributeEval estimates the worth of an attribute by calculating the Pearson's correlation coefficient between the attribute and the class. Then Ranker gives back the list of attributes in descending order, according to the Pearson's correlation coefficient. For the predicted transporter interactions as descriptors, they are all assigned with coefficient of 0 by SignificanceAttributeEval. With CorrelationAttributeEval the highest calculated Pearson's

correlation coefficient is 0.058 for OATP1B3 inhibition, thus suggesting very weak association with the DILI class label.

In order to further investigate any possible association between transporter inhibition and DILI, we performed a chi-squared test⁷⁵ for categorical variables. For this, the transporter predictions are transformed into categorical variables in analogy to the DILI class labels. For the transporters BSEP, BCRP and P-gp, if the prediction score is ≥ 0.5 , the compound is characterized as inhibitor, otherwise it is considered as non-inhibitor. For OATP1B1 and OATP1B3, if a compound is predicted as inhibitor by at least 2 out of 4 models, then it is considered as inhibitor. For OATPs, BSEP and P-gp we got p-values of > 0.05 , suggesting independence between the variables of transporter inhibition (inhibitor/non-inhibitor) and DILI (positive/negative). Interestingly, for BCRP the obtained p-value of 0.008 suggests dependence between the variables, i.e. association. Nevertheless, this might be less evident when the prediction is used as a float score. This is a probable explanation why BCRP inhibition is not considered as an important variable by attribute selection methods and does not improve the performance of the DILI classification model.

An additional reason for being unable to identify a strong association between DILI and transporter inhibition might be the fact that we did not include inhibition predictions for all liver transporters of potential relevance for DILI. Apart from BSEP, BCRP, P-gP and OATPs, it is known that more transporters can be involved in drug induced hepatotoxicity. Examples worth to be mentioned are MRP2^{15, 17, 76}, multidrug resistance protein (MDR3)^{17, 77} and MRP3 and MRP4^{13, 15, 17}. Unfortunately, due to the lack of experimental data, it is not possible to develop and validate high quality *in silico* models for these transporters in order to include them in the study.

Finally, it might be that the complexity of the DILI endpoint itself does not allow a strong association between liver transporters' inhibition with DILI. Apart from transporter inhibition, there are several more mechanisms that produce hepatotoxicity⁸, which are not taken into account in this study. In particular, the metabolizing liver enzymes of the cytochrome P450 family play a vital role for drug-induced liver injury, via the alteration of drugs' bio-availability and the formation of reactive metabolites.⁷⁸⁻⁸⁰ Glutathione adduct formation⁷⁹ and mitochondrial toxicity^{16, 79} also seem to play an important role.

Conclusions

Drug-induced liver injury is a major issue for patients and therefore also for the process of drug discovery. Within the last decade several attempts have taken place to predict DILI, with different grades of success. In this work we have compiled the majority of the available datasets in literature for drug-induced liver injury and subsequently stepwise curated the data regarding the chemotypes and the class label assignment. Starting from 1777 compounds, the curation reduced the size of the dataset to 1550 compounds and finally to 968 compounds. The particular curating procedure gradually improved our data quality and, consequently, the performance of the classification models. The final model, based on Random Forest and 2D MOE descriptors, showed AUC values in the range of 64% - 78% for the training set and two external test sets. We further investigated whether including predicted transporter inhibition profiles for BSEP, BCRP, P-gp, and OATP1B1 and 1B3 as descriptors will increase the classification performance. Interestingly, contradictory to literature suggestions, the transporters' contribution was not found statistically significant. Our results further indicate that in case of high quality data even basic classifier provide satisfactory results.

All in all, the current work once more stresses the significance of data quality. However, there might be still a substantial amount of miss-labeled compounds, as the conflicting class labels for overlapping compounds in the training and test sets show. One should not forget that the DILI dataset is basically based on toxicity reports from the original sources. The adverse event reporting system suffers from several issues. The most major is under-reporting,^{47, 52, 81} due to the voluntary character of the system.^{52, 82, 83} In general, it is quite difficult to obtain human toxicity data; very often they are proprietary and post-marketing data are difficult to procure.⁴⁷ Moreover, in most of the cases a causal relationship is not required to submit an adverse event.⁵² This is quite serious in the contemporary era of polypharmacology, where many people, especially the elderly, receive more than one different medication. Thus, in case of an adverse effect, the patient's doctor would report all the drugs administered to the patient, without identifying which one(s) caused the side-effect. This further strengthens the tremendous need for industry driven collaborative efforts to share data and to make them publicly available for mining and exploitation. Only large sets of high quality data will allow deriving predictive *in silico* models covering a broad chemical space.

ASSOCIATED CONTENT

Supporting Information.

- pdf file: a list with the subset of 93 2D MOE descriptors (Table S1) and the statistical performance of all final described models for the average of 10-fold cross validation for 50 iterations (Table S2) and for the external test sets (Table S3).

- zip file: training sets (1777, 1550 and 686 compounds) and external test sets (Liew et. al, Mulliner et al.); datasets provided in 5 sd files and an excel workbook containing all datasets; for each compound the structure, the source(s) of origin with the respective class(es), as well as the general DILI class after curation, are provided.

“This material is available free of charge via the Internet at <http://pubs.acs.org>.” For instructions on what should be included in the Supporting Information, as well as how to prepare this material for publication, refer to the journal’s Instructions for Authors.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

Acc: Accuracy, ADMET: absorption, distribution, metabolism, excretion, toxicity, AUC: area under the curve, BA: balanced accuracy, BCRP: breast cancer resistance protein, cpd(s): compound(s), CV: cross validation, DILI: drug-induced liver injury, EV: external validation, IV: internal validation, MCC: Matthews correlation coefficient, MDR3: multidrug resistance protein, MRP2: multidrug resistance-associated protein 2, MRP3: multidrug resistance-associated protein 3, OATP1B1: organic anion transporting polypeptide 1B1, OATP1B3: organic anion transporting polypeptide 1B3, P-gp: P-glycoprotein, RF: Random Forest, SMO: sequential minimal optimization, sd: standard deviation, Sen: sensitivity, Spec: specificity, SVM: support vector machines

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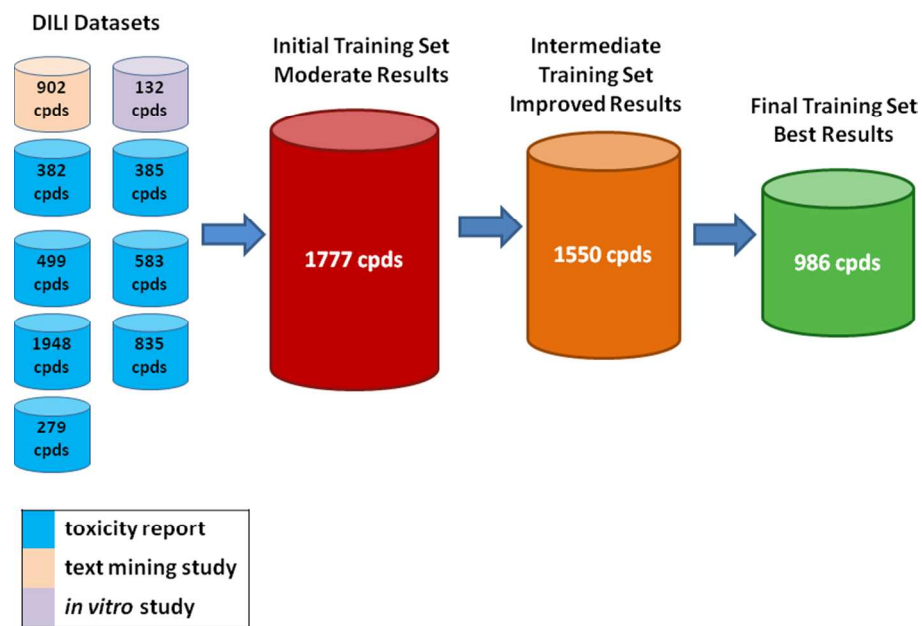
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Table of Contents Graphic



Chapter 5

Classification of Hyperbilirubinemia

Linking transporter interaction profiles to toxicity - the hyperbilirubinemia use case

Eleni Kotsampasakou, Sylvia E. Escher and Gerhard F. Ecker

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In this paper we report the development of two *in silico* classification models for hyperbilirubinemia – one based on human public data and one based on an animal dataset provided by the eTOX consortium. Since there is literature evidence for the association of OATP1B1 and OATP1B3 inhibition with hyperbilirubinemia, we used the compounds' predictions for OATP1B1 and OATP1B3 inhibition obtained by the models described in chapter 3, in combination with fingerprints and/or physicochemical descriptors, in order to generate the classification models. However, we didn't see strong association between OATP inhibition and hyperbilirubinemia. The models were validated through 10-fold cross-validation.

E. Kotsampasakou has compiled and curated the human training and curated the animal training set, calculated the transporters' inhibition predictions, generated the models, made the statistical analysis and wrote the manuscript, apart from the part "Methods – Animal data". S. E. Escher analyzed the animal dataset, provided the correct class labels for the compounds further used for modeling and wrote the part "Methods – Animal data" of the manuscript. G. F. Ecker supervised the conducted work, reviewed the manuscript and contributed to writing.

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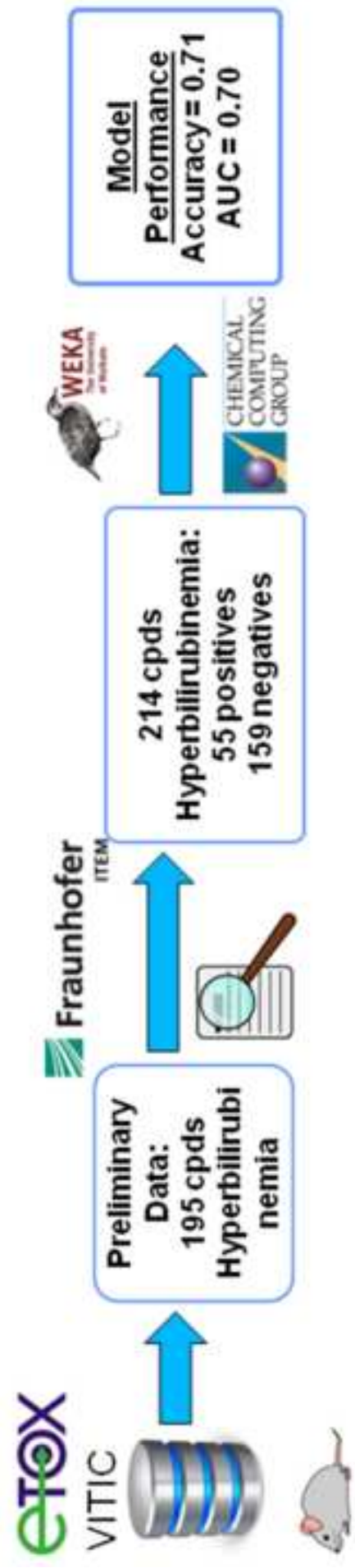
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Abstract: Hyperbilirubinemia is a pathological condition of excessive accumulation of conjugated or unconjugated bilirubin in blood. It has been associated with neurotoxicity and non-neural organ dysfunctions, while it can also be a warning of liver side effects. Hyperbilirubinemia can either be a result of overproduction of bilirubin due to hemolysis or dyserythropoiesis, or the outcome of impaired bilirubin elimination due to liver transporter malfunction or inhibition. There are several reports in literature that inhibition of organic anion transporting polypeptides 1B1 and 1B3 (OATP1B1 and OATP1B3) might lead to hyperbilirubinemia. In this study we created a set of classification models for hyperbilirubinemia, which, besides physicochemical descriptors, also include the output of classification models of OATP1B1 and 1B3 inhibition. Models were based on either human data derived from public toxicity reports or animal data extracted from the eTOX database VITIC. The generated models had satisfactory performance of 68% accuracy and area under the curve (AUC) for human data and 71% accuracy and 70% AUC for animal data. However, our results did not indicate strong association between OATP inhibition and hyperbilirubinemia, neither for humans nor for animals.

Human Data Workflow



Animal Data Workflow



Linking transporter interaction profiles to toxicity - the hyperbilirubinemia use case

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Linking transporter interaction profiles to toxicity - the hyperbilirubinemia use case

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Abstract

Hyperbilirubinemia is a pathological condition of excessive accumulation of conjugated or unconjugated bilirubin in blood. It has been associated with neurotoxicity and non-neural organ dysfunctions, while it can also be a warning of liver side effects. Hyperbilirubinemia can either be a result of overproduction of bilirubin due to hemolysis or dyserythropoiesis, or the outcome of impaired bilirubin elimination due to liver transporter malfunction or inhibition. There are several reports in literature that inhibition of organic anion transporting polypeptides 1B1 and 1B3 (OATP1B1 and OATP1B3) might lead to hyperbilirubinemia. In this study we created a set of classification models for hyperbilirubinemia, which, besides physicochemical descriptors, also include the output of classification models of OATP1B1 and 1B3 inhibition. Models were based on either human data derived from public toxicity reports or animal data extracted from the eTOX database VITIC. The generated models had satisfactory performance of 68% accuracy and area under the curve (AUC) for human data and 71% accuracy and 70% AUC for animal data. However, our results did not indicate strong association between OATP inhibition and hyperbilirubinemia, neither for humans nor for animals.

Keywords

Hyperbilirubinemia; liver; organic anion transporting polypeptide 1B1 (OATP1B1); organic anion transporting polypeptide 1B3 (OATP1B3); classification; support vector machines; decision trees

Abbreviations

OATP1B1: organic anion transporting polypeptide 1B1, OATP1B3: organic anion transporting polypeptide 1B3, NADPH: reduced nicotinamide adenine dinucleotide phosphate, UGT1A1: UDP-glucuronosyltransferase 1A1, MRP2: multidrug resistance-associated protein 2, MRP3: multidrug resistance-associated protein 3, BCRP: breast cancer resistance protein, SVM: support vector machines,

SMO: sequential minimal optimization, RF: Random Forest, MCC: Mathews correlation coefficient, ROC area: receiver operating characteristic area, AUC: area under the curve

1. Introduction

Hyperbilirubinemia is a pathological condition of excessive accumulation of conjugated or unconjugated bilirubin, an endogenous substance that is generated as result of heme catabolism (Billing and Black, 1969; Templeton et al., 2014; Tenhunen et al., 1968), in blood (Chang et al., 2013). Bilirubin is able to cross the blood brain barrier, thus hyperbilirubinemia has been associated with neurotoxicity. This is a special problem for the more susceptible newborn infants, since it affects the uptake of neurotransmitters (Dennerly et al., 2001; Fujiwara et al., 2010; Hansen, 2001; Tiribelli and Ostrow, 2005). Moreover, it is connected to non-neural organ dysfunctions, since it affects processes of protein/peptide phosphorylation also at other tissues apart from brain (Chang et al., 2013; Hansen, 2001). Furthermore, it can be regarded as a warning sign of potential side-effects of drugs, since it is accompanying severe liver conditions like hepatocellular drug induced liver injury (Bjornsson, 2014; Leise et al., 2014; Navarro and Senior, 2006; Ozer et al., 2008) and cholestasis (Padda et al., 2011; Pauli-Magnus and Meier, 2006). The reasons causing hyperbilirubinemia can be divided into two classes: i) increased production or ii) inadequate elimination of bilirubin. The latter has to do with inhibition or down-regulation of the transporting/metabolizing processes inside the hepatocyte and can be attributed to either genetical diseases or xenobiotics. The primary transporters/enzymes implicated in the cycle of bilirubin - OATP1B1/1B3 (uptake into the hepatocyte), UGT1A1 (glucuronidation), MRP2/BCRP (transport into bile for elimination) - are all of vital importance for the normal elimination of bilirubin (Campbell et al., 2004; Chang et al., 2013; Fevery, 2008; Keppler, 2014; Sticova and Jirsa, 2013; Templeton et al., 2014). Moreover, proper function of MRP3 serves as an extra measure of precaution against the pooling of bilirubin inside the hepatocyte (Cherrington et al., 2002; Keppler, 2014; Ogawa et al., 2000; Sticova and Jirsa, 2013). The bile salt export pump (BSEP), an ABC-transporter residing on the basolateral membrane of the hepatocyte, responsible for the excretion of bile salts into bile, is not directly affecting bilirubin elimination (Kullak-Ublick et al., 2004). However, potential inhibition of BSEP results in accumulation of bile salts that can cause cholestatic diseases, which are accompanied by high levels of bilirubin (Chang et al., 2013).

Furthermore, two hereditary diseases causing conjugated hyperbilirubinemia have been described. One of them is Dubin-Johnson syndrome, described in 1954 by Dubin et al. (Dubin and Johnson, 1954) and Sprinz et al. (Sprinz and Nelson, 1954) that is characterized by mild hyperbilirubinemia, due to absence

or deficiency of MRP2 (Toh 1999, Sticova 2013, Keppler 2014). Rotor syndrome, described in 1948 by Rotor et al. (Rotor et al., 1948), is also characterized by mild hyperbilirubinemia and it is attributed to total or partial lack of OATP1B1 and 1B3 expression (Keppler, 2014; Sticova and Jirsa, 2013; van de Steeg et al., 2012).

Regarding drug-induced hyperbilirubinemia, it is well known that hepatic transporters have a wide – and often overlapping- range of substrates and inhibitors, including both endogenous and exogenous substances (Faber et al., 2003; Hagenbuch and Stieger, 2013; Roth et al., 2011). Thus, it is quite likely that drug-like compounds interact with selected liver transporters, which would potentially result in hyperbilirubinemia. A recent study by Chang et al. (Chang et al., 2013) tried to evaluate the in vitro inhibition of drug transporters to identify drugs that cause hyperbilirubinemia by testing 6 known drugs (atazanavir, indinavir, ritonavir, nelfinavir, bromfenac, troglitazone, trovafloxacin) in vitro. Moreover, Liu and colleagues (Liu et al., 2011) included hyperbilirubinemia among the 13 hepatotoxic side effects (or HepSEs) as reference point to further predict drug-induced liver injury. During the study, a first attempt was made to model hyperbilirubinemia. However, the generated model was not extensively described or validated, since this was not the aim of that particular study.

Within the current study we aim at generating classification models for hyperbilirubinemia using both data from human toxicity reports as well as preclinical repeated dose studies in rat. Furthermore, we investigate if the inclusion of predicted OATP1B1 and 1B3 inhibition, as additional information, improves hyperbilirubinemia predictions.

2. Methods

2.1 Human data

The human data used in this work originate from the above publication of Liu (Liu et al., 2011). In their study, Liu et al. compiled several datasets for hepatotoxicity, including one dataset from SIDER (Kuhn et al., 2010)(<http://sideeffects.embl.de/>). This dataset consists of 888 compounds for 13 hepatotoxicity endpoints, among them also hyperbilirubinemia. We carefully curated the compounds according to the following rules:

- Inorganic compounds, salt parts, as well as compounds containing metals and rare or special atoms were removed and the chemotypes were standardized using the Standardiser tool created by Francis Atkinson (Atkinson, 2014).

- Duplicates and permanently charged compounds were removed using MOE 2014.09 (2015).
- 3D structures were generated using CORINA (version 3.4) (Sadowski et al., 1994), and their energy was minimized with MOE 2014.09, using default settings, but changing the gradient to 0.05 RMS kcal/mol/Å². In addition, the existing chirality was preserved.

After data curation 835 compounds (86 positives and 749 negatives) annotated for hyperbilirubinemia remained in the dataset. The compiled dataset is provided in the supplementary material as a csv file.

2.2 Animal data

Using Vitic Nexus 2.6 (Lhasa Limited), the eTOX 2015.1.0 database (Briggs et al., 2015) was reviewed and a number of compounds were examined for hyperbilirubinemia. The final dataset extracted, having the highest confidence regarding the compounds' class for hyperbilirubinemia, comprised 214 compounds (55 positives and 159 negatives).

In preclinical studies an increase of bilirubin is detected within the clinical chemistry examination. All compounds were classified to be positive showing a significant and treatment related increase of total bilirubin in the plasma of the tested species. Included species are predominately rodents such as rat and mice as well as studies with rabbits and dogs. All available study types were included, with the majority of studies being subacute with treatment periods of about 4 weeks. Findings reported at end of treatment were considered. Effects arising only in the recovery period were not included. Negative compounds did not show a bilirubin increase at any time point in any reported study, up to the reported highest tested dose.

The datasets above were the final ones used for descriptors calculation and modeling. Unfortunately, for confidentiality issues, we were not able to disclose this dataset in the supplementary material.

2.3 Generation of statistical models

2.3.1 Algorithms used

Classification models were built using the software package WEKA (Hall et al., 2009)(version 3.7.12). For both datasets logistic regression, tree methods (Random Forest and J48 tree), Support Vector Machines (SMO in WEKA with polynomial, RBF and Puk kernels), Naïve Bayes, and k-nearest neighbors were explored. Among meta-classifiers, we used MetaCost (Pedro, 1999) in order to artificially equilibrate our imbalanced datasets, since the ratio negatives:positives was approximately 3:1 for the animal dataset, and 8:1 for the human dataset, respectively. Additionally, we used ThresholdSelector in order to obtain

the optimal threshold for the classification, as well as attribute selection methods in order to select the most important descriptors and to evaluate their importance.

2.3.2 Molecular descriptors

For both datasets, several types of molecular descriptors have been calculated, such as all 2D MOE descriptors and the 3D volsurf series of descriptors (2015), PaDEL 2D and 3D descriptors (Yap, 2010) and several series of ECFPs (extended connectivity fingerprints; ECFP4 and ECFP6), using RDKit (<http://www.rdkit.org/>) (Landrum). Moreover, due to the potential association of OATP1B1 and OATP1B3 inhibition with hyperbilirubinemia, for both datasets, the predictions of OATP1B1 and 1B3 inhibition were calculated in WEKA based on the models published in a previous study (Kotsampasakou et al., 2015). Iteratively we tried out for each transporter: a) the consensus binary score out of six models (if a compound predicted as inhibitor for at least 3 classification models, it is considered as inhibitor), b) the sum of the binary scores (0 or 1, thus the sum score ranges between 0 and 6), and c) the sum of the float scores obtained from each model (the probability that each compound is an inhibitor ranges between 0.000 and 1.000, before it is translated into a binary/class score). The transporters predictions for the public human dataset are also included in the supplementary material.

2.3.4 Model validation

Due to lack of data –especially for the positive class- no external dataset was held aside for validation. Instead, we preferred to validate our models via 10-fold cross validation. Then, for the optimal models, in order reassure higher variance in our test set inside cross validation, 50 iterations were performed with change of the cross-validation seed. Thus, for our results, apart from the performance of the model for the default cross-validation seed, we also report the average of the 50 iteration $\pm$ the standard deviation.

The evaluated statistical performance metrics comprised Accuracy, Sensitivity (True Positive Rate), Specificity, Mathews Correlation Coefficient (MCC), Receiver Operating Characteristic (ROC) Area, and Precision and Weighted Average Precision (due to the imbalanced datasets). Moreover, in order to evaluate whether the performance of the models increases by including the transporters information, we performed a two-sample t-test in R , comparing the performance of the models (for the 50 iterations).

Even though the two datasets have small overlap (only 10 compounds), we considered it non-appropriate trying to validate the human model with animal data and vice versa, because of the low

concordance between humans and animals for hepatotoxicity endpoints (Liu et al., 2011; Olson et al., 2000).

3. Results and Discussion

3.1 Hyperbilirubinemia model based on human data

3.1.1 Descriptors, settings and performance

After generating multiple models with several combinations of classifiers and descriptors for the human dataset, the best model was retrieved using ECFP6 fingerprints. As a measure of OATP inhibition, the float score that was retrieved as a sum of the 6 individual models of OATP1B1 and OATP1B3 inhibition (Kotsampasakou et al., 2015) was used. Nevertheless, it should be noted that both the consensus binary score and the sum of the individual binary scores gave similar results in terms of the statistical performance of the models.

Regarding the classification scheme, as meta-classifiers we used MetaCost (Pedro, 1999) with a cost matrix of [0.0, 1.0; 10.0, 0.0] in order to artificially balance the two classes as well as AttributeSelectedClassifier with SignificanceAttributeEval (Ahmad and Dey, 2005) as evaluator and Ranker as search method for assessing the descriptor's importance. As base classifier we implemented SMO (the support vector machine option in WEKA) using the RBF kernel, while the buildLogisticModels setting was set to True, in order to transform the prediction into probabilities between 0 and 1. Table 1 provides an overview on the parameters and settings for the best models.

Table 2 shows in detail the statistical performance of the human model. All in all, considering the complexity of the endpoint and the low number of positive instances, the general statistical performance is quite satisfactory (AUC = 0.68 as average for 50 iterations). This was irrespective whether OATP inhibition was used as descriptor or not. The only statistics metric that appears quite low is the precision on actives (<0.2). However, this seems an artifact of the imbalanced class-composition of the dataset, as the weighted average precision, which equilibrates the value according to the two classes' population, is on average 0.85. In an effort to compare the statistical performance of the models with and without the information of transporters' inhibition, a two sample t-test was performed. The high p-values obtained (see table 2) imply that the performance of the two models is practically identical.

3.1.2 Correlation between OATP1B1/1B3 inhibition and hyperbilirubinemia

In order to assess the descriptors importance we applied AttributeSelectedClassifier inside the classification scheme. As evaluator the Significance AttributeEval was used, which evaluates the importance of an attribute by computing the Probabilistic Significance as a two-way function: attribute-classes and classes-attribute association (Ahmad and Dey, 2005). As a search method Ranker was applied, which ranks the attributes according to the score given by the evaluator (between 1 and -1). According to the attribute evaluator, out of the 1026 attributes (1024 ECFP6 fingerprints + OATP1B1 and 1B3 Inhibition prediction), OATP1B3 Inhibition ranked second, and the 21st most important attribute was OATP1B1 Inhibition. This was perceived as an indication that OATP inhibition is an important attribute for predicting hyperbilirubinemia. Additionally, after transforming the OATP information into categorical form (if a compound is predicted as inhibitor by at least 3 or more models, then the compound is considered as inhibitor; otherwise it is considered as non-inhibitor) we performed a chi-squared test (Agresti, 1990) regarding the independence of the categorical variables of OATP1B1/1B3 inhibition (inhibitor/non-inhibitor) and hyperbilirubinemia (positive/negative) as implemented in R. The obtained p-values were > 0.05 (p-value = 0.1594 for 1B1 and 0.0975 for 1B3), suggesting that the two variables are independent. Thus, both a chi-squared test of independency, and the two sample t-test comparing the performance of the two models are contradictory to the descriptors' evaluation by the classifier. A possible explanation would be that in contrast to what is suggested by literature, indeed OATP inhibition is not extremely important for predicting hyperbilirubinemia in a manner that would enhance the models' performance. However, in comparison to the amount of information enclosed in each individual fingerprint, the transporter's prediction is evaluated as quite important, since the particular models were actually built upon a set of 6 or 11 physicochemical descriptors. Therefore, potentially they carry more "molecular information", which leads to their high ranking among the list of 1024 fingerprints. Nevertheless, they do not outperform the ensemble of all 1024 fingerprints taken together.

Theoretically, it would be rather interesting to assess the importance of other transporters' inhibition for the endpoint of hyperbilirubinemia, such as MRP2, which is also considered important in the hyperbilirubinemia circle. Unfortunately, for the time being this is not possible due to relative lack of data for generating a robust MRP2 inhibition model.

Table 1: Parameters used for the best models

Species	Descriptors	Classifier
Human	ECFP6 (1024 binary fingerprints) with or without OATP inhibition	MetaCost (cost matrix of [0.0, 1.0; 10.0, 0.0]) + AttributeSelectedClassifier (SignificanceAttributeEval + Ranker) + SMO (RBF kernel, buildLogisticModels:True, rest default)
Animals	2D MOE descriptors (92 descriptors selected) with or without OATP inhibition	MetaCost (cost matrix of [0.0, 1.0; 4.0, 0.0]) + ThresholdSelector (measure:FMeasure, evaluationMode: N-Fold cross validation, numXValFolds:5, rest default) + AttributeSelectedClassifier (SignificanceAttributeEval + Ranker) + J48 tree

Table 2. Human model's performance with and without the OATP inhibition information.

	Accuracy	Sensitivity	Specificity	MCC	AUC	Precision	Weighted Avg Precision
Without OATPs (default)	0.679	0.674	0.680	0.225	0.685	0.195	0.870
Without OATPs (Average 50 iterations $\pm$ sd)	0.684 $\pm$ 0.036	0.585 $\pm$ 0.037	0.693 $\pm$ 0.0167	0.179 $\pm$ 0.022	0.679 $\pm$ 0.013	0.180 $\pm$ 0.010	0.858 $\pm$ 0.005
With OATPs (default)	0.675	0.651	0.678	0.209	0.687	0.189	0.866
With OATPs (Average 50 iterations $\pm$ sd)	0.683 $\pm$ 0.014	0.585 $\pm$ 0.039	0.688 $\pm$ 0.048	0.180 $\pm$ 0.024	0.679 $\pm$ 0.013	0.180 $\pm$ 0.011	0.854 $\pm$ 0.026
p-values (2-sample t-test)	0.808	0.962	0.482	0.911	0.952	0.817	0.362

Table 3. Animal model's performance with and without the OATP inhibition information.

	Accuracy	Sensitivity	Specificity	MCC	AUC	Precision	Weighted Avg Precision
Without OATPs (default)	0.72	0.6	0.761	0.335	0.725	0.465	0.748
Without OATPs (Average 50 iterations $\pm$ sd)	0.713 $\pm$ 0.027	0.629 $\pm$ 0.053	0.742 $\pm$ 0.038	0.340 $\pm$ 0.052	0.700 $\pm$ 0.031	0.460 $\pm$ 0.037	0.751 $\pm$ 0.021
With OATPs (default)	0.752	0.709	0.767	0.435	0.758	0.513	0.789
With OATPs (Average 50 iterations $\pm$ sd)	0.712 $\pm$ 0.033	0.630 $\pm$ 0.051	0.743 $\pm$ 0.045	0.343 $\pm$ 0.050	0.698 $\pm$ 0.031	0.462 $\pm$ 0.039	0.751 $\pm$ 0.025
p-values (2-sample t-test)	0.910	0.895	0.881	0.753	0.879	0.943	0.993

3.2 Hyprbilirubinemia model based on animal data

3.2.1 Descriptors, settings and performance

A similar approach as applied to the human data was followed for retrieving classification models for hyperbilirubinemia for the animal data. A full description of the best model obtained is outlined in Table 1. This time the best results were obtained using a preselected dataset of 92 2D MOE (2015) descriptors (the full list of the 92 used descriptors is provided in the supplementary material). As a measure of OATP inhibition the float score that was retrieved as a sum of the 6 individual models for each transporter (Kotsampasakou et al., 2015) was used. Also in this case the performance of the consensus binary score and the sum of binary scores was equal.

For balancing the dataset, MetaCost (Pedro, 1999) with a cost matrix of [0.0, 1.0; 4.0, 0.0] was applied. Moreover, ThresholdSelector (measure:FMeasure, evaluationMode: N-Fold cross validation, numXValFolds:5, rest default), which was tuned according to FMeasure (which is a measure of a model's accuracy, considering both precision and sensitivity)(Powers, 2011). In this case the optimization of the threshold was necessary, since the obtained probabilities for several classifiers, among them also decision trees, is not 0.5 (Niculescu-Mizil and Caruana, 2005; Zadrozny and Elkan, 2001). Finally, AttributeSelectedClassifier using SignificanceAttributeEval (Ahmad and Dey, 2005) as evaluator and Ranker as search method served for assessing the descriptors' importance. Among the base classifiers used, both Random Forest and J48 tree gave comparable results. Thus, we chose to use a single J48 tree due to its better interpretability in comparison to the more complex RF.

Similarly to the human data, the model's performance for the animal data was quite satisfactory for all statistics metrics (for details see Table 3). Here, it needs to be mentioned that the initial modeling approach for a preliminary dataset of 195 compounds that had not receive thorough curation was more or less random (ROC area rarely exceeding 0.5; data not shown). However, due to the subsequent careful curation by a toxicologist, and the use of several meta-classifiers to optimize the performance, we were able to obtain a final model with an improved accuracy and ROC area of 0.71 and 0.70, respectively. Of course, one should always keep in mind that the particular model was built upon a numerically restricted dataset, thus its applicability domain might be quite narrow.

As already observed for the human models, also in this case the statistical performance of the models with and without the information of transporters' inhibition is practically the same (Table 3). Moreover, the architecture of the trees with and without the use of OATP inhibition as attribute is quite similar. Comparing the two trees of Figure 1 (with the inclusion of OATP1B1/1B3 inhibition predictions) and Figure 2 (without) it reveals that the root and the upper branches of the two trees are exactly the same.

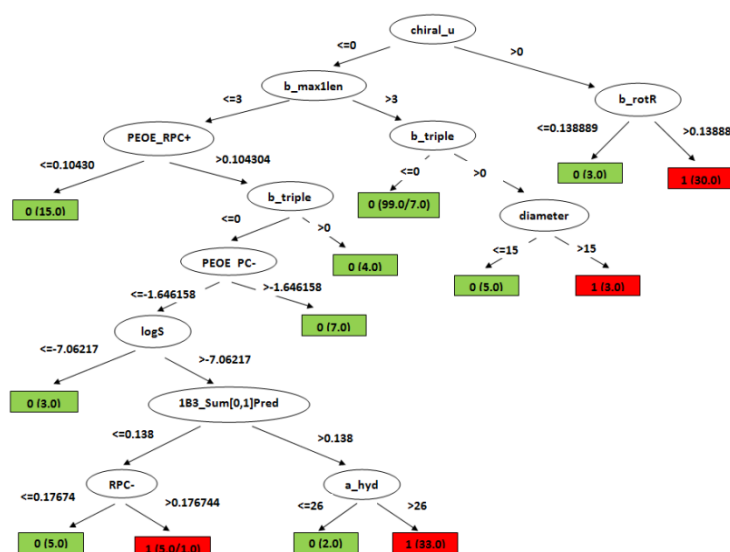


Figure 1. J48 tree for animal data with the inclusion of OATP1B1/1B3 inhibition predictions. The final leaves of the tree are colored in red for giving positives and green for negatives.

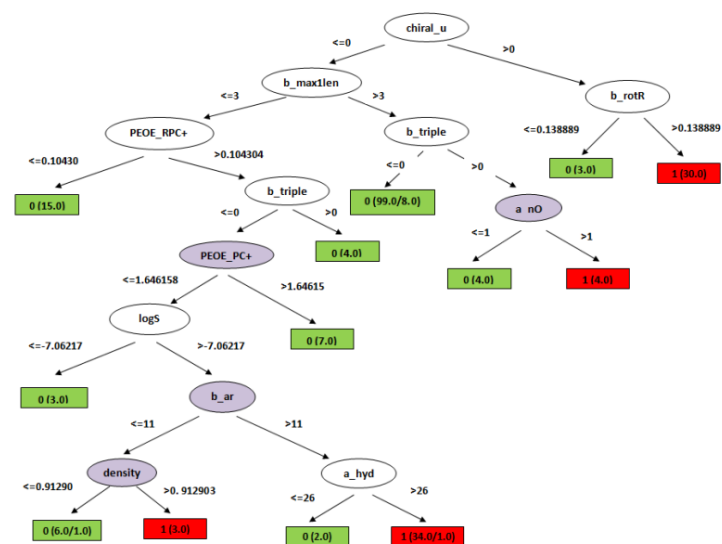


Figure 2. J48 tree for animal data without OATP1B1/1B3 inhibition predictions. The final leaves of the tree are colored in red for giving positives and green for negatives; light violet shows the branches that are different from Figure 1.

3.2.2 Descriptor's importance

Interestingly, the most important descriptors for the animal hyperbilirubinemia model were `chiral_u` (the number of unconstrained chiral centers) and `b_max1len` (maximum length of single bonds), as assessed both from the attribute evaluator and the ranking method, and also according to the tree architecture. Apart from that, statistical two-sample t-tests were performed, comparing the models' performance via the normal use of `chiral_u` and `b_max1len`, or with the values of `chiral_u` and `b_max1len` shuffled or missing. Both descriptors were evaluated as important, since the models' performance was decreasing and the p-values obtained were less than 0.05. Especially for the case of `chiral_u`, the model performance drops to random when it is missing or when its values are randomly shuffled. In addition, considering compounds having `chiral_u` > 0 as chiral and those having `chiral_u` = 0 as non chiral, we performed a chi-squared test regarding the independence of the categorical variables of chirality (chiral/non-chiral) and hyperbilirubinemia (positive/negative). The obtained p-value of $3.9 \cdot 10^{-12}$ suggests two highly dependent variables for the animal dataset. Strikingly, for the human dataset a p-value of 0.87 was obtained, thus showing independency. Having a closer look regarding the composition of the two datasets (human and animal) in terms of chiral compounds reveals, that for the human data for both hyperbilirubinemia classes (positives and negatives) the proportion of chiral compounds was equal and approximate to 60%, quite similar to the one reported for drugs in general (Leeson et al., 2010). Nevertheless, for the animal data 45% of the positives contain chiral centers (unconstrained), while the respective proportion among the negatives is only 5%. At this stage we cannot say if this large difference in the datasets' composition is a result of the different size of the datasets – the animal dataset is smaller, thus less representative –, or it is related to the fact that the two datasets are collected at different stages of the drug discovery and development process (preclinical compounds vs approved drugs).

3.2.3 Correlation between OATP1B1/1B3 inhibition and hyperbilirubinemia

For the animal data, OATP inhibition was not assessed by the attribute evaluator as being an important descriptor, which is in concordance to the results of the two sample t-test comparing the model's performance when OATP1B1/1B3 inhibition prediction is and is not used, respectively. Also a chi-squared test pointed towards independency of hyperbilirubinemia and OATP1B1/1B3 inhibition (p-values of 0.87 and 0.74 for 1B1 and 1B3 inhibition, respectively). Interestingly, even though OATP inhibition doesn't seem important for hyperbilirubinemia, it appears as a branch in the J48 classification

tree, though not in a prominent position. In addition, when OATP information is not used for model generation, it is replaced by the number of aromatic bonds at the same branch.

3.3 Association between OATP inhibition and hyperbilirubinemia - does it work?

All in all our results from both human and animal models do not indicate a strong association between OATP inhibition and hyperbilirubinemia. On the one hand, the human model suggests some sort of relationship, since OATP1B1 and especially OATP1B3 inhibition are evaluated as important descriptors from the Attribute Selection evaluator and ranker. However, this relationship was not strong enough to improve the performance of the model when the transporters' information is added to the structural information provided by the fingerprints. On the other hand, the animal model suggests even less strong relationship: OATP inhibition is not highly ranked among the descriptors – actually the assigned importance coefficient for both transporters inhibition is 0 - and the performance of the model is the same, regardless if transporters inhibition is used or not. Moreover, the tree architecture is very similar for both models and the existence of 1B3 inhibition as a brunch of the tree does not seem very important since it can be replaced by the number of aromatic bonds without pronounced differences in the model's predictions.

In case of the animal data, this lack of association between hyperbilirubinemia and transporter's inhibition is most probably due to the fact that the OATP inhibition models are based on human OATPs. Although in case of e.g. ABC-transporter mixing human with rat and mouse data seems appropriate due to their high sequence identity, for OATPs the situation is quite different. OATP1B1 and 1B3 in humans (sharing 80% sequence identity) are replaced by just one isoform (oatp1b2) in rats and mice, which are the prevalent species in the animal dataset. More particularly, according to a BLAST search (<http://blast.ncbi.nlm.nih.gov/>) (Altschul et al., 1990), the sequence identity of mouse oatp1b2 is only 66% and 64% for human OATP1B3 and 1B1, respectively. Rat oatp2b1, which shares 81% sequence identity with the respective mouse isoform, shows analogous values. Thus, it seems quite questionable if OATP inhibition data retrieved with human OATP1B1 and 1B3 can be transferred to mouse and rat oatp1b2. This is further supported by a recent publication (Huang et al., 2015), trying to model 10,000 chemical profiles for *in vivo* toxicity prediction and mechanism characterization. The authors found that models based on data from *in vitro* assays (human cell lines) were distinctly better in predicting human toxicity end points than to predict *in vivo* animal toxicity. Older studies (Martic-Kehl et al., 2012) also suggest that animal toxicity data only around half of the time successfully predict human outcomes.

Another reason that could attribute to the lack of association we found between OATP inhibition and hyperbilirubinemia are the technical difficulties (Templeton et al., 2014; Zhou et al., 2010) while measuring bilirubin, especially in its glucuronidated form:

- Bilirubin and its metabolites are unstable and photosensitive, thus prone to oxidation by reactive oxygen species.
- Bilirubin glucuronidation is a sequential reaction, which makes difficult to define the initial rate conditions or the authentic bilirubin glucuronides standards.
- Bilirubin is highly protein-bound (to albumin), so establishing its free fraction is practically hindered by its instability.

These difficulties can compromise the measurements' accuracy, both for the case of animal in vivo data and for the case of the human toxicity reports (which are apparently based in measuring the blood rates in humans).

Indeed, when inspecting the contingency tables 4a and 4b representing the relationship of positives and negatives for OATP1B1 /1B3 inhibition and hyperbilirubinemia, only around 1/3 of the OATP1B1 and 1B3 inhibitors are positives for hyperbilirubinemia. However, if some of the OATP1B inhibitors have actually been falsely measured as hyperbilirubinemia negatives, the landscape would change a lot, giving a higher correlation between OATP inhibition and hyperbilirubinemia.

Tables 4a and 4b: Contingency matrices presenting the number of a) OATP1B1 Inhibitors/Non Inhibitors vs positive and negative compounds for hyperbilirubinemia and b) OATP1B3 Inhibitors/Non Inhibitors vs positive and negative compounds for hyperbilirubinemia for animal data

Table 4a

	Hyperbilirubinemia negative	Hyperbilirubinemia positive	Total
OATP1B1 Inhibitor	53	17	70
OATP1B1 Non Inhibitor	106	38	144
Total	159	55	214

Table 4a

	Hyperbilirubinemia negative	Hyperbilirubinemia positive	Total
OATP1B3 Inhibitor	58	18	76
OATP1B3 Non Inhibitor	101	37	138
Total	159	55	214

Furthermore, even though OATP1B1 and OATP1B3 seem to be the most important transporters contributing to bilirubin uptake in the hepatocyte, there are more transporters/enzymes in the equation. There is strong evidence for the implication of MRP2 inhibition in hyperbilirubinemia (Sticova and Jirsa, 2013; Templeton et al., 2014). Nevertheless, as mentioned earlier, we are lacking data in order to develop a robust model for human MRP2 inhibition. The same applies for the enzyme UGT1A1. There are reports in the literature for its involvement in hyperbilirubinemia (Chang et al., 2013; Liu et al., 2011; Sticova and Jirsa, 2013; Templeton et al., 2014), but there are still not enough data available in order to generate a classification model for UGT1A1 inhibition. Finally, it should be noted that total impairment of both OATP1B1 and OATP1B3 doesn't seem to radically influence the physiological function of the liver. However, the fact that lack of both these transporters has an effect on the clearance of conjugated bilirubin might suggest the existence of one, or more, so far undetermined transporter(s) for unconjugated bilirubin, as suggested recently (Lin et al., 2015), which somehow might compensate the absence of OATP1B1 and 1B3.

Another possible mechanism of hyperbilirubinemia that is independent of OATP inhibition could be increased hemolysis/anemia, which would increase the amount of produced bilirubin (Feverly, 2008; Wickramasinghe and Wood, 2005). In order to check this hypothesis, we compared animal in vivo data for anemia with the respective ones for hyperbilirubinemia. More particularly, within the framework of the eTOX project, using Vitic Nexus 2.6 (Lhasa Limited), the eTOX 2015.1.0 database (Briggs et al., 2015) was reviewed and a dataset of 119 compounds positive for hemolytic anemia was retrieved (<http://www.lhasalimited.org/products/vitic-nexus.htm>). After thorough data curation, 115 compounds remained in the dataset. This dataset was compared to the respective one for hyperbilirubinemia, giving an overlap of 40 compounds (33 negatives and 7 positives for hyperbilirubinemia). Since the source for

both datasets was identical, the remaining 174 compounds of the hyperbilirubinemia dataset have to be considered as anemia negatives. The contingency matrix for the particular endpoints is shown in Table 5.

Table 5: Contingency matrix presenting the number of positive and negative compounds for anemia and hyperbilirubinemia, respectively.

	Hyperbilirubinemia negative	Hyperbilirubinemia positive	Total
Anemia negative	126	48	174
Anemia positive	33	7	40
Total	159	55	214

A chi-squared test of independence for the two categorical variables retrieved a p-value of 0.25, which indicates that the two variables are independent. Thus, at least for the particular dataset, anemia did not seem to be associated with hyperbilirubinemia.

Finally, with respect to the toxicity reports approach for the human data, the toxicity reporting system includes several drawbacks. First of all, it is done on voluntary basis (Chen et al., 2008; Hauben, 2004; Zhu and Kruhlak, 2014), and thus depends on the initiative of the patient and his/her physician. Moreover, most of the organizations gathering this information (e.g. FDA) do not require causal relationship between the drug and the side-effect (Zhu and Kruhlak, 2014). The contemporary phenomenon of polypharmacology is also not taken into account: a patient who expresses a particular side effect might be under the administration of 3 or more drugs concomitantly. Especially in elderly people this is quite often the case. All the particular drugs that the patient receives will be reported for this side effect. Since no causal effect is required, no discrimination will be done regarding which one(s) caused the particular side effect. As a result, all these ambiguities can introduce a considerable noise to the toxicity reporting system, which might compromise *in silico* modeling approaches.

4. Conclusions

Uncontrolled hyperbilirubinemia can be threatening for the human body, while it could also be a marker of underlying liver disease. The correlation between hyperbilirubinemia and liver transporters' inhibition has been widely discussed through literature, suggesting a positive association. In this study we present classification models for human and animal hyperbilirubinemia, which are based on carefully curated data. Our final hyperbilirubinemia models present a performance of 68% accuracy and area under the curve (AUC) for human data and 71% accuracy and 70% AUC for animal data, which is quite satisfactory considering the complexity of the endpoint and the lack of positives. Moreover, we investigated the potential association of OATP1B1 and 1B3 inhibition with hyperbilirubinemia. However, no significant correlation between hyperbilirubinemia and OATP inhibition was observed. This could be due to the wide variety of mechanisms implicated in hyperbilirubinemia, and potentially also due to the lack of positives in both datasets. Thus, to fully exploit a systems-based toxicology approach for hyperbilirubinemia, inclusion of additional transporters such as MRP2 as well as enzymes (UGT1A1) will be needed.

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Chapter 6

Classification of Cholestasis

Predicting drug-induced cholestasis with the help of hepatic transporters – an *in silico* modeling approach

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In the current paper we report the development of an *in silico* classification model for cholestasis. For the development of the model we compiled positives for cholestasis from several public sources and as negatives we used the negative compounds for DILI according to the procedure described in chapter 4. Moreover, we tried to use hepatic transporters' interaction profiles (BSEP, BCRP, P-gp, OATP1B1 and OATP1B3), in combination with physicochemical descriptors, in order to generate the classification model. This time, liver transporters' inhibition predictions contribute significantly in the prediction of DILI as their inclusion in the set of descriptors improves the statistical performance of the model. Interestingly, the increase in the performance is not directly matched to one particular transporter, but, as we show, it is a rather synergistic effect. The obtained model has been validated via 10-fold cross validation and on the basis of an external test set.

E. Kotsampasakou has compiled and curated the training and test set, calculated the transporters' inhibition predictions, generated the models, made the statistical analysis and wrote the manuscript. G.F. Ecker supervised the conducted work, reviewed the manuscript and contributed to writing.

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Predicting drug-induced cholestasis with the help of hepatic transporters – an in silico modeling approach

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KEYWORDS

cholestasis, DILI, drug-induced liver injury, IBk, k-nearest neighbors, machine learning, 2-class classification, liver transporters, BSEP, BCRP, P-glycoprotein, OATP1B1, OATP1B3, data curation

ABSTRACT

Cholestasis represents one out of three types of drug induced liver injury (DILI), which comprises a major challenge in drug development. In this study we applied a two-class classification scheme based on k-nearest neighbors in order to predict cholestasis, using a set of 93 2D physicochemical descriptors and predictions of selected hepatic transporters' inhibition (BSEP, BCRP, P-gp, OATP1B1 and OATP1B3). In order to assess the potential contribution of

transporter inhibition, we compared whether the inclusion of the transporters' inhibition predictions contributes to a significant increase in model performance in comparison to the plain use of the 93 2D physicochemical descriptors. Our findings were in agreement with literature findings, indicating a contribution not only from BSEP inhibition, but a rather synergistic effect deriving from the whole set of transporters. The final optimal model was validated via both 10-fold cross validation and external validation. It performs quite satisfactory resulting in 0.688 ± 0.011 for accuracy and 0.727 ± 0.014 for AUC for 10-fold cross-validation (mean $\pm$ standard deviation from 50 iterations).

Introduction

Drug induced liver injury (DILI) is a major issue worldwide, both for patients and health providers.^{1, 2} It is one of the primary causes for attrition during clinical and pre-clinical studies and the main reason for drug withdrawal from the market.³⁻⁶ DILI is divided into a: i) hepatocellular, ii) cholestatic or iii) mixed (hepatocellular and cholestatic) type, according to the type of liver damage and the clinical chemistry biomarkers alterations.⁷ The cholestatic and mixed hepatocellular and cholestatic type are the two most severe manifestations of DILI and yield almost half of the recorded cases of DILI.^{8,9}

Cholestatic liver injury, or more simply cholestasis, is the disruption of the bile flow, which might be either due to biliary tract obstruction or to complications in bile acid uptake. While the mechanistic basis for hepatocellular DILI is still a mystery for the majority of the cases, more knowledge exists for cholestatic DILI. There is growing evidence for a vast amount of

cholestasis cases pinpointing the important role of hepatic transporters.¹⁰ Hepatic transporters are classified into basolateral and canalicular ones. Basolateral transporters are responsible for the uptake of drugs and other endobiotics and xenobiotics from the blood, influencing the exposure of the hepatocyte to potential damage. Canalicular transporters regulate the hepatic clearance, as well as the secretion of bile salts and bile conjugates into bile.¹⁰⁻¹⁵ Any disturbance of the transporters' physiological function may result in the accumulation of potentially harmful bile products that can finally cause cholestasis.¹⁰ Figure 1 provides an overview on the respective location of hepatocyte transporters.

Several transporters' malfunction has been associated with cholestasis. The most important one, due to its pivotal role in bile salts clearance, is the bile salt export pump (BSEP).^{8, 10, 16-21} Apart from BSEP, there is evidence for the implication of other canalicular efflux transporters such as the multidrug resistance-associated protein 2 (MRP2),^{8-10, 22} breast cancer resistance protein (BCRP),⁸⁻¹⁰ multi-drug resistance protein 3 (MDR3)^{8, 10} and P-glycoprotein (P-gp)⁸⁻¹⁰. MDR3 functions as an ATP-dependent phospholipid flippase, translocating phosphatidylcholine from the inner to the outer canalicular membrane. Canalicular phospholipids are then solubilized by canalicular bile salts to form mixed micelles, protecting cholangiocytes from the detergent properties of bile salts. While P-gp is also not transporting bile salts, it is implicated in cholestasis because of its large amount of substrates and inhibitors which cause drug-drug interactions that disrupt the smooth function of the hepatocyte.¹⁰ The basolateral transporters play also an important role; both the uptake transporters, such as organic anion transporting polypeptides 1B1, 1B3 and 2B1 (OATP1B1, 1B3 and 2B1)⁸⁻¹⁰ and sodium (Na⁺) taurocolate co-transporter (NTCP),^{8-10, 23, 24} and the efflux transporters, like multidrug resistance-associated protein 3⁸⁻¹⁰ and 4^{8-10, 20} (MRP3 and MRP4). In particular, in cases of cholestasis, the basolateral

uptake transporters NTCP and OATP1B1 have been found down-regulated.^{10, 11} However, in this case, OATP1B3 is up-regulated as a compensatory mechanism for the elimination of xenobiotics from sinusoidal blood.^{10, 25, 26} On the contrary, in cases of cholestasis, MRP3 and MRP4 are up-regulated to facilitate the efflux of the toxic bile salts out of the hepatocyte.²⁷ Thus, simultaneous inhibition of several of these transporters could induce drug toxicity due to inadequate elimination from the blood or increase the cholestatic effect due to accumulation of bile salts in the hepatocyte.

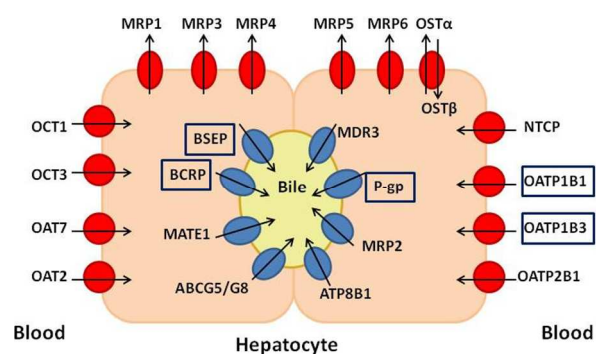


Figure 1: Transporters located on the hepatocyte. Blue symbols represent mainly the canalicular transporters and red symbols the basolateral ones. The arrows define the direction of transport. The transporters used in this study are presented within rectangular frames. The arrows show the direction of transport. MRP1-6: multidrug resistance-associated protein 1-6, OST α /OST β : organic solute transporter, BSEP: bile salt export pump, BCRP: breast cancer resistance protein, MATE1: multidrug and toxin extrusion transporter 1, ABCG5/G8: ATP-binding cassette sub-family G member 5/8, MDR3: multi-drug resistance protein 3, P-gp: P-glycoprotein, ATP8B1: ATPase-aminophospholipid transporter, OATP: organic anion transporting polypeptide, NTCP: sodium (Na^+) taurocolate co-transporting polypeptide, OCT: organic cation transporter 1, OAT: organic anion transporter.

Consequently, drug-induced liver injury and cholestasis are important toxicity alerts to be considered in drug development. Interestingly, there are only a few computational studies for the prediction of cholestasis reported in literature.^{28, 29} With respect to the involvement of hepatic transporter, there are some *in vitro* studies correlating cholestasis with transporter inhibition, such as BSEP^{17, 18, 30} MRP3 and MRP4²⁰ and NTCP³¹. Also several *in silico* studies for the identification of potentially cholestatic compounds via modeling of transporters and then associating them with the cholestatic effect of their inhibitors have been conducted. A characteristic example is the study by Greupink et al. in 2012, who developed a pharmacophore approach for NTCP²⁴ in order to identify potentially NTCP inhibitors. Under the same principles, in 2014 Ritschel and colleagues performed a 3D ligand-based pharmacophore model for BSEP inhibition. However, in most of these cases the amount of validated drugs is small and what is basically described is the association between transporter inhibition and cholestasis. Thus, as the respective is associated with cholestasis, it is assumed that an inhibitor is causing cholestasis. Most recently, Muller et al.³², in order to model DILI, also modeled some more hepatotoxicity endpoints, including cholestasis. Moreover, Mulliner et al.³³ presented a multilevel modeling approach for DILI, where cholestasis was also included as a morphological hepatobiliary finding. However, examining the liver transporters contribution was not within the scope of their work.

In this study we present a classification scheme in order to predict cholestasis from a public dataset, using physicochemical descriptors as well as predicted transporter inhibition profiles. For the latter we used our in house classification models for BSEP,³⁴ BCRP, P-gp,³⁵ OATP1B1 and OATP1B3³⁶.

Methods

Data Compilation

Training Set

For compiling the DILI training dataset we searched in PubMed (<http://www.ncbi.nlm.nih.gov/pubmed>),³⁷ Google,³⁸ Scopus (<https://www.scopus.com/>)³⁹ and the SIDER database v2^{40, 41} using the search terms: “drug-induced cholestasis” or “cholestasis”. The retrieved publications were then investigated manually for data, i.e. compounds that are positive or negative for drug-induced cholestasis. Unfortunately, cholestasis is an endpoint that is not widely examined in terms of experimental or *in silico* studies that would potentially guide us to big datasets. Thus, even though we were able to compile several drugs positive for cholestasis, there was almost no information in terms of the negatives. On the other hand, DILI in general is studied quite extensively and there are several respective datasets. Since cholestasis is a possible manifestation for DILI, we can consider safely that any compound negative for DILI will definitely be also negative for cholestasis. Thus, the negative compounds for DILI that we had compiled and curated in a previous work⁴² (just submitted in Chem Res Toxicol) were also used as negatives for this study. The dataset was carefully curated according to the following rules:

- All inorganic compounds were removed based on their chemical formula in MOE 2014.09.⁴³
- Salt parts and compounds containing metals and/or rare or special atoms were removed and the chemical structures were standardized using the Standardiser tool created by Francis Atkinson⁴⁴.
- Duplicates and permanently charged compounds were removed using MOE 2014.09⁴³.

- 3D structures were generated using CORINA (version 3.4)⁴⁵, and their energy was minimized with MOE 2014.09⁴³, using default settings, but changing the gradient to 0.05 RMS kcal/mol/Å². In addition, the existing chirality was preserved.

After these curation steps 154 compounds remained as positives for cholestasis. The negatives for DILI, and subsequently for cholestasis, were 468 compounds. However, when uniting the data, there were 38 compounds with contradictory class assignments. These compounds were removed from the dataset, yielding a dataset of in total 584 compounds (135 positives and 449 negatives). The compiled dataset is provided in the supporting information.

External test set

Very recently – and after having already compiled our training set for cholestasis and developed the respective model - a dataset covering multiple levels of hepatotoxicity was published by Mulliner and coworkers.³³ The data are hierarchically clustered by the authors into three levels of hepatotoxicity: level 0 corresponds to general hepatotoxicity, level 1 corresponds to clinical chemistry findings and morphological finding as distinguished parts of general hepatotoxicity and level 2 discriminates both clinical chemistry and morphological findings into hepatocellular and hepatobiliary injury. We use the data of morphological findings for hepatobiliary injury as an external test set for validating the developed cholestasis model. Once more, we performed chemical data curation and removed the compounds overlapping with the training set, which led to 1512 compounds (267 positives and 1245 negatives) as external test set.

Generation of statistical models

Molecular descriptors

For both datasets, several types of molecular descriptors have been calculated, such as all 93 2D MOE descriptors, the 3D Volsurf series of descriptors³², as well as ECFPs (extended connectivity fingerprints; ECFP6), using RDKit (<http://www.rdkit.org/>)⁴⁶. In addition to this, predicted hepatic transporter inhibition profiles were also included in the list of descriptors. The transporters investigated comprise BSEP, P-gp, BCRP, OATP1B1 and OATP1B3.

In particular, for basolateral transporters we calculated the predictions for 4 *in silico* classification models built upon PaDEL descriptors⁴⁷ for OATP1B1 and OATP1B3³⁶ inhibition. For obtaining the predictions we use the models' version implemented in eTOXlab⁴⁸, an open source modeling framework for implementing predictive models. Out of each model we got a binary result: positive or negative. For each transporter we use the sum of these binary scores, denoted "Sum binary score". The Sum binary score can take values between 0 (if all models predict the compound as negative) and 4 (if all models predict the compound as positive). For basolateral transporters, we used the continuous score obtained by the BSEP³⁴ inhibition prediction model. Float prediction-scores were also retrieved for P-glycoprotein³⁵ and BCRP³⁵ inhibition.

Algorithms used

The 2-class classification models were built using the software package WEKA (version 3.7.12).⁴⁹ We investigated the performance of several base classifiers, such as logistic regression, tree methods (Random Forest and J48 tree), Support Vector Machines (SMO in WEKA with polynomial, RBF and Puk kernels), Naïve Bayes, and k-nearest neighbors. Moreover, because the dataset is slightly imbalanced, in order to equilibrate the effect of the majority class on model

performance, we also applied the cost-sensitive meta-classifier MetaCost.⁵⁰ The cost matrix applied corresponds to the imbalance ratio of the data (3:1). Additionally, several meta-classifiers were explored for attribute selection (AttributeSelectedClassifier), as well as for improving the statistical performance, such as Bagging⁵¹ and Boosting^{52, 53}.

Model validation

The models were originally validated via 10-fold cross validation, which is considered a quite trustworthy method of validation.⁵⁴ The best models – according to 10-fold cross-validation evaluation - were further validated via using the dataset by Mulliner.³³ Subsequently, for the best obtained models, 50 iterations were performed by changing the cross-validation seed (for splitting the data within cross validation) and the respective performance parameters were calculated. In order to compare whether the inclusion of the transporters predictions in the descriptors set improves significantly the model's performance, a two-sample t-test was performed in R.⁵⁵

The statistics metrics taken into consideration were accuracy, sensitivity, specificity, Matthews Correlation Coefficient (MCC), area under the curve (AUC), precision and weighted average precision. Weighted average precision is the average precision obtained for the two classes but weighted from the total number of instances of the classes.⁴⁹ It is a quite helpful parameter in multi-class classification problems, as well as for imbalanced datasets where the number of negatives is greater than the number of positives. Especially for the latter case, due to the definition of precision [$PPV = TP / (TP + FP)$], its value for the positive class would be low, which not necessarily means that the total performance of the model is bad.

Results and Discussion

Generation of a cholestasis classification model

Several combinations of descriptors and classifiers were investigated and the optimal classification model was selected on basis of the results of 10-fold cross validation. With respect to the classifier, the best results were obtained using as base classifier IBk – the k nearest neighbors implementation in WEKA - with k=5. The meta-classifier MetaCost was also applied, with the application of the cost matrix [0.0, 1.0; 3.0, 0.0], i.e. weighting the minority class 3 times more than the majority class, in order to cope with the slightly imbalanced training set. 2D MOE descriptors were performing better than fingerprints and/or VolSurf descriptors. Apart from the 2D descriptors, we also included the predicted transporter inhibition profiles. In order to assess the importance and significance of this additional information individually, we used them in different combinations: all transporters, only BSEP, all transporters excluding either BSEP, or P-gp, or BCRP, or the OATPs. This led to in total 7 models (Table 1).

Table 1. Performance of the model for MetaCost [0.0, 1.0; 3.0, 0.0] + IBk (k=5), changing the descriptors settings via including or excluding particular transporters.

Model Settings		Validation	Accuracy	Sensitivity	Specificity	MCC	AUC	Precision	Weighted Average Precision
93 2D MOE dscrs		10 CV	0.684	0.570	0.658	0.221	0.677	0.352	0.728
		Test set	0.619	0.588	0.626	0.166	0.637	0.252	0.766
93 2D MOE dscrs + all transporters pred.		10 CV	0.714	0.704	0.717	0.366	0.762	0.428	0.783
		Test set	0.581	0.648	0.566	0.164	0.638	0.243	0.769
93 2D MOE dscrs + BSEP pred.		10 CV	0.664	0.570	0.693	0.230	0.682	0.358	0.731
		Test set	0.634	0.543	0.654	0.155	0.634	0.252	0.761
93 2D MOE dscrs + all transporters pred. without BSEP		10 CV	0.680	0.704	0.673	0.322	0.738	0.393	0.770
		Test set	0.574	0.637	0.561	0.151	0.633	0.237	0.765
93 2D MOE dscrs + all transporters pred. without P-gp		10 CV	0.695	0.652	0.708	0.314	0.752	0.402	0.763
		Test set	0.585	0.622	0.578	0.152	0.629	0.240	0.764
93 2D MOE dscrs + all transporters pred. without BCRP		10 CV	0.712	0.704	0.715	0.363	0.769	0.426	0.782

Test set		0.574	0.633	0.561	0.148	0.619	0.236	0.764	
93	2D MOE dscrs + all transporters pred. without OATPs	10 CV	0.688	0.585	0.719	0.269	0.681	0.385	0.744
Test set		0.632	0.562	0.647	0.163	0.648	0.254	0.764	

Inspecting the obtained results in Table 1, it becomes obvious that the best settings for the model are achieved with the inclusion of all transporter inhibition predictions in the list of descriptors. Both for 10-fold cross validation, as well as for the external validation, including predicted inhibitor profiles for all transporters yields higher sensitivity values. As we are dealing with prediction of toxicity, this is the parameter we are most interested in. Interestingly, the use of BSEP inhibition prediction stand-alone doesn't seem to be sufficient. There is a drop in the statistics - especially for sensitivity - in comparison to the use of the whole set of transporter predictions.

Statistical analysis of transporter predictions on the model's performance

In order to assess if the predicted transporter inhibition profiles indeed statistically significantly improve the models, we performed 50 iterations of 10 fold cross validation followed by a two sample t-test on the performance parameters. For this we used the models with 2D MOE descriptors, 2D MOE + all transporters, 2D MOE plus BSEP, and 2D MOE plus all transporters without BSEP (Table 2).

Table 2. Mean standard deviation values obtained from 50 iterations of 10-fold cross-validation for the statistics metrics of accuracy, sensitivity, specificity, MCC, AUC, precision and weighted average precision

Model Settings		Accuracy	Sensitivity	Specificity	MCC	AUC	Precision	Weighted Average Precision
93 2D MOE dsers	mean	0.656	0.540	0.691	0.202	0.665	0.344	0.720
	sd	±0.011	±0.028	±0.016	±0.022	±0.012	±0.012	±0.008

93 2D MOE dsers + all transporters pred.	mean	0.688	0.642	0.702	0.299	0.727	0.393	0.758
	sd	±0.011	±0.030	±0.013	±0.027	±0.014	±0.015	±0.010
93 2D MOE dsers + BSEP pred.	mean	0.671	0.527	0.714	0.214	0.667	0.356	0.723
	sd	±0.011	±0.028	±0.014	±0.023	±0.013	±0.014	±0.009
93 2D MOE dsers + all transporters pred. without BSEP	mean	0.676	0.658	0.682	0.293	0.714	0.384	0.757
	sd	±0.011	±0.032	±0.013	±0.026	±0.012	±0.013	±0.010

Analyzing the p-values for the pair-wise comparisons (supplementary information Table S2) the main conclusion is that indeed the use of liver transporter inhibition predictions contributes significantly to the models performance when compared to the use of only 2D physicochemical descriptors. Interestingly, it is not only the BSEP inhibition contribution, which matters. Of course, BSEP is important, as for the majority of the statistics metrics the model containing also the BSEP prediction information performs better than the respective one with only 2D physicochemical descriptors. Moreover, when BSEP is removed from the descriptors set, only sensitivity seems improving; the rest of the statistics metrics are unaltered or significantly better for the whole transporters set. This suggests that BSEP apparently contains important information. Nevertheless, it does not contain all the important information. When all transporters are included, for all statistical parameters apart from specificity, the performance of the model is superior in comparison to the case when only BSEP inhibition predictions are used. This suggests that information on transporter inhibition contributes important information for

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3 predicting cholestasis. This information is not specifically attributed to BSEP, which is the most
4 widely discussed transporter in literature with respect to cholestasis.^{8, 10, 16-21} The other
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6 transporters included in our study are in general not well described in literature via experimental
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8 procedures, but they are rather pinpointed due to the fact that they are transporting bile salts or
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10 bile conjugates (with the exception of P-gp, whose role is attributed mainly to drug-drug
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12 interactions). Thus, our study gives extra weight to literature indications concerning BCRP, P-gp,
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14 OATP1B1 and 1B3.
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20 Here we must note that there is also experimental evidence for the implication of the
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22 basolateral efflux transporters MRP3 and MRP4,^{8-10, 20} as well as for the canalicular efflux
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24 transporter MRP2.^{8-10, 22} We are aware of this fact, but for these transporters there are currently
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26 not sufficient data available to develop high quality models that can be further used for
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28 contributing to our cholestasis model. Additionally, we should pinpoint the fact that we are using
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30 predictions for the inhibition of transporters rather than real *in vitro* data. Nevertheless, the
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32 performance of the classification models from which we obtained the predictions provides us
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34 with enough confidence to use them in our input matrix.
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40 However, our final *in silico* models for cholestasis were extensively validated with 10-fold
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42 cross validation and statistical tests. Furthermore, the external validation set was of a significant
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44 size being even bigger than the training set. It is interesting to note that the external validation set
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46 had a contradiction rate of ~20% regarding the class labels of those compounds shared with the
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48 training set (49 out of 254 shared compounds had contradictory class labels). We assume that
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50 this is due to the drawbacks of the toxicity reporting system: under-reporting,⁵⁶⁻⁵⁸ voluntarily
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52 basis,⁵⁸⁻⁶⁰ difficulties to obtain the data, which are often proprietary,⁵⁶ as well as the lack of the
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54 prerequisite of a causal relationship between drug-adverse event.⁵⁸ In any case, despite these
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contradictions between the training and the test set, our model retained its satisfactory performance.

We had previously performed an analogous study for DILI.⁴² However, in that case the transporter inhibition predictions did not significantly improve the DILI model performance. This might be attributed to the fact that the mechanisms for development of DILI are wider, more general and incorporate several sub-mechanisms, where transporter inhibition is only one portion. An important role is also played by idiosyncratic^{61, 62} or immune^{63, 64} reactions, protein alkylation,^{62, 65} metabolizing enzymes inhibition,^{62, 64} depletion of glutathione,⁶⁴ generation of reactive metabolites,⁶²⁻⁶⁴ farnesoid X receptor (FXR) antagonism by non-steroidal anti-inflammatory drugs (NSAIDs)⁶⁶ and mitochondrial toxicity^{62, 63, 67}.

In contrast, cholestasis has a more specific mechanism of action, which is tightly dependent on the bile flow. Thus the transporting capacity of transporters having bile salts as substrates should be directly linked to this toxic endpoint. Furthermore, there is an active interplay between several transporters included in this study. This includes adjusting up- or down-regulation of their expression, as well as compensation mechanisms in case of inhibition of one specific transporter in order to reduce the respective damage.^{8, 10, 65} Consequently, inhibition of a group of transporters might have greater impact on the development of cholestatic DILI than just blockade of BSEP.

Conclusions

In this study we present a two-class classification model for the prediction of cholestasis (or cholestatic DILI) based on a public dataset of 584 compounds. The model is incorporating information both from 2D physicochemical descriptors, as well as predictions of inhibition of the

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3 hepatic transporters BSEP, BCRP, P-gp, OATP1B1 and OATP1B3. The performance of the
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5 resulting model is rather satisfactory and is validated both via 50 iterations of different 10-fold
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7 cross validations, as well as an external test set of over 1500 compounds.³³ Our results
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9 demonstrate that adding transporter predictions as additional descriptors to the list of 2D
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11 physicochemical descriptors is significantly improving model performance. This is in alignment
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13 with evidence from literature which shows that inhibition of selected hepatic transporters
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15 contributes to cholestasis.
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20 Interestingly, the increase in model performance cannot be attributed solely to BSEP
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22 inhibition, which is the transporter that is most correlated to cholestasis in literature. Although
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24 the performance of the model where BSEP predictions are used together with 2D descriptors is
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26 significantly better than when only the 2D descriptors are used, the performance increases even
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28 more when the whole list of transporter inhibition predictions is included. This result points
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30 towards a rather synergistic effect of several transporters, including the less elucidated role of
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32 OATPs, BCRP and P-gp in cholestasis.
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37 Our study is the first of its kind regarding combining physicochemical descriptors and
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39 predicted transporter information in order to predict cholestasis. This provides a useful extension
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41 to previous approaches for the prediction of cholestasis.
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ASSOCIATED CONTENT

Supporting Information

- pdf file: a list with the subset of 93 2D MOE descriptors (Table S1) and the p-values of the pair-wise statistical comparison of the models (Table S2) for 10-fold cross validation
- zip file: training set and external test set for cholestasis (chemical structures, compound names and descriptors) are provided in .csv and .sdf format

“This material is available free of charge via the Internet at <http://pubs.acs.org>.” For instructions on what should be included in the Supporting Information, as well as how to prepare this material for publication, refer to the journal’s Instructions for Authors.

Supporting Information for Review only

pdf file: Kotsampasakou, E.; Ecker, G. F., A simple 2-class classification model for drug-induced liver injury (DILI) – the importance of data curation. Manuscript submitted in Chem Res Toxicol, August 2016

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

AUC: area under the ROC curve, BCRP: breast cancer resistance protein, cpd(s): compound(s), DILI: drug-induced liver injury, dscr(s): descriptors, MCC: Matthews correlation coefficient, MDR3: multidrug resistance protein 3, MRP2: multidrug resistance-associated protein 2, MRP3: multidrug resistance-associated protein 3, OATP1B1: organic anion transporting polypeptide 1B1, OATP1B3: organic anion transporting polypeptide 1B3, P-gp: P-glycoprotein, transp: transporters

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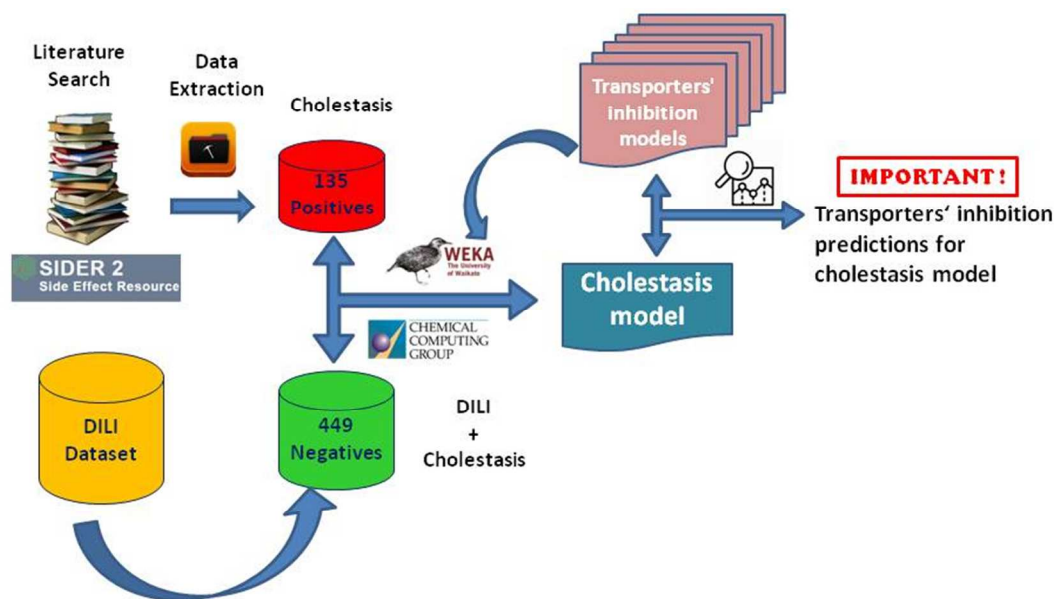
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Chapter 7

Case Studies-

Machine Learning Applications to Predict Hepatotoxicity Endpoints

7.1 A Case Study on eTOX Animal *in Vivo* Data – A Global Hepatotoxicity Model vs a 7-Endpoint Modeling Approach

Predicting drug-induced liver injury (DILI) for preclinical data: comparison of a single global hepatotoxicity model vs a 7-hepatotoxicity-endpoint ensemble modeling approach

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In the following paper we reported the development of 7 *in silico* classification models for 7 hepatotoxicity endpoints: 1) necrosis, 2) steatosis, 3) bile duct abnormalities, 4) preneoplastic effect, 5) inflammation as secondary effect, 6) hypertrophy and 7) glycogen decrease. Based on the predictions obtained from these models, we implemented a 7-endpoint ensemble modeling approach for predicting hepatotoxicity. Independently of the 7 endpoints' models, we also implemented a global hepatotoxicity model; for this model, the global hepatotoxicity class of the dataset was defined according to the true class labels of the compounds for the 7-hepatotoxicity endpoints. Finally, we compared the two

methods, on the basis of the performance, while we also comment on the applicability of the two approaches. The models were validated via 10-fold cross validation and via splitting the dataset into 80% training set and 20% test set.

E. Kotsampasakou curated the training set, generated the 80% training/ 20% test subsets, generated the models, made the statistical analysis and wrote the manuscript. A. Amberg contributed in defining the term clusters for eTOX data and provided advice on toxicological matters throughout the study. J. Funk contributed in defining the term clusters for eTOX data. Manuela Stolte, D. Mulinner and L. Anger contributed in the preparation and analysis of the data used for modeling. G.F. Ecker supervised the conducted *in silico* work and reviewed the manuscript.

Predicting drug-induced liver injury (DILI) for preclinical data: comparison of a single global hepatotoxicity model vs a 7-hepatotoxicity-endpoint ensemble modeling approach

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Abstract

DILI is a main challenge for drug development in the pharmaceutical industry as one of the main causes for attrition during clinical and pre-clinical studies. In this study we tried to predict DILI for preclinical data via a dual approach: first, a global single hepatotoxicity model and second, the development of 7 individual *in silico* classification models for 7 distinctive hepatotoxicity endpoints that would be used in a cooperative fashion to predict general hepatotoxicity. The modeling data were obtained from eTOX database. In total, 7 histopathological terms of similar mechanisms were extracted, yielding 764 compounds for rat data. In order to generate the global hepatotoxicity model, we considered a compound toxic if it was reported as positive for at least one hepatotoxicity endpoint. For the consensus approach, we developed 7 individual *in silico* classification models for 1) necrosis, 2) steatosis, 3) bile duct abnormalities, 4) preneoplastic effect, 5) inflammation as secondary effect, 6) hypertrophy and 7) glycogen decrease, applying the best performing classification scheme for each endpoint. To predict general hepatotoxicity (sensitivity vs specificity trade off) using the 7 individual classification models as an ensemble, the optimal threshold was the prerequisite of having positive predictions for more than one hepatotoxicity endpoints to consider the prediction positive for global hepatotoxicity. The models were validated via 10-fold cross validation, as well as by splitting the data into 80% for training and 20% for testing, repeating the procedure 10 times and calculating the average statistics. All models yield quite satisfactory results and the two approaches are equal for most statistics metrics. However the 7 consensus model approach gives marginally better (p-value = 0.0494) sensitivity of 0.68 vs 0.61 for the unique global hepatotoxicity model, while it also allows to trace back the potential mechanism of hepatotoxicity.

Keywords

Bile duct abnormalities, 2-class classification, consensus modeling, DILI, drug-induced liver injury, eTOX database, glycogen decrease, hepatotoxicity, hypertrophy, inflammation as a secondary effect, machine learning, necrosis, preneoplastic effect, rat, steatosis

Abbreviation list

AUC: area under the curve, BCRP: breast cancer resistance protein, BSEP: bile salt export pump, cpd(s): compound(s), DILI: drug-induced liver injury, MCC: Matthews correlation coefficient, MRP2: multidrug resistance-associated protein 2, RF: Random Forest, SMO: sequential minimal optimization, SVM: support vector machines,

Introduction

Drug-induced liver injury (DILI) or hepatotoxicity is a significant challenge for drug development in the pharmaceutical industry. It is considered one of the major causes for attrition during clinical and pre-clinical studies and the main reason for drug withdrawal from the market or labeling with a black box warning (Ballet 1997; Chen et al. 2011; O'Brien et al. 2006). Subsequently, there is a lot at stake. The financial cost for pharmaceutical industries during drug development procedure is very high and it is extremely costly a drug withdrawal towards the last stages of drug development or –even worse- when it is already in the market (Greener 2005). But most important, it is crucial for the safety and health of patients, since DILI is the most frequent event of liver failure and it is a potentially fatal adverse event (Weiler et al. 2015).

Thus, there is great effort from the pharmaceutical industry to predict a possible DILI side effect as early as possible during drug development. There are several *in vitro* tests and biomarkers for testing the potential risk of DILI (Schadt et al. 2015). Worthy to mention are glutathione (GSH) adduct formation (Sakatis et al. 2012), covalent binding to proteins (Nakayama et al. 2009), CYP3A time-dependent inhibition (Zimmerlin et al. 2011) and inhibition of bile salt export pump (BSEP) (Aleo et al. 2014; Dawson et al. 2011). Moreover, extremely important are mitochondrial (Aleo et al. 2014), lysosomal (Nadanaciva et al. 2011) and inflammatory (Cosgrove et al. 2009) effects, as well as cytotoxicity assays

on human (Schadt et al. 2015) and murine (Marroquin et al. 2007) hepatocytes. Actually, Thomson and coworkers have proposed a combination of assays for cytotoxicity (Thompson et al. 2012). Particularly, they suggest the use of a hazard matrix, based on covalent binding, in combination with an array of five *in vitro* assays, addressing cytotoxicity in different cell lines and inhibition of the canalicular transporters BSEP and multidrug resistance-associated protein 2 (MRP2), with individual cut-off values for each assay.

Nevertheless, despite the development of several *in vitro* approaches, still one of the most crucial screens against toxicity is –inevitably– testing on animals. The knowledge gained from preclinical models assists in the development of new mechanistic biomarkers and is critical for the interpretation of biomarker data in clinical samples (McGill and Jaeschke 2014). Furthermore, as we move towards more complex systems, like living organisms, interactions may occur that are not identified with *in vitro* models. Unfortunately, the animal testing is quite costly, and it is also accompanied by ethical dilemmas. All in all, the 3Rs principle (Replacement, Reduction, Refinement) is guiding the drug discovery procedure in terms of animal use. This attitude is a compromise between the costs (i.e. the harm on the animal) and benefits for the welfare of animals vs the costs and benefits for the welfare of humans (Graham and Prescott 2015). Of course, the total elimination of experiments on animals during the drug discovery procedure is kind of utopic, however there is more and more pressure for reducing them. Towards this direction play important role the development of *in silico* models. In general, in the area of *in silico* modeling, more effort is put towards developing computational models for human hepatotoxicity. It might seem trivial trying to generate models for lab animals; however it can be financially efficient having a first indication of toxicity before sacrificing animals on experiments. It can also have ethical impact.

To our knowledge, there are only two *in silico* approaches predicting animal toxicity. The first was a study by Fourches et al. who developed classification QSAR models with data obtained by text mining techniques (Fourches et al. 2010). A second one was published recently by Mulliner and colleagues, concerning the development of hepatotoxicity classification models using SVM on the basis of 3 levels: starting from general hepatotoxicity (level 0) to more particular endpoints (levels 1 and 2)(Mulliner et al. 2016). We should also note that both studies performed modeling also for human data of hepatotoxicity.

In this study, conducted under the framework of the eTOX project, we made use of compounds collected from eTOX database (Briggs et al. 2015) concerning rat data for seven terms of hepatotoxicity. Using these data, we developed 7 models for each one of the 7 hepatotoxicity endpoints and we used

them synergistically, as an ensemble modeling approach, to predict global hepatotoxicity. Moreover, we developed a single global hepatotoxicity model and compared the two approaches regarding their performance.

Methods

Datasets

Training set

Using Vitic Nexus 2.6 (Lhasa Limited), the eTOX 2015.1.0 database (Briggs et al. 2015) was reviewed for data extraction. The compounds of interest concern non-confidential *in vivo* data from rats treated orally for maximum 4 weeks. Originally, all treatment-related – i.e. the observed effect was consequence of the administered drug- standardized data, with histopathological terms related to liver were extracted yielding a sum of 78 terms with at least one treatment-related finding for liver. The different histopathology terms were initially grouped into 9 clusters of similar mechanisms with sufficient compounds to allow model development. The idea behind this cluster-merge was that there should be a sufficient number of compounds (both positives and negatives) in each cluster to allow modeling.

The initial data consisted of continuous and NA values that were further transformed into binary form (0/negative and 1/positive) appropriate for classification. All the NA values, indicating that the particular effect was not inspected, were transformed into 0/negatives. All the continuous numbers, referring to lowest observed effect level (LOEL), i.e. the lowest dose an effect is observed, were transformed into 1. In general, the positives were a minority class for all term clusters. Term clusters with less than 40 positive compounds were excluded. Thus, further inspection of the data, led us to the reduction of term clusters from 9 to 7 because for some clusters the number of positive compounds was below the threshold. In total 793 compounds were collected for the 7 histopathological term clusters: 1) necrosis, 2) steatosis, 3) bile duct abnormalities, 4) preneoplastic effect, 5) inflammation as secondary effect, 6) hypertrophy, and 7) glycogen decrease. In supporting information are provided into a table (S1) the histopathological terms organized into 7 clusters of sufficient number of positives, having the same mechanism.

The dataset was carefully curated according to the following rules:

- All inorganic compounds are removed according to their chemical formula in MOE 2014.09.(Molecular Operating Environment (MOE) 2015)

- Salt parts and compounds containing metals and/or rare or special atoms were removed and the chemotypes were standardized – i.e. the same chemical representations are used for the same chemical structures- using the Standardiser tool (Atkinson 2014).
- Duplicates and permanently charged compounds were removed using MOE 2014.09 (Molecular Operating Environment (MOE) 2015).
- 3D structures were generated using CORINA (version 3.4) (Sadowski et al. 1994), and their energy was minimized with MOE 2014.09 (Molecular Operating Environment (MOE) 2015), using default settings, but changing the gradient to 0.05 RMS kcal/mol/Å². In addition, the existing chirality was preserved.

The curation procedure reduced the total dataset to 764 compounds. The distribution of the data for each term cluster/hepatotoxicity endpoint was various and it is presented in table 1. It must be noted that for all endpoints the dataset is imbalanced.

Table 1. Number of positives and negatives for each hepatotoxicity endpoint and for global hepatotoxicity for the total of 764 compounds.

N	Hepatotoxicity Endpoint	# Positives	# Negatives
1	Necrosis	86	678
2	Steatosis	97	668
3	Bile duct abnormalities	45	719
4	Preneoplastic effect	65	699
5	Inflammation as 2 nd effect	74	690
6	Hypertrophy	143	621
7	Glycogen decrease	46	718
8	Global Hepatotoxicity	198	556

Definition of the class for global hepatotoxicity

In order to generate the global hepatotoxicity model we had to define the class for global hepatotoxicity. Initially we considered that if a compound was positive for at least one hepatotoxicity endpoint, it should be considered as positive for hepatotoxicity. However, this class assignment originally led to models with poor to moderate performance. This, prompted us to inspect further the composition of the dataset. We realized that there were several compounds that were positive only for hypertrophy and none of the other hepatotoxicity endpoints. This was quite interesting, since hypertrophy, if it is not accompanied by any other morphological or clinical chemistry finding, is most probably an adaptive, reversible phenomenon, rather than an indication of hepatotoxicity (Hall et al.

2012). Thus, we considered as positive for global hepatotoxicity all compounds that are positive for at least one hepatotoxicity endpoint, unless this endpoint is only hypertrophy; in this case more than one hepatotoxicity endpoints should be positive to assign a positive label for hepatotoxicity on a compound. This approach summed up 198 positives and 556 negatives for general hepatotoxicity (Table 1).

External test set

In an earlier work, Mulliner and colleagues (Mulliner et al. 2016) had published a DILI dataset for both clinical and preclinical data. From the preclinical data, we used only those referred to a dose < 500 mg/kg, since our training sets includes also restrictions in terms of period of administration and dose. The same principles for data curation were applied, as for the training set, resulting in the compilation of 1509 compounds, 650 positives and 859 negatives for DILI. The compounds shared with the training set were further removed, resulting in 1490 compounds, 619 positives and 851 negatives for DILI. This dataset was used for external validation. The external test set is provided in the supporting information.

Generation of statistical models

Definition of the thresholds for predicting hepatotoxicity

I. A global hepatotoxicity model

According to the global hepatotoxicity class assignment, a single global hepatotoxicity classification model was created for distinguishing hepatotoxic from non-hepatotoxic compounds. If a compound is predicted as positive (probability score ≥ 0.5) it is hepatotoxic, while when predicted as negative (probability score < 0.5) it is non-hepatotoxic.

II. A 7-hepatotoxicity-endpoint consensus modeling approach

A more complex approach was followed. A set of 7 models, one for each of the 7 hepatotoxicity endpoints is created. This set of models was used in parallel during toxicological *in silico* screening, in order to distinguish hepatotoxic vs non-hepatotoxic compounds. In order to make this distinction, several thresholds are applied on the number of positively predicted endpoints. The optimal threshold –giving the best sensitivity vs specificity trade off– was considering a compound hepatotoxic when it is predicted as positive for at least two hepatotoxicity endpoints.

Molecular descriptors

For both datasets, several types of molecular descriptors have been calculated, such as all 2D MOE descriptors and the 3D volsurf series of descriptors (Molecular Operating Environment (MOE) 2015), PaDEL 2D and 3D descriptors (Yap 2010) and ECFPs (extended connectivity fingerprints; ECFP6), using RDKit (<http://www.rdkit.org/>) (Landrum).

Algorithms used

Classification models were built using the software package WEKA (Hall et al. 2009) (version 3.7.12). Several algorithms were investigated, including logistic regression, tree methods (Random Forest and J48 tree), Support Vector Machines (SMO in WEKA with polynomial, RBF and Puk kernels), Naïve Bayes, and k-nearest neighbors. Among meta-classifiers, MetaCost (Pedro 1999) was used, in order to artificially equilibrate our imbalanced datasets, since the ratio of negatives to positives was ranging from ~4:1 (for the most balanced dataset for hypertrophy) to 16:1 (for the most imbalanced dataset for bile duct abnormalities). Additionally, ThresholdSelector was used to obtain the optimal threshold for the classification when MetaCost was not sufficient, as well as attribute selection methods to select the most important descriptors and to evaluate their importance.

Several combinations of descriptors and classifiers were investigated and the best classification model was selected for each hepatotoxicity endpoint and for global hepatotoxicity. Apparently, for each individual endpoint as well as for global hepatotoxicity, there was a different optimal classification scheme.

Model validation

Due to lack of external test sets for the 7 hepatotoxicity endpoints and the restricted amount of positive that would complicate the splitting of the dataset into training and test set, we applied 10-fold cross validation in order to validate the 7 individual hepatotoxicity end-point models. The same approach was also applied for the generation of the global hepatotoxicity model.

However, 10-fold cross-validation, despite being a very robust indicator for the quality of a model (Gutlein et al. 2013), is not efficient for letting us compare which approach is better: the use of a single global hepatotoxicity endpoint model or the ensemble approach. For this comparison, an external test set would fit more.

For external validation, the dataset by Mulliner et al. was initially used. However, there was a high level of contradiction in the labels for the data also present in our training set. This is probably a result of the different way the class assignment was performed for the two datasets. Under these circumstances,

trying to predict the external set based on the training set makes not much sense. Thus, we also splitted the original dataset into 80% -which would be used for training- and 20% -which would be used for testing. The splitting was stratified and performed on the basis on the global hepatotoxicity class label. The whole procedure was repeated 10 times, resulting in 10 different subsets of 80-20% splits. Based on these 10 subsets, all models were generated and tested externally –including global hepatotoxicity model and the 7 individual hepatotoxicity endpoint models- and the average statistics and standard deviation are reported . The statistics metrics from both approaches (global vs consensus one) were collected and a two-pair t-test was performed in R for each statistics metric in order to compare the two approaches.(R Core Team (2013). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL <http://www.R-project.org/>.

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Heatmaps and Clustering

In order to have an overview of the dataset distribution across the 7 hepatotoxicity endpoints, heatmaps and cluster dendrograms were generated. The heatmap was generated in R with the heatmap.2 function of gplots package and hierarchical clustering was done with hclust function of cluster package. Most of the conventional hierarchical clustering methods were investigated, such as complete linkage, single linkage, group average and Ward's method.(Murtagh 1983) In both cases, the data clustering and visualization was done column-wise, depending on whether a compound is positive or negative for each one of the 7 separate endpoints, thus according to the compound class across the 7 hepatotoxicity endpoints. No information regarding chemical structure or physicochemical properties of the compounds is taken into account for this task.

Results and Discussion

Generation of the individual models for hepatotoxicity endpoints

Several combinations of descriptors with meta- and base classifiers were investigated until ending up to an optimal classification scheme for each hepatotoxicity endpoint and for the single model for global hepatotoxicity. In general, for most of the cases, 2D MOE descriptors were yielding the best statistical performances. Thus, for reasons of homogeneity, we decided to implement all our models using the 2D MOE descriptors, applying WEKA's meta-classifiers for attribute selection when appropriate.

For all toxicity endpoints the datasets were slightly or even highly imbalanced -as seen in table 1- which requires the use of special meta-classifiers in order to handle the data and equilibrate the sensitivity-specificity trade off. For this purpose we used in most of the cases the cost-sensitive meta-classifier MetaCost (Domingos 1999) which is a combination of a cost-sensitive classifier and Bagging. It introduces cost-sensitivity by reweighting training instances according to the total cost assigned to each class. Sometimes the application of MetaCost alone is not sufficient; therefore we additionally apply the meta-classifier ThresholdSelector, which was tuned according to FMeasure -a measure of a model's accuracy, considering both precision and sensitivity.(Powers 2011) Threshold selection finds the optimal threshold that would yield the highest FMeasure value, and the classification is done according to it, instead of the default 0.5 threshold on the model score that would be applied otherwise.

In Table 2 are detailed the settings used for the generation of the models for the 7 hepatotoxicity endpoint, as well as for global hepatotoxicity.

Table 2. Best models settings for each individual hepatotoxicity endpoint and global hepatotoxicity. In parenthesis are reported the particular setting/options selected for each particular base- or meta-classifier

Toxicity Endpoint	Best Model's Settings
Necrosis	AttributeSelectedClassifier (InfoGainAttributeEval + Ranker, 100 descriptors selected) + Naïve Bayes
Steatosis	MetaCost (cost matrix of [0.0, 1.0; 10.0, 0.0]) + ThresholdSelector (measure:FMeasure, evaluationMode: N-Fold cross validation, numXValFolds:5, rest default) + Random Forest (100 trees, default WEKA settings)
Bile duct abnormalities	MetaCost (cost matrix of [0.0, 1.0; 25.0, 0.0]) + ThresholdSelector (measure:FMeasure, evaluationMode: N-Fold cross validation, numXValFolds:5, rest default) + AttributeSelectedClassifier (InfoGainAttributeEval + Ranker, 100 descriptors selected) + SMO (Puk kernel, buildLogisticModels:True, rest default)
Glycogen decrease	MetaCost (cost matrix of [0.0, 1.0; 17.0, 0.0]) + ThresholdSelector (measure:FMeasure, evaluationMode: N-Fold cross validation, numXValFolds:5, rest default) + AttributeSelectedClassifier (InfoGainAttributeEval + Ranker, 50 descriptors selected) + Random Forest (100 trees, default WEKA settings)
Inflammation as 2nd effect	MetaCost (cost matrix of [0.0, 1.0; 10.0, 0.0]) + ThresholdSelector (measure:FMeasure, evaluationMode: N-Fold cross validation, numXValFolds:5, rest default) + SMO (Puk kernel, buildLogisticModels:True, rest default)
Preneoplastic effect	MetaCost (cost matrix of [0.0, 1.0; 16.0, 0.0]) + ThresholdSelector (measure:FMeasure, evaluationMode: N-Fold cross validation, numXValFolds:5, rest default) + AttributeSelectedClassifier (InfoGainAttributeEval + Ranker, 50 descriptors selected) +

	Random Forest (100 trees, default WEKA settings)
Hypertrophy	MetaCost (cost matrix of [0.0, 1.0; 6.0, 0.0]) + ThresholdSelector (measure:FMeasure, evaluationMode: N-Fold cross validation, numXValFolds:5, rest default) + Random Forest (100 trees, default WEKA settings)
Global hepatotoxicity	MetaCost (cost matrix of [0.0, 1.0; 3.0, 0.0]) + AttributeSelectedClassifier (CfsSubsetEval + BestFirst)+ IBk (kNN=5, rest default)

Validation of the individual models for hepatotoxicity endpoints

All models described above have been validated via 10-fold cross validation. Because our datasets are quite imbalanced and not very large, we performed, for each model, 10 iterations of 10-fold cross validation, in order to be more confident regarding the stability of the models' performance. The statistical performance for the individual endpoint models ranges between 0.570-0.753 for accuracy and 0.588-0.724 for the area under the curve (AUC). In general, we tried to compromise the sensitivity-specificity trade off, to avoid values below 0.5. However, we were definitely in favor of higher sensitivity vs specificity, when this was possible. We took this decision because we are dealing with toxicity endpoints; therefore it is of higher importance to miss as few true positives as possible, even if this would result in higher false positive rate. Nevertheless, in any case, neither sensitivity nor specificity should be less than 0.5.

The statistical performance for each individual endpoint model is reported in Table 3, as the mean of 10 iterations together with the standard deviation, for accuracy, sensitivity, specificity, Matthews correlation coefficient (MCC), AUC, precision and weighted average precision. Weighted average precision is the average precision of the two classes, weighted by the total number of instances for each class. We also use this metric since all the datasets for all modeling cases are very imbalanced, thus precision is always low due to the way it is defined.

Generation and validation of the single model for global hepatotoxicity

In order to generate the model for global hepatotoxicity, we should first assign the respective class label based on the classes of the individual hepatotoxicity endpoints. Our first impression was that if a compound would be positive for at least one individual hepatotoxicity endpoint, it should be automatically be considered as hepatotoxic, i.e positive for global hepatotoxicity. Nevertheless, the first modeling attempts gave moderate results for 20 iterations of cross validation, with mean values of

accuracy of 0.597 and AUC of 0.609, while sensitivity was even lower to 0.528. This prompted us to reconsider our labeling strategy for global hepatotoxicity. After thoroughly inspecting the compounds being positive for only one hepatotoxicity endpoint - 83 compounds in total - we observed that 42/83 compounds, i.e. ~50%, were positive only for hypertrophy. However, it has been recognized by the scientific community that if hypertrophy is not accompanied by other morphological, histological or clinical chemistry alteration, then it should be considered as an adaptive response, rather than an indication of hepatotoxicity (Hall et al. 2012).

Thus, we modified our threshold: we considered one compound as hepatotoxic if it is positive for at least one hepatotoxicity endpoint, unless if this endpoint is hypertrophy; in this case it should be accompanied by at least another hepatotoxicity endpoint to be considered as positive. This has improved the global hepatotoxicity model's performance to 0.629 for sensitivity and 0.646 for AUC for 10-fold cross validation, while the accuracy was retained. A statistical two sample paired t-test between the statistics values of the two global hepatotoxicity models showed that this improved approach indeed yields significantly higher sensitivity, AUC, MCC and weighted precision, while accuracy is equal. A more detailed report of the models' statistics for the two different thresholds can be found in table 3.

Table 3. Average performance of the individual toxicity endpoint models and the global hepatotoxicity model for 10-fold cross validation and standard deviation. The performance corresponds to the mean values of accuracy, sensitivity, specificity, MCC, AUC, precision and weighted precision for 10 iterations for each one of the 7 endpoint models and 20 iteration for the two models of global hepatotoxicity. In bold are depicted the statistics metrics that are significantly better when comparing the two models for hepatotoxicity.

Toxicity endpoint	Accuracy	Sensitivity	Specificity	MCC	AUC	Precision	Weighted Average Precision
Necrosis	0.570	0.628	0.563	0.121	0.626	0.154	0.836
sd	0.008	0.017	0.010	0.010	0.011	0.003	0.003
Steatosis	0.574	0.664	0.561	0.150	0.647	0.181	0.826
sd	0.022	0.040	0.029	0.021	0.010	0.008	0.007
Bile duct abnormalities	0.753	0.644	0.760	0.214	0.724	0.152	0.922
sd	0.035	0.059	0.039	0.022	0.017	0.031	0.003

Glycogen decrease	0.686	0.598	0.692	0.143	0.723	0.109	0.912
sd	0.013	0.032	0.015	0.012	0.013	0.005	0.002
Inflammation as 2nd effect	0.691	0.565	0.704	0.175	0.665	0.177	0.864
sd	0.073	0.088	0.088	0.048	0.020	0.035	0.009
Preneoplastic effect	0.638	0.622	0.639	0.149	0.701	0.138	0.879
sd	0.013	0.038	0.017	0.015	0.014	0.005	0.004
Hypertrophy	0.579	0.554	0.585	0.110	0.588	0.236	0.736
sd	0.021	0.023	0.026	0.023	0.014	0.012	0.008
Global hepatotoxicity (Positives ≥ 1)	0.597	0.528	0.628	0.147	0.609	0.394	0.634
sd	0.016	0.026	0.020	0.031	0.021	0.018	0.014
Global hepatotoxicity (Positives > 1, if hypertrophy positive)	0.596	0.629	0.582	0.190	0.646	0.347	0.697
sd	0.015	0.029	0.017	0.026	0.013	0.013	0.011

Development of the ensemble modeling approach of 7-hepatotoxicity-endpoint models

Since we have actually assigned the class labels for global hepatotoxicity based on the classes for the individual hepatotoxicity endpoints, we thought that we could respectively develop an ensemble modeling approach that would be based on the 7 individual hepatotoxicity endpoint models. More particularly, a new compound of unknown hepatotoxicity status would pass through the array of the 7 hepatotoxicity endpoint models and according to the sum of individual predictions, the prediction for global hepatotoxicity would be defined. In order to automatize this procedure, we implemented our models in KNIME (v. 2.10.4) (Berthold et al. 2007)

However, the question that arises with this kind of approach is what the optimal predictions' threshold is, in order to consider a compound as positive prediction. The initial thought was setting the threshold accordingly to the threshold of the global hepatotoxicity model. In particular, if at least one model gives a positive prediction, then the compound should be considered predicted as hepatotoxic, unless the positive prediction comes only for hypertrophy; in this case the prediction threshold should be adjusted

to more than one model. However, this assumption might be a bit arbitrary, since we are talking about predictions and not actual experimental values. Thus, the optimal threshold is something that should be investigated via the use of an external test set.

External Validation

As mentioned before, using an external test set is of vital importance in order to assign the optimal threshold and validate the consensus modeling approach of the 7-hepatotoxicity-endpoint models ensemble. Moreover, external validation would give an extra insight regarding the predicting capacity of the global hepatotoxicity endpoint model. For this purpose we initially used the preclinical data for DILI published by Mulliner et al. (Mulliner et al. 2016). The dataset, after removing the shared with the training set compounds, was comprised of 1490 small molecules, 619 positives and 851 negatives for DILI.

However, the results on the external test set were quite poor, both for the global hepatotoxicity model, as well as for ensemble 7-model approach. In particular, for the global hepatotoxicity model, the predictions on the external test set were yielding low sensitivity (0.321) and quite high specificity (0.721), resulting in quite poor accuracy and AUC. For the ensemble modeling approach, regardless the threshold applied, we were unable to achieve a satisfactory sensitivity-specificity tradeoff; the overall statistics were also quite poor. The best option would be setting the threshold for general hepatotoxicity to at least one positively predicted endpoint (regardless if the positive would be for hypertrophy or any other endpoint). This condition would yield a sensitivity of 0.607 and specificity of 0.492. A detailed report on the statistics of the external test set predictions is presented in the supporting information.

The poor performance of the models for the external validation was initially quite puzzling. We had a closer look on the shared compounds of the training set and the external test set. For the total of 39 shared compounds, only 11 compounds had the same class label among the two datasets. The other 28/39 compounds (~69%) have contradictory class assignments between the two datasets. More particularly, there is one compound with a positive class label in the training set and negative for the external test set and the remaining 27 contradictory compounds are negatives in the training set and positives in the external test set. Of course, the shared compounds have been removed from the external test set prior to the external validation. Additionally, this amount of compounds is quite small to directly influence the predictions for a dataset of 1490 compounds *per se*. However, considering the percentage of the contradictory compounds, maybe we should search deeper into the way the datasets were compiled and the class label was assigned.

One of the characteristics of the eTOX database is that the findings for the *in vivo* endpoints are assigned with a 3-label code: 0, 1 and 2, apart from the continuous values for an endpoint. Code 0 corresponds to a compound negative for this endpoint and the result is treatment relevant. Code 2 corresponds to a positive, treatment relative finding. But code 1 is assigned to findings that are not considered treatment relevant, regardless of the inspected effect (increase/decrease, positive/negative). This 3-label code might cause complications when attempted to be transformed into binary (positive/negative, 1/0) form, since -by default- code 1 will be transformed into negatives, no matter what is the observed effect. Moreover, the compilation of the data has been done from the internal repositories of several pharmaceutical companies, thus there might be a small difference regarding the standards each company uses in order to characterize the toxicological findings.

Furthermore, the training dataset includes findings exclusively for rats, while the external test set concerns also –apart from rats- mice, dogs and monkeys. This wider variance of species might also be the reason of class disagreement between the two datasets, since the differences between species for hepatotoxicity is something well-known (Martignoni et al. 2006; Olson et al. 2000).

On the other hand, searching for further information on the contradictory compounds, we found out that several compounds had ambiguous effect, depending on the type of the assay or the species of the animals. For example furosemide was found to cause hepatotoxicity to mice, but not rats (Williams et al. 2007). There were also compounds reported to have hepatoprotective effect against other hepatotoxic agents. For example rimonabant has hepatoprotective effect against isoniazid (Noorani et al. 2010), pentoxifylline protects against CCl₄-induced fibrosis (Desmouliere et al. 1999), agomelatine is protective against paracetamol-induced hepatotoxicity (Karakus et al. 2013) and rosiglitazone was found to reverse hepatic damage (Hockings et al. 2003). All the compounds mentioned were negatives for the training set, but positives for the external test set. Therefore, the findings above might be an indication that the external test set is actually over-flagging as hepatotoxicants some ambiguous compounds.

Splitting the initial training dataset into 80% for training and 20% for testing

As explained above, it seems that the external testing was not the most optimal approach for external validation, because the compilation of the data was not done in a homogenous way for the two datasets. In order to overcome this problem we decided to split our original training set into 80% for

training and 20% for testing, repeating the procedure 10 times. In order to automatize the procedure, we used KNIME (v. 2.10.4) for splitting the dataset and retraining the models, as well as for making the predictions. The data split was done in a stratified manner –which means that each subset contains equal proportion of the two classes as the initial dataset- according to the global hepatotoxicity class label (as defined for the optimal global hepatotoxicity models evaluated via 10-fold cross validation). This way we have 10 different data subsets for training and 10 subsets for testing, respectively, that are of the same consistency. With these subsets we repeated the training and the testing of the models for 10 times – one with each subset- and we collected the statistical results of the predictions to extract the average. Moreover, this way we can compare the single global hepatotoxicity model vs the ensemble modeling approach, since both will be tested against the same test sets ensemble.

The retraining and retesting was performed for the global hepatotoxicity single model, as well as for the 7 individual hepatotoxicity endpoint models, in order to evaluate the performance of the ensemble modeling approach. The applied model settings that have been used were exactly the same ones used while validating via 10-fold cross validation (Table 2). The only drawback of the procedure is the way the stratification is done -according to the global hepatotoxicity class label. This way the distribution of positives and negatives of the training subsets for the global hepatotoxicity model is the same among each individual subset, as well as with the initial training set. However, for the subsets used for training the 7 individual hepatotoxicity endpoint models the stratification was not done according to each hepatotoxicity endpoint class label. Thus, the 10 training and test sets are not of the same consistency regarding the class label in question each time, which might result in some paradoxically low or high performance for each training-testing round. Nevertheless, since the procedure is repeated 10 times, we expect that the differences in performance will be relatively balanced, even though most probably the initial 7 hepatotoxicity endpoint models generated (and validated via 10-fold cross validation) are expected to be more robust.

Validation of the single model for global hepatotoxicity vs the ensemble modeling approach of the 7-hepatotoxicity-endpoint models

The validation of the single global hepatotoxicity model is quite simple: we generated 10 models for the 10 training subsets, using the same model settings as the original model and then validated with the respective 10 test sets. The results were gathered and the mean values and standard deviations were

calculated for accuracy, sensitivity, specificity, AUC and precision. The approach is schematically represented in Figure 1.

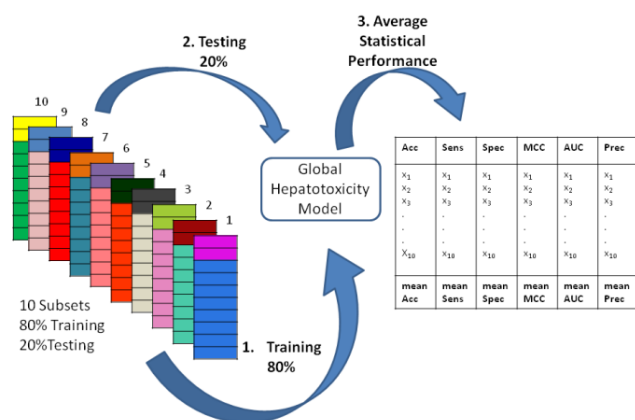


Figure 1. Validation of the global hepatotoxicity model after splitting the data into 80%-training and 20%-testing.

For the ensemble modeling approach, similarly, 10 models were generated for the 10 training subsets and then tested against the 10 respective subsets. The predictions for each training-test set pair were summed up as an ensemble for all 7 hepatotoxicity endpoints. Then, for each one of the 10 subsets the confusion matrix for general hepatotoxicity was calculated, according to the following set thresholds:

- If the number of positive predictions is equal or greater than 1 out of 7 hepatotoxicity endpoints, then a compound is predicted as positive for global hepatotoxicity.
- If the number of positive predictions is equal or greater than 1 out of 7 hepatotoxicity endpoints, then a compound is predicted as positive, unless the positive prediction concerns the endpoint of hypertrophy; in this case the number of positive predictions must be greater than 1 to be considered as positive prediction for global hepatotoxicity.
- If the number of positive predictions is greater than 1 out of 7 hepatotoxicity endpoints (regardless the endpoint), then a compound is predicted as positive for global hepatotoxicity.
- If the number of positive predictions is greater than 2 out of 7 hepatotoxicity endpoints (regardless the endpoint), then a compound is predicted as positive for global hepatotoxicity.

After the generation of the respective confusion matrices for each testing subset and for each threshold, the statistical performance was calculated for accuracy, sensitivity, specificity, AUC and precision. Then, the overall performance for all 10 testing subsets for each threshold was averaged

and the standard deviation values were calculated. The followed approach for the ensemble modeling approach is schematically represented in Figure 2. In Table 4 are reported the mean values with standard deviations for the performance of the single global hepatotoxicity model, as well as for the ensemble 7-endpoint modeling approach for the 4 different thresholds defining global hepatotoxicity.

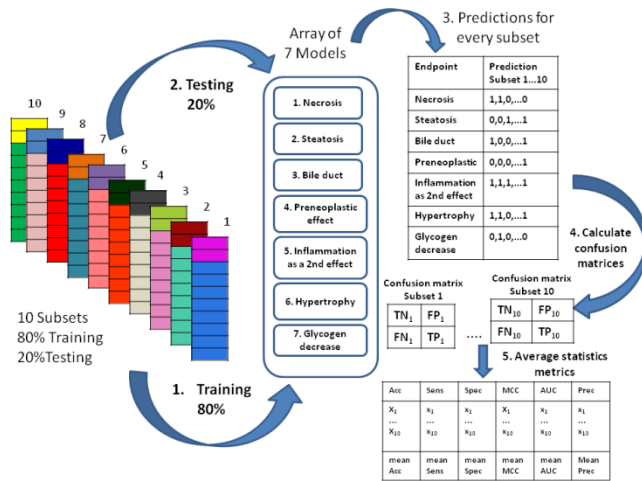


Figure 2. Validation of the ensemble modeling approach after splitting the data into 80% - training and 20%-testing.

Table 4. Performance of the single global hepatotoxicity model as well as for the ensemble 7-endpoint modeling approach for the 4 different thresholds defining global hepatotoxicity. The global hepatotoxicity model and the optimal ensemble modeling approach are depicted in bold.

Model	Accuracy	Sensitivity	Specificity	MCC	AUC	Precision
Global hepatotoxicity-Single model	0.592	0.608	0.587	0.172	0.596	0.344
	±0.039	±0.070	±0.058	±0.062	±0.034	±0.032
Ensemble modeling approach-threshold: positives≥1	0.447	0.850	0.305	0.149	0.579	0.303
	±0.073	±0.070	±0.115	±0.075	±0.039	±0.023
Ensemble modeling approach-threshold: positives≥1, except for hypertrophy: positives>1	0.477	0.808	0.360	0.155	0.584	0.312
	±0.086	±0.086	±0.141	±0.078	±0.043	±0.027
Ensemble modeling approach-threshold: positives>1	0.571	0.680	0.532	0.190	0.614	0.347
	±0.078	±0.083	±0.128	±0.069	±0.038	±0.048
Ensemble modeling approach-threshold: positives>2	0.631	0.521	0.670	0.178	0.595	0.366
	±0.065	±0.121	±0.116	±0.079	±0.042	±0.058

Selection of the optimal threshold for the ensemble modeling approach

As shown in Table 4, the lowest threshold of at least 1 positive prediction to consider a prediction positive for global hepatotoxicity, with the inclusion or exclusion of hypertrophy as positives (approaches i and ii), yield high sensitivity values of >0.8 , while the specificity is significantly low (<0.4) and the overall accuracy is < 0.5 . On the other hand, for the highest threshold of more than 2 positive predictions to consider a prediction positive for global hepatotoxicity (approach iv), the overall effect is exactly the opposite: specificity increases to 0.67 while sensitivity drops to 0.521. The overall accuracy is quite satisfactory –equal to 0.631- because negatives are the prevalent class, thus since specificity is favored (high number of true negatives), the overall accuracy is by default high. All in all, accuracy is highest for the threshold of >2 positive predictions to consider a prediction positive for global hepatotoxicity and the rest of the statistics metrics are quite satisfactory. Nevertheless, we were more in favor of selecting the threshold of > 1 positive predictions to consider a prediction positive for global hepatotoxicity. Thus, sensitivity is favored over specificity, which we consider more important, since we are dealing with a toxicity classification problem; there are more at stake if a compound is classified as safe and finally prove toxic, than the other way round.

Here we must also note that, theoretically, the optimal threshold for the ensemble modeling approach would be expected to be the same as the one used for the class assignment for the case of the global hepatotoxicity model. Namely, the number of positive predictions out of 7 hepatotoxicity endpoints should be ≥ 1 for a compound to be predicted as positive, unless the positive prediction is for the endpoint of hypertrophy; in this case the number of positive predictions must be >1 to be considered as positive prediction for global hepatotoxicity (approach ii). However, this threshold was actually favoring sensitivity, instead of presenting a balanced sensitivity – specificity trade off. We assume that this was the result of our initial decision to slightly favor sensitivity with respect to specificity for most of the individual hepatotoxicity endpoints. This was accumulated for the ensemble modeling approach, giving significantly greater sensitivity than specificity in the final results.

In any case, the advantage with this ensemble modeling approach is that it can be individualized according to the end user's needs. If the user wants to be even stricter the threshold can be decreased to ≥ 1 positive predictions out of 7 endpoints –including or excluding hypertrophy. The other way round, if the user is for some reason more interested in high accuracy and specificity, the threshold can be increased to >2 positive predictions. Furthermore, the ensemble approach can provide feedback

regarding the underlying mechanism(s) of hepatotoxicity, depending on which particular endpoint(s) was (were) predicted as positive.

Comparison of the single model for global hepatotoxicity vs the ensemble modeling approach of the 7-hepatotoxicity-endpoint models

In order to compare the performance between the single global hepatotoxicity model and the ensemble 7-hepatotoxicity-endpoint model for the optimal threshold (approach iii) -of >1 positive predictions for considering predicted global hepatotoxicity- we selected , we performed a two sample t-test in R(R Core Team (2013). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL <http://www.R-project.org/>).

). The differences are very slight for most of the statistic metrics, thus we had to investigate whether they are statistically significant. The retrieved from the t-test p-values are quite high for all statistic metrics apart from sensitivity, indicating that for accuracy, specificity, MCC, AUC and precision the 2 modeling approaches are performing equally. However, for sensitivity the p-value is 0.049, which is < 0.05. This means that in terms of sensitivity the ensemble modeling approach is superior. In Table 5 are depicted the obtained p-values for the two sample t-test.

Table 5. Mean, standard deviation and p-values obtained for the two sample t-test comparing the single global hepatotoxicity model vs the ensemble modeling approach for threshold predicted positives >1. The significantly different values for sensitivity are depicted in bold.

Model	Accuracy	Sensitivity	Specificity	MCC	AUC	Precision
Global hepatotoxicity- Single model	0.592 ±0.039	0.608 ±0.070	0.587 ±0.058	0.172 ±0.062	0.596 ±0.034	0.344 ±0.032
Ensemble modeling approach- threshold: positives>1	0.571 ±0.078	0.680 ±0.083	0.532 ±0.128	0.190 ±0.069	0.614 ±0.038	0.347 ±0.048
p-value	0.448	0.049	0.239	0.545	0.265	0.876

How could we interpret the results of the t-test? Well, once more it is up to the end user. For a “quick and dirty” screening, we would recommend the use of the single global hepatotoxicity model; it is performing equally for almost all of the statistics metrics with the ensemble approach, while it is at the same time simpler and faster. However, if the user is interested especially for sensitivity, it would be better to use the ensemble approach and test against all 7 individual models, in order to be more confident that he will not miss any true positives for hepatotoxicity. The same issues in case the end

user is interested in the particular mechanism(s) causing hepatotoxicity, which can be elucidated by the predictions for the 7 hepatotoxicity endpoints.

Heatmaps and Clustering

In order to obtain an overview of the compounds class (positive or negative) across the 7 hepatotoxicity endpoints, we generated heatmaps. In figures 3a and 3b are depicted the heatmap of a) all 764 compounds of the dataset for all 7 hepatotoxicity endpoints and b) of the 240 compounds that are positive for at least one hepatotoxicity endpoint. With green are depicted the negatives and with red the positives. The rows correspond to the compounds, while the columns correspond to the toxicity endpoints. A small isolated red area in figure 3a, like an outlier, represents the compounds that are positive only for hypertrophy.

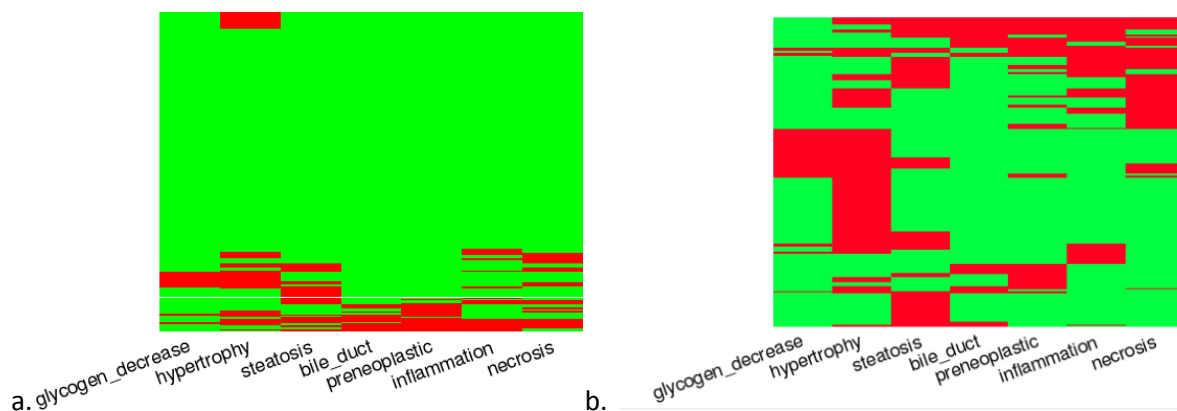


Figure 3a and 3b. Heatmap of a) all 764 compounds of the dataset for all 7 hepatotoxicity endpoints and b) of the 240 compounds that are positive for at least one hepatotoxicity endpoint. With green are depicted the negatives and with red the positives.

Moreover, to investigate if there are any trends among compounds and toxicity endpoints, as well as the association/similarities between the toxicity endpoints themselves, we performed hierarchical clustering. The clustering was done according to a compounds class over the 7 hepatotoxicity endpoints. In principle compounds with the same toxicity profile, i.e. that are positive for the same toxicity endpoints, should be binned in the same cluster. Thus higher in the dendrogram would be compounds being positive for several toxicity endpoints while in the edge of the tree are clustered those that are positive only for one particular endpoint. The other way round, endpoints that have a lot of shared positive compounds will be neighboring in the tree.

In figure 4a-4d are depicted the retrieved cluster dendrograms after performing hierarchical clustering with single linkage method (4a), complete linkage method (4b), Ward's method (4c) and average method (4d).(Murtagh 1983)

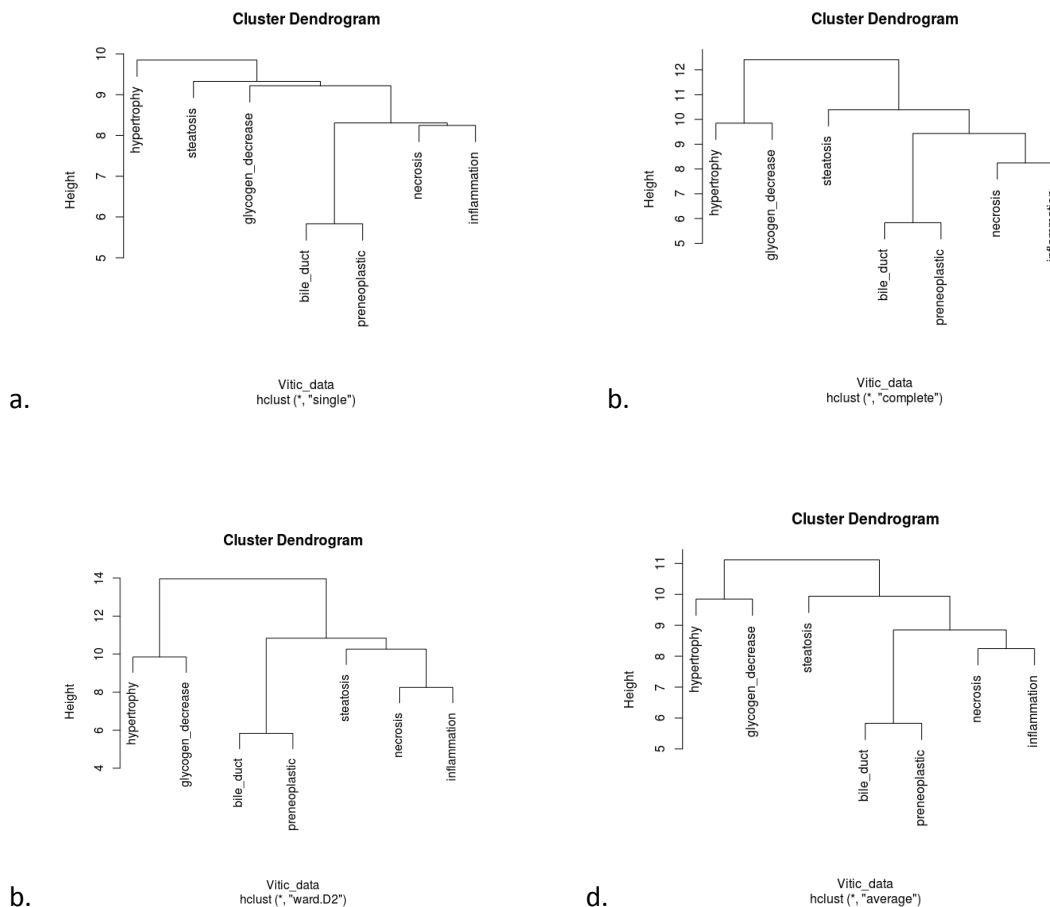


Figure 4. Cluster dendrograms for hierarchical clustering with 4a) single linkage method, 4b) complete linkage method, 4c) Ward's method and 4d) average method.

Regarding the clustering, it is quite interesting that all methods give quite similar dendrograms. In particular, for all methods, necrosis is clustered close to inflammation and the bile duct abnormalities cluster is placed close to preneoplastic cluster. This makes sense since also in literature hepatocyte necrosis has been associated with inflammation (Iimuro et al. 1997; Scaffidi et al. 2002; Thoolen et al. 2010), while there has also been evidence regarding bile duct abnormalities with preneoplastic effects (Thoolen et al. 2010). For three out of four clustering methods hypertrophy is clustered close to

glycogen decrease, which is also quite expected, since liver hypertrophy is very often accompanied by glycogen decrease (Hall et al. 2012).

This sort of clustering could also be helpful in terms of predicting toxicity endpoint. Since there is tight association for some particular endpoints, knowledge/experimental data for a compound for a particular endpoint could be used for predicting another closely associated endpoint. However, this approach involves some level of extrapolation, thus probably needs some further investigation.

Conclusions

Drug-induced hepatotoxicity is one of the major issues in drug discovery. In this work we developed two classification approaches to predict drug-induced hepatotoxicity for rat data obtained from eTOX database. One of the developed approaches consists of a single global hepatotoxicity model, and the other one combines 7 individual models for 7 hepatotoxicity endpoints that work synergistically to predict global hepatotoxicity. All generated models give a reasonable performance, considering the complexity of the endpoint(s) and the relatively small number of positives in each dataset.

The results showed that the two approaches are equal for all validation metrics, apart from sensitivity; in terms of sensitivity, the ensemble modeling approach performs better. Thus, if speed and simplicity are of interest, using the single hepatotoxicity is preferred. However, when special attention is required to avoid missing any positives, thus, potential hepatotoxic compounds, the use the ensemble 7-model approach is highly recommended. Moreover, having an ensemble of 7 hepatotoxicity models can elucidate the mechanistic basis of the potentially developed hepatotoxicity, depending on which particular endpoint(s) was (were) positively predicted.

Additionally, using hierarchical clustering we showed that there are some hepatotoxicity endpoints that are more closely associated with each other. Thus, experimental findings for a drug regarding a hepatotoxicity endpoint could be used as basis for a quick estimation of the outcome for another highly associated endpoint.

We aspire that our developed approaches will have a multiple contribution. On the one hand, they will help towards the replacement, or at least the reduction animal experiments for prediction of hepatotoxicity. An *in silico* approach of this kind would have dual benefit: i) from financial point of view, since experiments on animals have a high cost, and ii) from ethical point of view, since the use of animals –with all the suffering they sustain- will be reduced. On the other hand, the ensemble of 7 hepatotoxicity endpoint models, apart from a general prediction for hepatotoxicity, can allow a better

understanding of the underlying mechanistic cause of hepatotoxicity. This way, we hope that our models will be of use for the eTOX partners, as well as from the rest of the scientific community, as part of drug development.

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7.2 A Case Study on Imbalanced Data: Comparing the performance of widely used meta-classifiers

Comparing the performance of meta-classifiers – A case study on imbalanced data

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In the following study we compare the performance of 7 widely-used meta-classifiers: 1) Bagging, 2) Under-sampled stratified bagging, 3) Cost-sensitive classifier, 4) MetaCost, 5) Threshold Selection, 6) SMOTE and 7) ClassBalancer on coping with imbalanced datasets. As a base-classifier, for all cases Random Forest is used. The performance is investigated for 4 different datasets that are directly (cholestasis) or indirectly (organic anion transporting polypeptide 1B1 and 1B3 inhibition) associated with toxicity. The imbalance ratio ranges between 4:1 and 20:1 for negatives : positives: i) OATP1B1 inhibition (8:1), ii) OATP1B3 inhibition (13:1), iii) human cholestasis (4:1) and animal cholestasis (20:1). The performance of the methods is compared upon 3 different sets of descriptors, using 10-fold cross validation as well as an external validation set (for datasets (i) to (iii)).

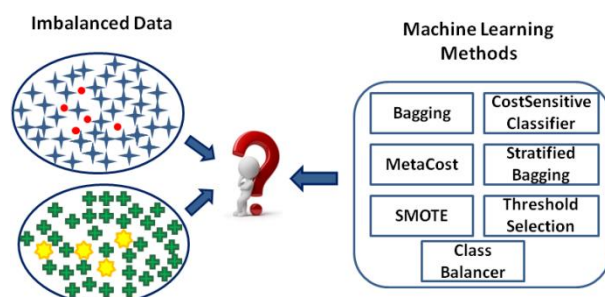
E. Kotsampasakou compiled the datasets, generated the models developed in WEKA (for Random Forest, Cost-sensitive classifier, MetaCost, Threshold Selection, SMOTE and ClassBalancer), did the statistical testing and wrote the manuscript. S. Jain performed the modeling on OCHEM for Bagging and Stratified Bagging, wrote the R code to generated the plots for the manuscript and reviewed the manuscript. G. F. Ecker supervised the experimental work and reviewed the manuscript. All three authors participated in the original design of the study.

Comparing the performance of meta-classifiers – A case study on imbalanced data

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Graphical Abstract



Abstract

In the current study we compare the performance of 7 meta-classifiers: 1) Bagging, 2) Under-sampled stratified bagging, 3) Cost-sensitive classifier, 4) MetaCost, 5) Threshold Selection, 6) SMOTE and 7) ClassBalancer for their ability to handle imbalanced datasets. As a base-classifier, for all cases, Random Forest is used. The performance is compared on the basis of 4 different datasets that are directly (cholestasis) or indirectly (organic anion transporting polypeptide 1B1 and 1B3 inhibition) associated with toxicity. The imbalance ratio is ranging between 4:1 and 20:1 for negatives : positives: i) OATP1B1 inhibition 8:1, ii) OATP1B3 inhibition 13:1, iii) human cholestasis 4:1 and animal cholestasis 20:1. The performance of the methods is evaluated upon 3 different sets of descriptors, using 10-fold cross validation, as well as an external validation set (for datasets (i) to (iii)). As the most important statistics metrics for the evaluation are considered in principle sensitivity and secondarily balanced accuracy, i.e. the average of sensitivity and specificity. As best performing methods, we evaluated Stratified Bagging, MetaCost and CostSensitiveClassifier. In principle, as MetaCost and CostSensitiveClassifier were yielding better sensitivity values, while Stratified Bagging outperformed for balanced accuracy.

Keywords:

Bagging, cholestasis, cost sensitive classifier, imbalanced data, machine learning, MetaCost, OATP1B1, OATP1B3, random forest, SMOTE, threshold selection

Abbreviations list

AUC: area under the ROC curve, HTS: high throughput screening, MCC: Matthews correlation coefficient, OATP1B1: organic anion transporting polypeptide 1B1, OATP1B3: anion transporting polypeptide 1B3, RF: Random Forest, sd: standard deviation, SMOTE: Synthetic Minority Over-Sampling TEchnique, SVM: support vector machines

Introduction

In many classification problems, the number of instances belonging to each class can be different. When a class is represented by a big number of instances and the other(s) by substantially less, we are talking about imbalanced datasets.^[1-3] In many cases of real-world classification problems the data are mainly representative of only one out of the two or more classes. Thus, the issue of imbalanced datasets has gained more and more attention during the recent years from the domains of machine learning and pattern recognition.^[1, 2, 4-6] There are several applications dealing with imbalanced datasets, such as face recognition, fault diagnosis, anomaly detection,^[1, 2] detection of fraudulent telephone calls, text classification,^[7, 8] telecommunications, World Wide Web (WWW), finances and ecology^[3] and many others. Moreover, imbalanced datasets significantly concern biomedical areas, such as clinical diagnostic tests of rare diseases and drug-induced toxicity.^[9]

For the broad scientific community, the root-problem of classification with imbalanced datasets lies within the fact that most base-algorithms maximize the overall number of correct predictions (accuracy), assuming that the overall misclassification cost for each class is the same.^[1, 3, 8-10] However, as it has been pointed out by other authors, a skewed distribution of the data should not be the main obstacle for a classifiers learning task^[6, 11] – actually there are reported cases, where actually good classification results were obtained with imbalanced data^[10], like the Sick dataset.^[12] For others,^[1, 6, 13-15] the limitation with imbalanced datasets derives from class overlapping, small sample size or small disjuncts within the datasets that deteriorate the learning process.

Due to the importance of the problem, as well as the wide occurrence of imbalanced datasets in real-world applications, several methods have been developed, in order to address the issue. These methods are

roughly divided into three main categories: 1) data-oriented or re-sampling techniques, 2) algorithm-oriented and 3) combinatorial/ensemble/hybrid techniques.^[1, 2, 6, 7, 9]

Within the data-oriented methods belong over-sampling, under-sampling and feature selection. Over-sampling tries to increase the minority class instances, while under-sampling reduces the number of instances for the majority class.^[2, 6, 7] A very popular technique for over-sampling is SMOTE^[8] (Synthetic Minority Over-Sampling TEchnique), developed by Chawla et al., which increases the minority class by generating new “synthetic” instances. Regarding feature selection, it is suggested that the current methods for feature selection are not the best choice for imbalanced data.^[1, 2] Alternatively Zheng et al. proposed separate feature selection for the majority and minority class and combination of them afterwards^[16], while some more recent feature selection approaches for imbalanced data are described in a recent review (2015) by Ali and colleagues.^[1]

Regarding the algorithm-oriented methods, these are focusing in optimizing parameters of the base classifier, adjusting it to fit more for the imbalanced learning purpose.^[1, 2, 7, 17] Furthermore, they include threshold adjustment for the classifier’s decision^[2, 18], one-class learning where only one class is taken into account for learning^[1, 2, 7, 19] and cost-sensitive learning that imposes on a base classifier a different cost for misclassifying an instance in a particular class in contrast to the other class.^[1-3, 7, 20]

Finally, in the combinatorial/ensemble/hybrid methods, i) the cases that combine several classical base-classifiers, in order to complement each other and ii) the ensemble-based techniques that concern meta-classifiers performing multiple functions simultaneously, are assigned.^[2, 6] The most famous base-algorithm that belongs in the ensemble methods is Random Forest,^[21] a base-classifier that is constituted by a number of several single decision trees. Among the well known ensemble meta-classifiers are AdaBoost,^[22] a boosting method to increase the performance of the base classifier, and Bagging^[23] –also known as Bootstrap Aggregating–that technically aggregates a predictor and uses the average performance. Last, but not least, we should mention MetaCost,^[24] a meta-classifier that combines a cost-sensitive technique with bagging (more information about metaCost will be provided in the methods section).

In the last years, in the domain of life sciences, there has been an intense interest towards imbalanced data and the methods to handle them. This was mainly an outcome of the generation of large amounts of high throughput screening (HTS)^[9, 20, 25, 26] and gene sequencing^[27, 28] data that need to be analyzed. Moreover, it was covering the emerging need to extract information out of risk assessment^[9] (Lin 2011) and medical/healthcare data^[29-31] that are currently available, in order to increase efficacy in drug development and reduce health risk and costs. Regarding the cases mentioned above, in a 2009 study Li et al. used granular SVM with repetitive under-sampling to predict luciferase bioassay HTS data.^[25] In 2014, Wan and

colleagues proposed a new learning method, named RankCost, to classify imbalanced medical data without using a priori cost. Instead, it translates the imbalanced classification problem into a partial ranking problem.^[31] Earlier in 2016 Anaissi and coworkers used ensemble SVM-recursive feature elimination (ESVM-RFE); the classifier follows the concepts of ensemble and bagging (like in case of Random Forest), but implements a backward elimination strategy.^[27] Wang and colleagues -in the same year- developed MatFind to handle imbalanced microRNA sequences. MatFind is used for identifying 5' mature microRNAs candidates from their pre-microRNA, based on ensemble SVM classifiers with idea of AdaBoost.^[28]

There were also studies comparing different methods and classifiers for handling imbalanced datasets of this kind. A study by Schierz and colleagues compares 4 WEKA classifiers (Naïve Bayes, SVM, Random Forest and J48 tree) with the application of costs for predicting several bioassay datasets of diverse sizes – approximately 60,000 to 60 compounds.^[32] Lin and Chen compared four classifiers for dealing with class-imbalanced HTS data. The four classifiers include: (i) diagonal linear discriminant analysis (DLDA), (ii) random forests (RFs), (iii) SVM - each one coupled with an ensemble correction strategy - and (iv) support vector machines (SVM)-based correction classifier with threshold adjustment (SVM-THR).^[9] Zakarov and colleagues used several imbalanced PubChem HTS assays to test and develop robust QSAR models in the program GUSAR, using different descriptor types (chemical and biological). The methods he used to handle the imbalanced data were based on under-sampling and threshold selection techniques.^[26] In a recent study of 2016, Razzaghi et al. compared classification results of multilevel SVM-based algorithms on public benchmark datasets having imbalanced classes and missing values and real data in health applications, with conventional SVM, weighted SVM, neural networks, linear regression, Naïve Bayes and C4.5 tree.^[30] Around the same time, Li S. and coworkers tried to predict the risk of tuberculosis for a patient to convert into multidrug-resistance tuberculosis, by comparing the performance of several methods to handle the imbalanced medical data: i) CART, ii) CART and Bagging, iii) CART with Under-sampling and Bagging, and iv) CART and EasyEnsemble.^[29]

Aim of this work was to evaluate how several well-established meta-classifiers perform while dealing with several datasets of different imbalance ratio, for different sets of descriptors and considering that the base classifier is the same. The meta-classifiers examined belong to all three categories of techniques to deal with imbalanced data, while the base-classifier was Random Forest. In total 4 different datasets of chemical compounds -mainly drugs- from the domain of biomedical sciences have been evaluated. The imbalance ratio of the datasets was 4:1, 8:1, 13:1 and 20:1 for negatives : positives, while the total number of compounds for each dataset was ranging between 1578 and 1766 compounds.

Methods

Training Datasets

In total 4 different datasets have been used for this study, coming from the domain of biomedical sciences. Two of the datasets concern transporters inhibition: organic anion transporting polypeptide 1B1 (OATP1B1) and 1B3 (OATP1B3) inhibition in particular. Those datasets were compiled during a previous study in our lab and were used as training sets for the development of OATP1B1 and 1B3 inhibition classification models.^[33] These data were originally retrieved from a study by De Bruyn and colleagues^[34] and they were further curated until obtaining their final form. The OATP1B1 inhibition dataset is consisted of 1708 compounds (1518 negatives and 190 positives) and had an imbalance ratio of $\sim 8:1$. In the OATP1B3 inhibition dataset there are 1725 compounds in total (1601 negatives and 124 positives), while having an imbalance ratio of 13:1.

The other two datasets come from the toxicology domain and concern drug-induced cholestasis for human data and for animal data respectively. Both datasets were recently published in a study by Mulliner and colleagues,^[35] where they developed computational models for hepatotoxicity and other liver toxicity endpoints, for both humans and animals. The human cholestasis dataset is comprised of 1766 compounds (1419 negatives and 347 positives) with an imbalance ratio of 4:1. The animal cholestasis dataset includes 1578 compounds (1512 negatives and 75 positives), having an imbalance ratio of 20:1.

External Testing Datasets

Apart from training data, for the training datasets of OATP1B1, OATP1B3 and human cholestasis mentioned above, there are also available test sets for external validation. The test sets for OATP1B1 and 1B3 were also used as external test sets in a previous study in our lab^[33] and they were compiled and curated from an original dataset published by Karlgren et al.^[36] The test set for OATP1B1 was consisted of 201 compounds (137 negatives and 64 positives) and the test set for OATP1B3 was consisted of 209 compounds (169 negatives and 40 positives). The test set for human cholestasis was compiled in two stages during two previous studies.^[37, 38] The positives for human cholestasis were compiled from literature^[39-42] and SIDER v2 database^[43, 44] while collecting data for a cholestasis modeling approach. As negatives for cholestasis were used the negatives for drug-induced liver injury compiled on a previous study,^[37] since compounds that are negative for DILI in general, will also be negative for cholestasis, one of the three types of DILI. The human cholestasis external dataset is consisted of 231 compounds (178 negatives and 53 positives). For the animal cholestasis dataset there were no data available to use. Moreover we were uneager to divide this dataset

into training and testing, due to the small number of positives; further reducing the 75 positives for animal cholestasis would complicate the model development. Table 1 presents the outlook of the datasets (both training and test), including the origin of the dataset, the total number of compounds, the number of compounds for each class and the imbalance ratio.

Table 1: Datasets overview

Dataset name	Total number of compounds	Number of positives	Number of negatives	Imbalance Ratio (negatives: positives)	Source
OATP1B1 Inhibition Training	1708	190	1518	8:1	Kotsampasakou et al. ^[33]
OATP1B1 Inhibition Testing	201	64	137	2:1	Kotsampasakou et al. ^[33]
OATP1B3 Inhibition Training	1725	124	1601	13:1	Kotsampasakou et al. ^[33]
OATP1B3 Inhibition Testing	209	40	169	4:1	Kotsampasakou et al. ^[33]
Cholestasis Human Training	1766	347	1419	4:1	Mulliner et al. ^[35]
Cholestasis Human Testing	231	53	178	3:1	In preparation
Cholestasis Animal Training	1578	75	1578	20:1	Mulliner et al. ^[35]

For all 4 datasets was applied careful curation of the chemotypes:

- All inorganic compounds are removed according to chemical formula in MOE 2014.09.^[45]

- Salt parts and compounds containing metals and/or rare or special atoms were removed and the chemical structures were standardized, using the Standardiser tool created by Francis Atkinson.^[46] (<https://github.com/flatkinson/standardiser/tree/1.0.1>).
- Duplicates and permanently charged compounds were removed using MOE 2014.09^[47]. Obviously, the duplicates between the training and the test set were also removed.
- 3D structures were generated using CORINA (version 3.4)^[48], and their energy was minimized with MOE 2014.09^[45], using default settings (Forcefield MMF94x), but changing the gradient to 0.05 RMS kcal/mol/Å². In addition, the existing chirality was preserved.

Descriptors

In total three different sets of descriptors were calculated for each one of the datasets and the generated models were based upon them:

- 1) All 2D MOE^[45] descriptors (192 descriptors in total).
- 2) ECFP6 fingerprints (1023 bits) calculated with RDKit.^[49]
- 3) MACCS fingerprints (166 bits), calculated with PaDEL software.^[50]

Algorithms

As base-classifier for all generated models is used Random Forest^[21] that is an ensemble method on its own, with 100 trees (rest settings at default) according to the respective implementation in WEKA software.^[51] The number of trees is arbitrarily set to 100, since it has been shown that the optimal number of trees is usually 64-128, while further increasing the number of trees does not necessarily improve the model's performance.^[52] This is also the default number of trees for the current WEKA version.

Concerning the methods used for dealing with the imbalance of the data, the following meta-classifiers are investigated: 1) Bagging, 2) Under-sampled stratified bagging, 3) Cost-sensitive classifier, 4) MetaCost, 5) Threshold Selection, 6) SMOTE and 7) ClassBalancer.

1) Bagging (Bootstrap AGGREGatING) is a machine learning technique that is based on an ensemble of models developed using multiple training datasets sampled from the original training set; it calculates several models and averages them to produce a final ensemble model.^[23] A traditional bagging method generates multiple copies of the training set by selecting the molecules with replacement from training set in a random fashion. Because of random sampling, about 37% of the molecules are not selected and left

out at each run. These samples create the “out-of-the-bag” sets, which could be used for testing the performance of the final model. A total of 64 models were used for our analysis, since it was shown in a previous study^[53] that larger numbers of models per ensemble (i.e., 128, 256, 512 and 1024) did not significantly increase the balanced accuracy of models. The same seed value was used to initialize the random generator in order that all the methods will be developed using the same datasets.

2) Under-sampled stratified bagging^[11] uses minority class samples to create the training set of positive samples using traditional bagging approach and after that randomly selects the same amount of samples from the majority class. Therefore, the total bagging training set size was double the number of the minority class molecules. E.g., in case of OATP1B1 inhibition training dataset from 1708 (190 positives) compounds, only about 22% of the compounds $((190+190)/1708)$ from the initial dataset were used to build each individual bagging model. Although a small set of samples was selected each time, the majority of molecules contributed to the overall bagging procedure, since the datasets were generated randomly. The performance of the developed models is tested with molecules from the “out-of-the-bag” set.^[54] Since only one way of stratified learning, i.e., under-sampling stratified bagging, was used in the study, we refer to it as “stratified bagging” by avoiding “under-sampling”.

Bagging and Stratified bagging were used according to their implementation in the online platform OCHEM^[55] for model generation. The rest of the meta-classifiers are used according to their implementation in WEKA(v. 3-7-12).^[51]

3) Cost sensitive classifier is a meta-classifier that makes its base classifier cost-sensitive.^[1-3, 7, 20] Two methods can be used to introduce cost-sensitivity: i) reweighting training instances according to the total cost assigned to each class or ii) predicting the class with minimum expected misclassification cost (rather than the most likely class). In our case, the cost sensitivity is introduced according to method (i) using the CostSensitiveClassifier from the set of meta-classifiers of WEKA software.

4) MetaCost^[24] is a combination of Cost-sensitive meta-classifier and Bagging. In principle, it should produce similar result to one created by passing the base learner to Bagging, which is in turn passed to a CostSensitiveClassifier operating on minimum expected cost. The advantage of MetaCost is the generation of a single cost-sensitive classifier of the base learner, giving the benefits of fast classification and interpretable output (if the base learner itself is interpretable). This implementation uses all bagging iterations when reclassifying training data (Domingos and colleagues^[24] report a marginal improvement when only those iterations containing each training instance are used in reclassifying that instance).

For both CostSensitiveClassifier and MetaCost, several trials of different cost matrices were applied, until a satisfactory outcome was retrieved.

5) ThresholdSelector is a meta-classifier in WEKA that sets a threshold on the probability output of a base-classifier. By default, the WEKA probability threshold to assign a class is 0.5. If an instance is attributed with a probability of equal or less than 0.5, it is classified as negative for the respective class, while if it is greater than 0.5, the instance is classified as positive. As mentioned earlier, threshold adjustment for the classifier's decision is one of the used methods for dealing with imbalanced datasets.^[2, 18] For our study, the optimal threshold was selected automatically by the meta-classifier by applying internal 5-fold cross validation to optimize the threshold according to FMeasure, a measure of a model's accuracy, considering both precision and sensitivity (FMeasure or F1 score = $\frac{2TP}{2TP + FP + FN}$).^[56]

6) SMOTE^[8] (Synthetic Minority Over-Sampling TEchnique) increases the minority class by generating new "synthetic" instances. SMOTE is used as a filter option of the FilteredClassifier –one of WEKA's meta-classifier. This way, it is applied within the cross-validation loop, since its application as a filter on the training set prior to model generation and cross validation would introduce bias and yield over-optimistic results.

7) ClassBalancer reweights the instances in the data so that each class has the same total weight. In principle, it is a filter in WEKA, but like SMOTE, it can also be used as a filter option of the FilteredClassifier, providing one more way to treat imbalance datasets.

Validation

All models generated are validated via 10-fold cross validation, except for the case of Bagging and Stratified Bagging. In these cases, multiple copies of the training set are generated by selecting the molecules with replacement from training set in a random fashion. Because of random sampling, about 37% of the molecules are not selected and left out at each run. These samples constitute the "out-of-the-bag" sets that are used for testing the performance of the final model. A total of 64 models were used for this analysis, since it was found to be the optimal trade-off between satisfactory performance and computational cost.

For the data for which there are complementary external test sets available, external validation is also performed.

Selection of the optimal method

Prior to selecting the optimal method, the optimal model out of each meta-classifier should be selected. In general, a model is considered eligible for selection if for 10-fold cross validation it has sensitivity equal or greater than 0.5. Sensitivity is evaluated as the most important statistics metric, since we are dealing with

cases of toxicity (cholestasis) or inhibition of transporters that are associated with toxicity phenomena^[57] (hyperbilirubinemia). Then, we also consider in mind balanced accuracy, which is the average of sensitivity and specificity, i.e. $\text{balanced accuracy} = (\text{sensitivity} + \text{specificity}) / 2$. Of course, the overall accuracy of a model is important. However, since the datasets are imbalanced, without much effort the classifier will give high accuracy, of 99% with ease over one class and fail to correctly classify the rare examples. Therefore the accuracy measure may be misleading on the highly imbalanced datasets. Thus, the solution of balanced accuracy seems more appealing, since it contains information for both sensitivity and specificity. Subsequently, as best representative model for each method, the model having the best sensitivity is selected, but without letting specificity drop lower than 0.5. Then, among models with equal sensitivity, those having the best balanced accuracy were prioritized for the final selection.

After selecting the best representative model for each method, this model is validated also on an external test set (for those datasets that there was an external test set available). Furthermore, for those models that gave for 10-fold cross validation sensitivity ≥ 0.5 , 20 iterations are performed, in order to obtain the mean and standard validation values. For Bagging and Stratified Bagging the 20 iterations were performed by changing the random seed for the Random Forest generation by assigning values from 1 (default) to 20. For the rest of the methods, the seed for cross validation is changed by assigning values from 1 (default) to 20. The best method is then evaluated by performing a statistical t-test in R^[58], as well as on the basis of the performance on the external test set. Once more, as most important metrics for the model's performance are evaluated sensitivity and specificity.

Results and Discussion

Best Representative Models for each Method

The best models representing each meta-classifier were selected on the basis of high sensitivity –at least equal with 0.5- as a first criterion and on the basis of high balanced accuracy as a second criterion. Moreover, satisfactory specificity of at least equal to 0.5 is considered as a prerequisite. The number of trees for the base classifier of Random Forest was arbitrarily set to the default setting of WEKA 3-7-12, since literature suggests that the optimal number of trees is between 64 and 128.^[52] For some meta-classifiers there was no parameter selection, like for the case of ClassBalancer; there was no need to tune any parameters, since it automatically sets the weights for the two classes to be equal. For the cases of

Bagging and Stratified Bagging, the only parameter that could be changed is the number of bags; since a previous study^[53] showed that the generation of 64 models gives satisfactory results without exponentially increasing the computational cost, this number of bags was used. For the ThresholdSelector, the selection of the optimal threshold was also done automatically by the software, by applying internal 5-fold cross validation, before the final model selection. As criterion for threshold selection was set FMeasure, which is the harmonic mean of precision and sensitivity. For the case of CostSensitiveClassifier and MetaCost, the applied cost for the misclassification of the minority class was applied initially according to the imbalance ratio. If this was not able to give a sensitivity of at least 0.5, it was further increased, until satisfying our prerequisites of a good model. For the case of SMOTE similar principles were applied: initially the number of the created synthetic instances was set in order to balance the two classes. If this was not sufficient, it was further increased up to the point that there was no further improvement in sensitivity, but merely a lack in specificity. The exact settings of best performing models for each method are provided in the supporting information.

For all models accuracy, balanced accuracy, sensitivity, specificity, Matthews correlation coefficient (MCC), area under the curve (AUC), precision and weighted average precision, i.e the average values of precision for the two classes, weighted by the number of instances of each class, were calculated.

Tables S2-S5 in Supporting information report the statistics metrics of accuracy, balanced accuracy, sensitivity, specificity MCC, AUC, precision and weighted average precision for the datasets of OATP1B1 inhibition, OATP1B3 inhibition, human cholestasis and animal cholestasis respectively. The results concern all three sets of descriptors: 2D MOE descriptors, ECFP6 fingerprints and MACCS fingerprints, and for all the investigated methods. Apart from the meta-classifier methods of Bagging, Stratified Bagging, CostSensitiveClassifier, MetaCost, ThresholdSelector, SMOTE and ClassBalancer, also the performance of the base-classifier (Random Forest) is reported for comparison reasons. Then, for the best performing methods for each dataset (shown in bold in Tables S2-S5), the mean and the standard deviation values out of the 20 performed iterations are reported in Tables S6-S9 for the respective datasets. For the best performing methods, also a statistical t-test was performed in R, to evaluate if the differences between the mean values of the statistics metrics between the different methods are statistically significant. For evaluating the best technique for classification with imbalanced datasets we considered: a) the performance of the different methods on the external validation and b) the mean performance for 10-fold cross validation out of 20 iterations, in association with the statistical test result (whether the difference in performance is statistically significant or not).

In figures 1-4 the performance for each dataset: OATP1B1 inhibition, OATP1B3 inhibition, human cholestasis and animal cholestasis of each method, for all three set of descriptors, is graphically represented. Figures (a) correspond to the performance on external validation on the test set and (b) show the result of 1 round of 10-fold cross validation. On x axon is depicted the balanced accuracy, while on y axon the sensitivity, i.e. the two statistics metrics we are mostly interested in and crucially influenced the selection of the final model. The rectangular-shaped points correspond to MOE descriptors, the triangle-shaped points correspond to ECFP6 fingerprints and the circular ones correspond to MACCS fingerprints. Each classifier is depicted in a different color: red for RF standalone, green for Bagging, blue for Stratified Bagging, dark pink for CostSensitiveClassifier, cyan for MetaCost, yellow for ThresholdSelector, orange for SMOTE and dark violet for ClassBalancer. The plots were generated in R and the code is provided in the supporting information.

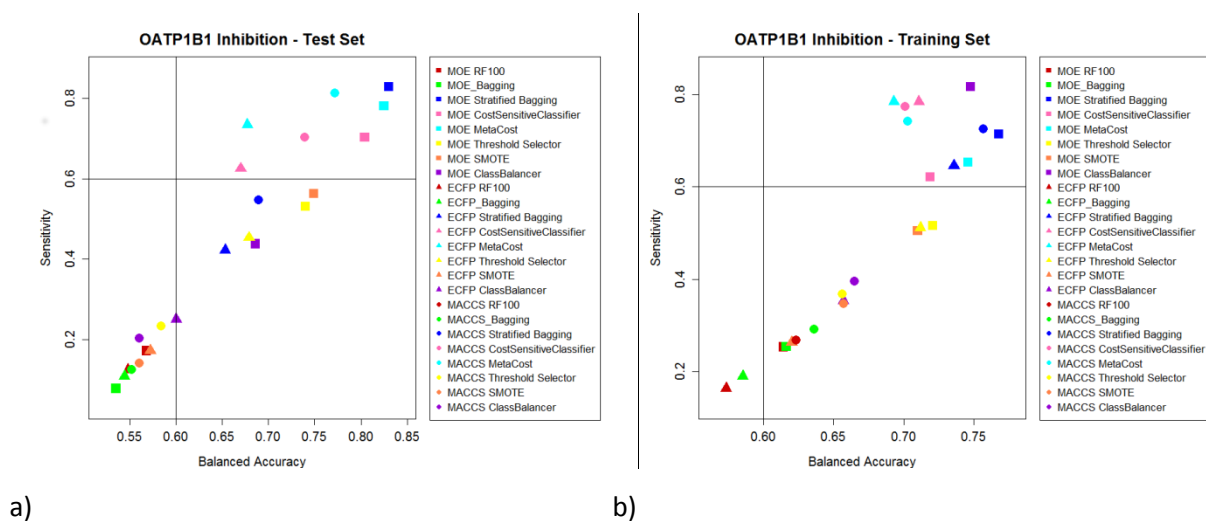


Figure 1. Performance of OATP1B1 inhibition a) on the test set and b) on the training set for one round of 10-fold cross validation. Please note that the scaling for the two axes is not the same.

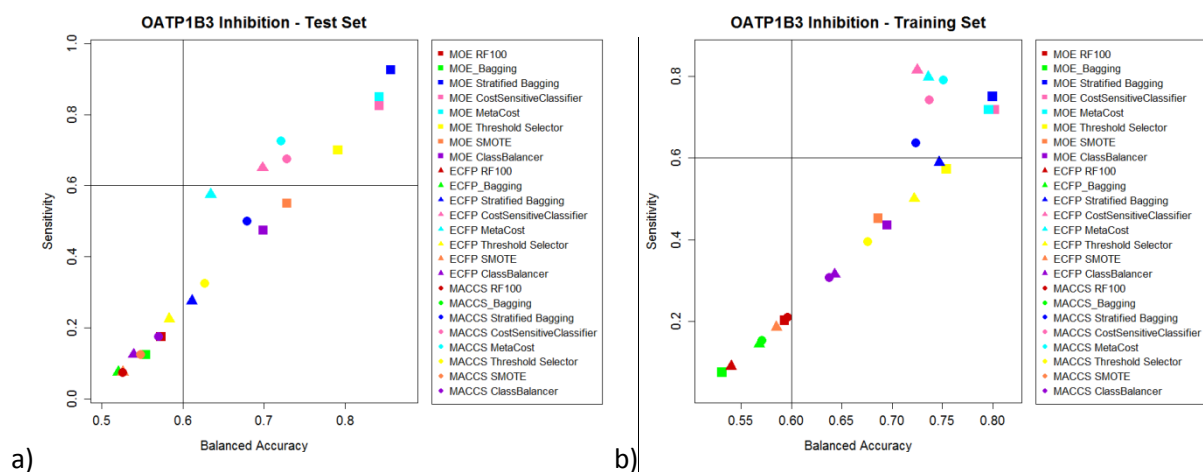


Figure 2. Performance of OATP1B3 inhibition a) on the test set and b) on the training set for one round of 10-fold cross validation. Please note that the scaling for the two axes is not the same.

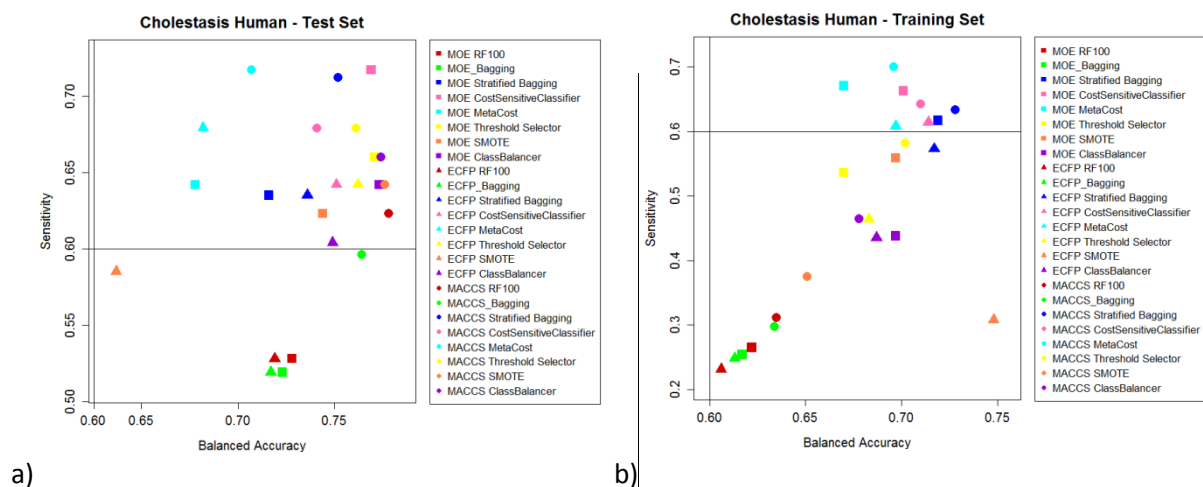


Figure 3. Performance of human cholestasis a) on the test set and b) on the training set for one round of 10-fold cross validation. Please note that the scaling for the two axes is not the same.

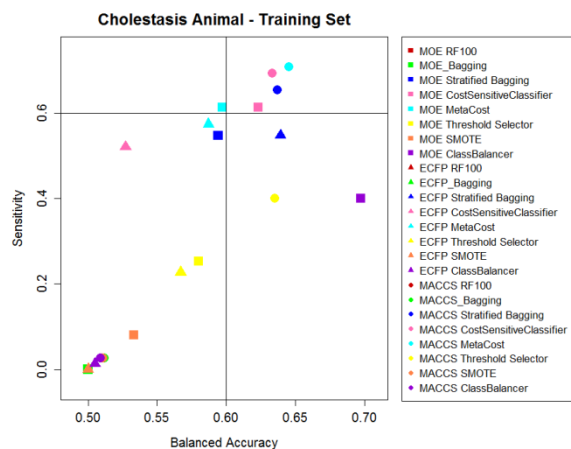


Figure 4. Performance of human cholestasis on the training set for one round of 10-fold cross validation (there was no test set available). Please note that the scaling for the two axes is not the same.

Figure 4. Performance of human cholestasis on the training set for one round of 10-fold cross validation (there was no test set available). Please note that the scaling for the two axes is not the same.

By definition, the best performing classifiers are gathered in the upper right corner of the graphs, while the weakest ones are lying on the bottom left corner. To make it visually more straightforward, we considered a threshold over 0.6 for both sensitivity and balanced accuracy and draw on the graphs the respective vertical lines to both axes. The methods gathered on the upper right rectangle that is formed were the most robust, since they yield high values for both metrics. For all cases of datasets and descriptors, Random Forest standalone yielded the weakest performance, which is rather expected – for all the others methods the base classifier is assisted by a meta-classifier which improved the performance.

In principle, apart from the case of the test set performance for human cholestasis, RF standalone was not able to yield a sensitivity of over 0.5 on its own. This shows that for almost all cases of datasets, some assistance on the base-classifier is necessary to obtain more satisfactory predictions. Here, it should also be noted that the human cholestasis datasets were compiled mainly on the basis of toxicity reports. However, the toxicity reporting system has several drawbacks. The major is under-reporting,^[59-61] due to the voluntary character of the system.^[61-63] Moreover, it is pretty hard to obtain human toxicity data; very often they are proprietary and post-marketing data are difficult to procure.^[59] Finally, a causal relationship is usually not required to submit an adverse event,^[61] which is of crucial importance for the case of a patient who receives several different medications or suffers from underlying chronic disease(s). It should also be stressed out that –most probably due to these reasons- the shared compounds between the training and the test set for human cholestasis show a contradiction of approximately 20% (49 out of 254 shared compounds) regarding the class label, which might also apply the rest of the datasets. Thus, for the case of this dataset, where the performance on the external test set is higher than for 10-fold cross validation, the results might be slightly over-optimistic. But, apart from that, the overall tendency also for this dataset is similar to the others, regarding the best performing methods.

As a general trend for all datasets, both for the test set validation and the 10-fold cross validation, the constantly best performing classifiers were Stratified Bagging, CostSensitiveClassifier and MetaCost. This conclusion became obvious from the graphs, the good performance on the external test sets and 10-fold cross validation. Moreover it has also been verified with the statistical t-test on the basis of 95% confidence interval (exact p-values not shown here). The statistical test was performed pair-wise for all the obtained statistics metrics of the models, while more focus was given for the metrics of sensitivity and balanced accuracy. Regarding these three best techniques, for almost all datasets and validation methods, both MetaCost and CostSensitiveClassifier tended to yield higher sensitivity than Stratified Bagging. Stratified Bagging on the other hand was superior for a greater number of statistics metrics, including accuracy, specificity, MCC value, and quite often balanced accuracy and AUC. An advantage of Stratified Bagging is that it is rather automatized and robust. There are not many parameters for the end-user to tune, thus its use is rather straightforward; even an inexperienced user of the method is hard to introduce bias in the results. On the other hand, it's hard to individualize according to one's needs, in case for example very high weight should be put on sensitivity; this is feasible with the cost-sensitive approaches. It must also be noted that, even though MetaCost and CostSensitiveClassifier were performing quite equally, the required cost to be applied in CostSensitiveClassifier was far greater than the respective one applied for the case of MetaCost. So, if this is also taken into account, we could say that MetaCost is "equilibrating" the dataset more easily. This can be attributed to the fact that MetaCost is actually a hybrid classifier: it combines Bagging with the application of a cost. On the other hand, the computational cost for MetaCost is higher than CostSensitiveClassifier. Stratified bagging is also not computationally demanding (for the optimal parameter of 64 bags). Each bag is double the size of minority class, thus the calculation of models using Stratified Bagging requires less computational time, compared to the models built using a Bagging approach (the bags are of the same size as the training set), or MetaCost (which includes both bagging and weighting). For the case of cholestasis (both animal and humans) Stratified Bagging was combined with the application of a slight cost of 2:1 in favor of the minority class, since Stratified Bagging on its own was not able to handle the two dataset in such satisfactory way. Interestingly, this was necessary only for the cholestasis datasets. For animal cholestasis, since it was actually the dataset with the highest imbalance ratio, this could be quite justified. However, for the case of human cholestasis the imbalance ratio was only 4:1 for negatives:positives, far less than OATP1B1 (8:1) and OATP1B3 (13:1) inhibition.

Here comes once more the general difficulty of modeling toxicity endpoints, where the assignment of a class label is in some cases on the basis of subjective factors. On the contrary, an in vitro experiment of a transporter inhibition is more standardized. It is noteworthy that for the case of OATP1B1 and 1B3, the contradiction of class labels for compounds shared between the training and the test set was minimal; for the few contradictions, the deviation was usually $\pm 10\%$ of the threshold of 50% inhibition. After the 3 best methods, we would rank threshold selection. In some - but not all- of the cases, it was able to handle the imbalance of the dataset. However, even for the successful cases, sensitivity was still quite low in comparison to other methods. This is due to the way thresholds were selected: on the basis of FMeasure, i.e. the harmonic measure of sensitivity and precision. For highly imbalanced datasets, the impact of the positive class is still prominent. Nevertheless, FMeasure is the optimal parameter for selecting the threshold. Accuracy and specificity are definitely not suitable, due to the high impact of the majority class. On the other hand, if the selection is done on the basis of sensitivity, the model tends to yield very high sensitivity (0.8-1.0) with radical decrease of specificity (0.2-0.0). Threshold selection was giving very good results in combination with a second meta-classifier. However, since our aim was to compare these particular single meta-classifiers, we did not investigate this trend in depth. SMOTE and ClassBalancer were able only in very few cases to handle successfully the imbalanced datasets, in order to give sensitivity of at least 0.5 for both test set and 10-fold cross validation. The poor performance of SMOTE was a bit of a surprise to us, considering its very good reputation. A possible assumption for this failure could be the size of the datasets. The particular datasets are quite big for the area of drug design and life sciences. However, they cannot be compared with the data obtained from high throughput screening, or other scientific fields, like statistics or economics, where there are datasets of hundreds of thousands or even millions of instances. For datasets of this size, even if the imbalance ratio would be 100:1, there would still be sufficient instances of the minority class, upon which SMOTE would generate the synthetic instances. In our case, since the actual number of instances of the minority class is quite small, there is probably not so much information for the generation of quite diverse synthetic instances that would allow a successful classification of a new “unseen” dataset. Finally, the worst performance was yielded for the case of Bagging, since it simply does re-sampling, without having any means of balancing or weighting the two classes. Furthermore, the trends regarding the performance of the classifiers were preserved not only across datasets, but also across the sets of descriptors. Thus, in most of the cases Stratified Bagging, CostSensitiveClassifier and MetaCost were the best performing methods for all three sets of descriptors and for all four datasets, both for the external test validation and 10-fold cross validation.

However, for all four datasets, the best results were obtained either with MOE 2D descriptors (in most cases) or with MACCS fingerprints. Interestingly, the worst performing descriptors were ECFP6 fingerprints. This is quite curious considering that ECFP6 are comprised for 1023 bits to describe each molecule, much more in comparison to the 192 physicochemical descriptors for MOE and 166 bits for MACCS). We assume that this was random due to the individual datasets and has nothing to do with the quality of ECFPs. Moreover, it could be an indication that even simpler or smaller sets of descriptors might be able to give equal results to more complex or highly populated descriptors. Subsequently, for the 7 examined methods for the 4 different datasets, became evident that the most powerful classifiers perform better regardless the type of dataset (toxicity endpoint, i.e. general or *in vitro* endpoint, i.e. specific), the type or number of the descriptors (physicochemical descriptors or fingerprints) or the level of imbalance between the datasets (slightly or highly imbalanced). Of course, for the case of a “difficult” dataset of a toxicity endpoint that is highly imbalanced, like the animal cholestasis dataset, the obtained performance was lower compared to the other datasets that are less imbalanced or/and simpler in terms of the endpoint. Nevertheless, still the ranking of the methods was retained. Moreover, the more sophisticated meta-classifiers, like Stratified Bagging and MetaCost -that combine re-sampling and some way to weight the two classes, either via under/over-sampling or cost assignments- were performing in principle better than Bagging (simple re-sampling) or ClassBalancer (simple re-weighting of classes until becoming equal).

Conclusions

The problem of imbalanced datasets is an important inhibitory factor for classification problems. The most classifiers tend by default to predict correct the majority class, yielding high accuracy values, while the minority class is highly misclassified. However, in several cases -like for the prediction of toxicity or active compounds against a molecular target- what is of highest interest is the minority class.

In the current study we compared the performance of 7 meta-classifiers: 1) Bagging, 2) Under-sampled stratified bagging, 3) Cost-sensitive classifier, 4) MetaCost, 5) Threshold Selection, 6) SMOTE and 7) ClassBalancer for their ability to handle 4 imbalanced datasets.

We showed that for all datasets and both for external validation and 10-fold cross validation the best performing methods were Stratified Bagging, MetaCost and CostSensitiveClassifier. MetaCost and CostSensitiveClassifier were tending to give better sensitivity values, while Stratified Bagging outperformed for the other statistics metrics, like balanced accuracy, accuracy and specificity. On the contrary, simpler classifiers like the base-classifier Random Forest standalone and Bagging were in general unable to handle

the imbalance problem. Interestingly, the performance of SMOTE, which is considered a quite sophisticated classifier, ranged between average and poor. This can potentially be attributed to the small size of the minority class and the whole datasets in general. The type of descriptors did not play a substantial role for the ranking of the different methods' performance, however for our case the 2D MOE descriptors and the MACCS fingerprints performed better than ECFP6 fingerprints.

All in all, what we should always keep in mind is that the best method to be used depends always on the type of datasets for classification, both in terms of endpoint and imbalance ratio. In general, more sophisticated methods for more complex problems tend to perform better. The computational cost can also be considered—methods that require extensive re-sampling are computationally more expensive. One can select a method that compromises the complexity of the algorithm with the computational cost, to retrieve a satisfactory result. Finally, of crucial important is the aim of the study; this way one can prioritize which class is the most important and which statistics metric is of primary interest. The procedure for handling the imbalanced data should be designed differently if the aim is avoiding toxicity in comparison to pursuing high biological activity.

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Chapter 8

Concluding Discussion

Approaching the end, it can be said that this work lies within the intersection of hepatotoxicity endpoints and hepatic transporters and tries to interpret the ways they affect each other. Three main hepatotoxicity endpoints were investigated: drug induced liver injury (DILI), hyperbilirubinemia and cholestasis, in association with five transporters: the basolateral uptake transporters OATP1B1 and OATP1B3, and the canalicular efflux transporters BSEP, P-gp and BCRP. Since it is extremely difficult to retrieve experimental data for all hepatotoxicity endpoints and for all transporters, for the case of the hepatotoxicity endpoints we made use of human toxicity reports or animal *in vivo* histopathological data curated by toxicologists. For the case of transporters we made use of the in-house *in silico* classification models of transporters' inhibition available in our lab.

Chapters 1 and 2 are mainly introductory. Chapter 1 discusses the reasons why drug-induced hepatotoxicity attracted our attention and we decided to investigate it in association with the role of some major hepatic transporters. It also mentions the individual contributions of this thesis. Chapter 2 provides the biological background of the hepatic transporters, with major focus on their pathological role and how they are implicated in several liver conditions. Moreover, it gives some special focus on the five individual transporters, whose role in drug induced hepatotoxicity will be more thoroughly investigated.

Chapter 3 discusses the development of classification models for OATP1B1 and OATP1B3 inhibition. It points out that for a clear biological endpoint, if the training datasets are sufficiently big and properly curated, the resulting models can be of high quality, even if they are built on a small set of physicochemical descriptors. Indeed, the high quality of these models was evaluated via cross-validation and with an external test set. As further proof, a blind test was performed by biologically testing a new set of 10 compounds for OATP1B1 and 1B3 inhibition, yielding an accuracy of predictions of 90% for OATP1B1 and 80% for OATP1B3. This high quality models can be further used in multilayer modeling approaches, where the predictions of one internal model are utilized as descriptors for the predictions of the external model.

Chapters 4, 5 and 6 describe the multilayer modeling approaches for hepatotoxicity endpoints. In particular in each chapter classification models for DILI, hyperbilirubinemia and cholestasis are

described that utilize, apart from molecular descriptors, also predictions of hepatic transporters' inhibition. They also investigate the role of the hepatic transporters for the particular endpoint.

For the case of DILI in chapter 4, special emphasis is given in careful curation of the data, not only from the chemotypes point of view, but also in respect to the class labels assignment. We propose a final high quality dataset for DILI, obtained from several public sources, that is used for the development of the final model for DILI. Our proposed model is a rather simple random forest, proving that with high quality data even simpler classifiers can give satisfactory performance. Moreover, the role of BSEP, BCRP, P-gp, OATP1B1 and OATP1B3 inhibition is investigated. The predictions for transporter inhibition were obtained from already available in-house classification models for the 3 ABC efflux transporters. For the basolateral OATPs, we use the models developed in chapter 3. Unfortunately, we are not able to show strong association between DILI and the hepatic transporters inhibition. This is mainly attributed to the complexity of DILI as well as the drawback of toxicity reporting system.

Chapter 5 discusses the development of one human and one animal model for hyperbilirubinemia, based on human data from toxicity reports and animal clinical chemistry data, respectively. It further investigates the role of OATP1B1 and OATP1B3 inhibition for the development of hyperbilirubinemia. The required transporter inhibition predictions are obtained from the classification models described in chapter 3. For the case of the animal model, we show no association with OATPs inhibition, which is quite normal since the predictions concerned human transporters and the data upon the model is built concern mainly rodents. However, surprisingly also for the case of humans, we inspect only minor association. The transporters' inhibition predictions are evaluated as important descriptors from the used attribute selection method, however, including them in the list of the molecular fingerprints does not significantly improve the models performance. Moreover, the performed chi-square test failed to show dependence between the class label for hyperbilirubinemia and the respective one for OATP1B1 and 1B3 inhibition.

In chapter 6 the classification model for cholestasis and the investigation of the hepatic transporters' association for the particular endpoint is described. The data concern once more toxicity reports for cholestasis from public sources. Due to lack of available negative data in literature for the particular endpoint, we use the negative compounds compiled for the DILI case in chapter 4. Since cholestasis is actually a form of DILI, negatives for DILI are subsequently negatives for cholestasis too. Of course the same deduction cannot be applied also for the positives for DILI-cholestasis. This time including the transporters' predictions in the set of descriptors significantly improved the models' performance. Interestingly, this observation did not only apply for BSEP- which is considered in literature as the most

important transporter for the development of cholestasis- but it was a rather synergistic effect. We hope that this will emphasize on the important role the integrity and function of the whole liver transportome has for a proper liver function.

What should also be noted, for all chapters 4, 5 and 6, is that we should always keep in mind that actually transporters inhibition predictions and not real *in vitro* data were used. Even though the performance of the original transporter models is rather satisfactory, there is always the possibility of getting different results with experimental data. And even for *in vitro* data, there still might be a different outcome on the level of a whole organism, where the interplay of different transporters takes place. Moreover, there are more transporters localized in the liver, as well as several metabolizing enzymes; their inhibition could have significant impact for all the aforementioned forms of hepatotoxicity. Unfortunately, mainly due to lack of data for modeling the particular transporters and/or enzymes, as well as because of time restrictions, we were not able to include them in the study. Nevertheless, even under these circumstances, if the mechanistic basis of a toxicity endpoint is simpler, like for the case of cholestasis, still an association can be observed. On the contrary, for more mechanistically complex endpoints, like DILI, it is even harder to draw any conclusions or seeing a clear trend.

Finally, in chapter 7 two case studies are discussed. The first case study concerns the development of two approaches to predict hepatotoxicity for animal data: one single global hepatotoxicity classification model and one 7-endpoint ensemble modeling approach. The latter one is based on 7 classification models for hepatotoxicity endpoints: 1) necrosis, 2) steatosis, 3) bile duct abnormalities, 4) glycogen decrease, 5) inflammation as a 2nd effect, 6) preneoplastic effect and 7) hypertrophy. The two developed methods are compared. Our results show the two methods as equal for all statistic metrics, apart from sensitivity, where the 7-endpoint consensus model approach prevails. Thus, we conclude that when convenience and speed matter, it is OK to use the single hepatotoxicity model. However, when special attention should be given to sensitivity, the consensus modeling approach seems preferable. Moreover, we hope that the 7-endpoint ensemble model will offer a more mechanistic understanding of experimental or predicted hepatotoxicity.

The second case study in chapter 7 compares several meta-classifiers for 4 different imbalanced datasets for 3 different sets of descriptors. In total, we compare 7 meta-classifiers: 1) Bagging, 2) Under-sampled stratified bagging, 3) Cost-sensitive classifier, 4) MetaCost, 5) Threshold Selection, 6) SMOTE and 7) ClassBalancer. Meta-classifiers are always used in combination with random forest, which is the

base-classifier. The 3 sets of descriptors are all 1D and 2D MOE descriptors, MACCS keys and ECFP6 fingerprints. The 4 datasets concern:

- i. one human dataset for cholestasis of 1766 compounds, having an imbalance ratio of 4:1 - negatives: positives, which is used as an external dataset for the cholestasis model in chapter 6 (after removing the compounds overlapping with the respective training set)
- ii. an animal dataset for cholestasis of 1578 compounds, having an imbalance ratio of 20:1- negatives: positives
- iii. the OATP1B1 training set (used in chapter 3) of 1708 compounds, having an imbalance ratio of 8:1 – negatives: positives
- iv. the OATP1B3 training set (used in chapter 3) of 1725 compounds, having an imbalance ratio of 13:1 – negatives: positives

Among the meta-classifiers used, Stratified Bagging, MetaCost and CostSensitiveClassifier are evaluated as the best performing ones; they are able to yield high sensitivity values, which is the statistics metric of primary interest, retaining also the rest of the statistics metrics quite high. Interestingly, these were the best methods for all datasets and for all sets of descriptors, both for 10-fold cross validation and for external validation. This suggests that regardless the descriptors or the imbalance ratio of the data, more sophisticated methods are usually more capable of handling imbalanced data.

Coming to the end, hopefully this thesis has made a contribution towards the important issue of predicting liver toxicity endpoints while associating them with hepatic transporters inhibition and provides good classification models for OATP1B1 and OATP1B3. Apart from “successful stories”, it also points out issues that hinder the process of hepatotoxicity prediction, such as lack of data, the nature of toxicity reporting system, the low concordance between humans and animals for hepatotoxicity, as well as the multiple underlying mechanisms of hepatotoxicity endpoints. This finally leads to the main conclusions/take-home messages:

- 1) Simpler endpoints/molecular targets, such as transporters (and thereafter enzymes and receptors) are more easily to model than complex endpoints, with multiple underlying mechanisms, such as hepatotoxicity.
- 2) Proper data curation and extended validation of the resulted models can yield satisfactory results, even with less fancy algorithms and smaller sets of descriptors.
- 3) Even the most sophisticated algorithmically methods face the risk of failure, when the quality of the underlying data is compromised.

Appendix

1. Supplements to Chapter 2

The following book chapter entitled: “Organic Anion Transporting Polypeptides as Drug Targets”, by Eleni Kotsampasakou and Gerhard F. Ecker is part of the book “Transporters as Drug Targets”. E. Kotsampasakou did the literature search and wrote the manuscript. G.F. Ecker critically reviewed the manuscript.

Organic Anion Transporting Polypeptides as Drug Targets

Eleni Kotsampasakou and Gerhard F. Ecker

Abbreviation List

ADT: androgen deprivation therapy, BBB: blood-brain barrier, BCRP: breast cancer resistance protein, BOPTA: gadobenate dimeglumine, BSP: Bromosulphophthalein, CCK-8: cholecystokinin octapeptide, CKD: chronic kidney disease, CRPC: castration resistant prostate cancer, DHEA: dehydroepiandrosterone, DHEAS: dehydroepiandrosterone-3-sulfate, DPDPE: [D-Pen(2),D-Pen(5)]-enkephalin, EOB-DTPA: gadoxetate dimeglumine, FXR/BAR: Farnesoid X Receptor/Bile Acid Receptor, GK: glucokinase, GKA: glucokinase activator, HMG-CoA: 3-hydroxy-3-methylglutaryl coenzyme A, HNF-1a: Hepatic Nuclear Factor 1a, MATE: multidrug and toxin extrusion transporters MRI: magnetic resonance imaging, MRP: multidrug resistance-associated protein, NIR: near-infrared, OAT: organic anion transporter, OATP: Organic anion transporting polypeptide, OCT: organic cation transporter, rT3: reverse triiodothyronine, SCD1: Stearoyl-CoA desaturase-1, SCLC: small cell lung cancer, SLC: Solute Carriers, STS: steroid sulfatase, T3: triiodothyronine, T4: thyroxine

Introduction

Transmembrane transporters are regulating the uptake and efflux of several important endobiotics, such as nucleotides, amino acids, sugars and inorganic ions, as well as of xenobiotics, such as drugs and toxins.¹⁻⁴ Specific membrane transporters are expressed in the basolateral and/or canalicular membrane

of hepatocytes, enterocytes, renal tubular epithelial cells, as well as important body barriers, such as the blood-brain barrier, blood-testis barrier and the placental barrier.¹ Due to their involvement in intestinal absorption, tissue distribution, as well as biliary and urinary excretion of drugs, distinct transporters are also inherently linked to the ADME profile of many drugs. Thus, transporters can affect the efficacy, as well as toxicity of drugs and drug candidates.^{1, 3-9}

Organic anion transporting polypeptides (humans: OATPs, rodents: Oatps) are attracting more and more attention across the scientific community, due to their vital role in transport of endobiotics and xenobiotics.^{2, 9, 10} They are expressed in several epithelia throughout the body, such as blood-brain barrier, liver, intestine, kidney, lungs, skeletal muscles, testis and placenta.¹¹⁻¹³ Almost all OATP family members are localized to the basolateral membrane of polarized cells. Interestingly, in addition to their basolateral localization in liver and placenta, OATP2B1 and OATP1A2 have been detected in the apical membrane of enterocytes in the human small intestine, where they play a significant role in the intestinal absorption of drugs.¹ They are responsible for the sodium-independent transport of a wide variety of endogenous amphipathic compounds, such as bile salts, bilirubin, organic dyes, steroids and its conjugates, thyroid hormones, anionic oligopeptides, cyclic and linear peptides, mushroom toxins and food constituents.^{2, 10, 11, 14, 15} Although the majority of the substrates are anions, many OATPs can transport also neutral or even cationic compounds.¹¹ Additionally, among their substrates are several drugs, such as statins, angiotensin-converting enzyme inhibitors, angiotensin receptor blockers, antibiotics, antihistaminics, antihypertensive and anticancer drugs (Table 1).^{10, 11, 13, 16}

Table 1 : Substrate classes of human OATPs¹⁰

OATP	Endogenous Substrates	Xenobiotic Substrates	Model Substrates
OATP1A2	bile salts, bilirubin, steroid hormone metabolites, thyroid hormones and metabolites	liver function markers, β -blockers, statins, antiviral drugs, antibiotics, mushroom toxins, neuropeptides, anticancer drugs	Bromosulphophthalein Estrone-3-sulfate Fexofenadine
OATP1B1	bile salts, bilirubin, steroid hormone metabolites, thyroid hormones and metabolites, inflammatory mediators	liver function markers, mushroom toxins, statins, sartanes, antibiotics, antiviral drugs, anticancer drugs	Bromosulphophthalein Estradiol-17 β -glucuronide Estrone-3-sulfate Pitavastatin Atorvastatin Pravastatin Rosuvastatin Valsartan
OATP1B3	bile salts, bilirubin, steroid hormone metabolites, thyroid hormones, inflammatory	liver function markers, mushroom toxins, statins, sartanes,	Bromosulphophthalein Cholecystokinin octapeptide Estradiol-17 β -glucuronide

	mediators	antibiotics, antiviral drugs, anticancer drugs, peptides	Valsartan
OATP1C1	thyroid hormones and metabolites, steroid hormone metabolites	liver function markers	Thyroxine
OATP2A1	inflammatory mediators		Prostaglandines (PGE1, PGE2, PGE2 α)
OATP2B1	steroid hormone metabolites, inflammatory mediators, thyroid hormones	liver function markers, statins	Bromosulphophthalein Estron-3-sulfate
OATP3A1	steroid hormone metabolites, inflammatory mediators, thyroid hormones		PGE1 PGE2
OATP4A1	bile salts, steroid hormone metabolites, thyroid hormones		Triiodothyronine Taurocholate
OATP4C1	bile salts, steroid hormone metabolites, thyroid hormones		Estrone-3-sulfate Digoxin

The exact mechanism(s) of OATP transport is unknown in detail. It is well defined that the transport is ATP-independent and there is no implication of sodium, potassium or chloride gradients, but the exact driving force of transport is still controversial.^{8, 10, 11} However, an accumulating amount of evidence suggests the implication of an anion exchange transport mechanism.¹⁰ More particularly, OATPs are capable of bi-directional transport and several studies suggest that they are acting as electroneutral exchangers. Evidence suggests that OATPs/Oatps may exchange their substrates for intracellular bicarbonate, glutathione or glutathione conjugates.^{10, 11} Nevertheless, there might be differences in transport mechanism across different OATP members. For example, OATP1B1 and OATP1B3 mediated transport is not affected by glutathione.^{11, 17} Additionally, OATP transport can also be affected by the pH (e.g. OATP1B1 transport is increased under acidic conditions), but this is not a universal rule, since the pH-influence is modulated by substrate affinity and membrane potential.^{11, 18} It has also been suggested that OATPs transport substrates through a positively charged pore via a rocker-switch mechanism.^{11, 19} Unfortunately, any effort for *in silico* studies is hindered by the high complexity of these systems: OATPs have multiple binding sites and/or translocation pathways.^{10, 11, 13}

In general, human OATPs range in size between 643 to 724 amino acids, with the exception of the still uncharacterized OATP5A1, which contains 848 amino acids.^{11, 16} Considering that all mammalian OATPs share at least 30% similarity with their most distant relative, it is highly possible that they share great

similarities among their transmembrane domains.¹⁰ Even though there is still no crystal structure for any OATPs –like the vast majority of mammalian transporters- several studies have shed light into the structural characteristics of this superfamily of transporters.^{11, 13} Nevertheless, the exact architecture of the substrate binding site of OATPs remains unclear. However, there is growing evidence that there exist more than one binding site, even though this does not apply for every OATP.^{10, 13, 20} OATPs are believed to contain 12 transmembrane domains, with both the amino-terminal and C-terminal edges located intracellular.^{10, 11, 13} Despite the fact that hydropathy models predict a 10- or 12-transmembrane domain architecture, the theory of the 12-transmembrane domain has been confirmed by Wang et al. (2008) for Oatp1a1.^{10, 11} Additionally, the second and the fifth extracellular loop contain several predicted and/or confirmed N-glycosylation sites, which are believed to be conserved for several members of the family.^{10, 11, 16} The fifth extracellular loop contains many conserved cysteins, that are believed to play an important role for the formation of disulfide bonds, which are essential for the expression of functional proteins.^{10, 11, 13, 16} The most highly conserved part of the OATPs family signature is located at the extracellular border of loop 3 in transmembrane domain 6. This sequence contains three conserved tryptophan residues and is used to discriminate Oatps in the different databases. However it is not clear if this sequence is essential for function or proper membrane targeting.¹⁶ Moreover, homology modeling techniques have been used to identify important amino acids for the transporting capability of different OATP-members, but more has to be done towards this direction.^{10, 11, 13}

The first Oatp was identified and isolated via expression cloning from rat liver in 1994, using the *Xenopus laevis* oocyte system.^{7, 13, 15, 16} The following years several more OATPs/Oatps were cloned from different species, using homology screening, either by hybridization experiments, or *in silico*, including human, rat, mouse, cow, horse, pig, chicken, quail and frog, as well as, fruitflies, bees, nematodes, sea urchins, zebrafish, catfish and pufferfish.^{15, 16} Compared to the 52 members of OATP family reported in 2004, today more than 300 members from over 40 species are identified or predicted.^{10, 13} The first human superfamily member identified in 1995 was OATP1A2, followed by the description of OATP1B1.^{1, 9, 15} Interestingly, no OATP homologues have been found in bacteria or yeast, suggesting that they are specific only for the animal kingdom, and particularly for animals that belong to bilaterians, i.e. species that belong to the clade of protostomia (e.g. arthropods, nematodes), or to the clade of deuterostomia (e.g. vertebrates, echinoderms).^{13, 15}

OATPs are encoded by the genes of *SLCO/Slco* (*SLCO* for humans/*Slco* for rodents) superfamily.^{2, 6, 11, 13, 15} The particular superfamily was originally named *SLC21A*, however the nomenclature of its members was

updated and standardized in 2004 on the basis of phylogenetics relationships, resulting in renaming it *SLCO*, the solute carrier family of OATPs.^{6, 11, 13, 15} The new nomenclature system, introduced and approved by the HUGO Gene Nomenclature Committee, allow the naming of any newly identified OATP with a unique name, if it is a unique member of the family, or with the name of its already known orthologue.^{6, 13} According to the rules of this classification system, proteins with more than 40% sequence identity belong to the same family and proteins with more than 60% sequence identity belong to the same subfamily.^{7, 13, 16} Up to now 11 human OATPs have been identified, which are assigned into 6 distinct families: OATP1, OATP2, OATP3, OATP4, OATP5 and OATP6, while there are also subfamilies, within the same family: OATP1A, OATP1B and OATP1C.^{3, 7, 10, 13, 16} The different proteins are named OATP, followed by the number of the family (e.g. OATP1, OATP2), followed by the subfamily letter (e.g. OATP1A, OATP1B) while a consecutive number, identifying the different subfamily members is following, which is indicating the chronological order of the member identification.^{11, 13}

Table 2 provides an overview on the human and rodent members of the OATP/SLCO superfamily, including a summary of new and old classification/nomenclature, the predominant substrates, the tissue distribution/subcellular expression, any link to a disease, gene localization, sequence accession id and the number of splice variants.^{13, 15}

Table 2: Human and rodent members of the OATP/SLCO superfamily. Summary of new and old classification/nomenclature, predominant substrates, tissue distribution/subcellular expression, link to a disease, gene localization, sequence accession ID and number of splice variants.^{13, 15}

New gene symbol ^a	New protein name ^a	Old gene symbol	Old protein name	Predominant substrates	Tissue distribution-Cellular/Subcellular expression	Link to a disease	Gene locus ^b	Sequence accession id	Splice Variants
<i>Slco1a1</i>	Oatp1a1	<i>Slc21a1</i>	Oatp1, Oatp	Bile salts, organic anions, organic cations	Liver (basolateral membrane of the hepatocyte), kidney (apical membrane of proximal tubule), choroid plexus (apical)	-	4q44 (r) 6A3-A5 (m)	NM_017111 NM_013797	
<i>SLCO1A1</i>	OATP1A2	<i>SLC21A3</i>	OATP-A, OATP	Bile salts, organic anions, organic cations	Brain (endothelial cells), kidney (apical), liver (cholangiocytes), eye (ciliary body)	-	12p12(h)	NM_021094 NM_134431	2
<i>Slco1a3</i>	Oatp1a3_v1 Oatp1a3_v2	<i>Slc21a4</i>	OAT-K1 OAT-K2	Bile salts, organic anions	Kidney	-	4q44 (r)	NM_030837	1
<i>Slco1a4</i>	Oatp1a4	<i>Slc21a5</i>	Oatp2	Digoxin, bile salts, organic anions, organic cations	Liver, blood-brain barrier, choroid plexus, ciliary body, retina	-	4 (r) 6G2 (m)	NM_131906 NM_030687	
<i>Slco1a5</i>	Oatp1a5	<i>Slc21a7</i>	Oatp3	Bile salts, organic anions	Jejunum, choroid plexus	-	4q44 (r) 6G2 (m)	NM_030838 NM_130861	
<i>Slco1a6</i>	Oatp1a6	<i>Slc21a13</i>	Oatp5	-	-	-	4q44 (r) 6G2 (m)	NM_130736 NM_023718	
<i>SLCO1B1</i>	OATP1B1	<i>SLC21A6</i>	OATP-C, LST-1, OATP2	Bile salts, organic anions	Liver (hepatocytes)	Statin-induced myopathy, Rotor Syndrome	12p12(h)	NM_006446	

<i>Slco1b2</i>	Oatp1b2	<i>Slc21a10</i>	Oatp4, Lst-1	Bile salts, organic anions	Liver, ciliary body	4q44 (r) 6G2 (m)	NM_031650 NM_020495
SLC01B3	OATP1B3	SLC21A8	OATP8	Bile salts, organic anions	Liver (hepatocytes)	12p12(h)	NM_019844
SLC01C1	OATP1C1	SLC21A14	OATP-F, OATP-RP5	T4, T3, rT3, BSP	Brain (blood–brain barrier), testis (Leydig cells)	12p12(h)	NM_017435
<i>Slco1c1</i>	Oatp1c1 Oatp1c1	<i>Slc21a14</i> <i>Slc21a14</i>	Oatp14, BSAT1, Oatp2	As above	As above	4q44 (r) 6G1 (m)	NM_053441 NM_021471
SLC02A1	OATP2A1	SLC21A2	hPGT	Prostaglandins	Ubiquitous	3q21 (h)	NM_005621
<i>Slco2a1</i>	Oatp2a1 Oatp2a1	<i>Slc21a2</i>	rPGT mPGT	As above	As above	8q32 (r) 9F1 (m)	NM_022667 NM_033314
SLC02B1	OATP2B1	SLC21A9	OATP-B, OATP-RP2	E-3-S, DHEAS, BSP	Liver (hepatocytes), placenta, intestine (apical), eye (ciliary body)	11q13 (h)	NM_007256
<i>Slco2b1</i>	Oatp2b1	<i>Slc21a9</i>	Oatp9, moat1	As above	As above	-	NM_080786
SLC03A1	OATP3A1	SLC21A11	OATP-D, OATP-RP3	E-3-S, prostaglandin	Testis, heart, brain, ovary	15q26 (h)	NM_013272
<i>Slco3a1</i>	Oatp3a1 Oatp3a1	<i>Slc21a11</i> <i>Slc21a11</i>	OATP11 MJAM	As above	As above	7D1 (m)	AF239219 NM_023908
SLC04A1	OATP4A1	SLC21A12	OATP-E, OATP-RP1	Taurocholate, T3, prostaglandin	Ubiquitous	20q13.1 (h)	NM_016354
<i>SLC04a1</i>	Oatp4a1	<i>Slc21a12</i>	Oatp12, oatpE	As above	As above	2H4 (m)	NM_133608 NM_148933
SLC04C1	OATP4C1	SLC21A20	OATP-H	Digoxin, ouabain, thyroid hormones, methotrexate	Kidney (basolateral)	5q21 (h)	AY273896

SLC05A1	OATP5A1	SLC21A15	OATP-J, OATP-RP4	-	-	8q13.1 (h)	NM_030958	3
SLC06A1	OATP6A1	SLC21A19	OATP-I, GST	-	Testis	5q21 (h)	NM_173488	
<i>Slco6b1</i>	<i>Oatp6b1</i>	<i>Slc21a16</i>	<i>Oatp16, TST-1, GST-1</i>	Taurocholate, T3, T4, DHEAS	Testis, epididymis, ovary, adrenal gland	9 (r) 1 (m)	NM_133412 AK006249	
<i>Slco6c1</i>	<i>Oatp6c1</i>	<i>Slc21a18</i>	<i>Oatp18, TST-2, GST-2</i>	Taurocholate, T3, T4, DHEAS	Testis	9 (r) 1 (m)	NM_173338 AK016647	
<i>Slco6d1</i>	<i>Oatp6d1</i>	<i>Slc21a17</i>	<i>Oatp17</i>	-	-	1 (m)	AK014872	

^a Capital letters correspond to human genes and proteins while lower case symbols correspond to lower case symbols. Because there are some exceptions for the old protein names that may correspond to rodent proteins, human entries are also represented with bold symbols.

^b (h) human, (r) rat, (m) mouse

The following section comprises a short description of the characteristics of each OATP family and its individual members:

Family OATP1

OATP1 contains four human subfamilies: OATP1A2, OATP1B1, OATP1B3 and OATP1C1. It is the best described family and the largest one, containing 27 members.^{11, 13, 15}

Subfamily OATP1A

OATP1A2 is the only member of the OATP1A subfamily that contains several rat and mouse members that have probably arose through gene duplication.^{15, 16} Human OATP1A2 is a glycoprotein of 670 amino acids, having a molecular mass of approximately 85 kDa in the liver and 65 kDa in the brain, due to incomplete glycosylation.¹⁶ Messenger RNA of SLCO encoding OATP1A2 has been found in several tissues throughout the body, including brain, liver, kidneys, intestine, lungs, testes, prostate and placenta.^{11, 13} Having this wide distribution across organs, it is thought to possess a crucial role in the absorption, distribution and excretion of xenobiotics. At protein level, OATP1A2 has been found to be expressed in endothelial cells of the blood-brain barrier^{13, 16} and in cholangiocytes, but not in hepatocytes in the liver.^{11, 13, 16} OATP1A2 is also localized in the brush border membrane in the distal nephron (kidney)^{11, 13, 16} and in the apical membrane of the enterocytes (intestine)¹⁶. However, it must be noted that a study by Meier et al. (2007) found no detectable OATP2A1 mRNA in duodenum.⁷ Among substrates of OATP1A2 are bile salts and bromosulfophthalein (BSP), steroid conjugates, the thyroid hormones like T3 (triiodothyronine), T4 (thyroxine), rT3 (reverse triiodothyronine), prostaglandin E2, the endothelin receptor antagonist BQ-123, the thrombin inhibitor CRC-220, the opioid receptor antagonist DPDPE and deltorphin II, fexofenadine, certain magnetic resonance imaging contrast agents, ouabain, the lipophilic organic cations N-(4,4-azo-n-pentyl)-21-ajmalinium, N-methyl-quinine and -quinidine, and the cyanobacteria toxin microcystin. In general, it has to be marked out that human OATP1A2 transports the largest number of amphipathic compounds of all human OATPs.¹⁵

Subfamily OATP1B

Subfamily OATP1B is consisted of two human members: **OATP1B1** and **OATP1B3**.^{11, 13, 15, 16}

OATP1B1 was cloned from human liver and it is a glycoprotein consisted of 691 amino acids. It shares 80% sequence identity with OATP1B3,^{9, 15} the other member of the subfamily, but only 64%/65% sequence identity with the rat/mouse orthologue Oatp1b2. Also several polymorphisms have been described for SLCO1B1 that encodes OATP1B1.¹⁵ Its apparent molecular mass is 84 kDa, which is reduced after de-glycosylation to 54 kDa.^{15, 16} Under normal conditions, it is almost selectively expressed in the basolateral (sinusoidal) membrane of the hepatocytes in the liver, throughout the lobule.^{5, 9, 11, 13, 15, 16, 21} It was cloned by human liver cDNA libraries by three different groups (Abe et al., Hsiang et al. and König et al.) in 1999.¹⁶ The almost exclusive expression of this transporter in the human hepatocyte suggests that it plays an important role in the uptake and hepatic clearance of albumin-bound amphipathic organic compounds.^{15, 16} It has a wide range of substrates, including endogenous substances, such as bile salts, conjugated and unconjugated bilirubin, thyroid hormones, eicosanoids, BSP, steroid conjugates, cyclic and linear peptides, natural toxins such as microcystin and phalloidin, as well as drugs of several therapeutic classes, like antibacterial drugs, anticancer drugs, statins and cardiovascular drugs.^{9, 11, 13, 15}

OATP1B3 was also cloned from human liver. It is a glycoprotein of 702 amino acids, with a molecular mass of 120 kDa that is reduced to 65 kDa after deglycosylation.^{15, 16} Like OATP1B1, OATP1B3 is also, considered almost a liver specific transporter. Under normal conditions it is expressed on the basolateral membrane of the hepatocyte^{5, 11, 13, 15, 16}, but in comparison to OATP1B1, it is primarily expressed around the central vein and not the portal vein.^{11, 13, 16} Whether this specific expression serves a particular physiological function is not known yet.¹⁶ This difference in expression between OATP1B1 and OATP1B3 has been used to explain the experimental findings (Michalski et al. 2002, Briz et al. 2006) where mRNA levels of OATp1B1 are higher in liver homogenate than the respective levels of OATP1B3 mRNA.¹¹ However, a more recent study (Ji et al. 2012) reports that both proteins are found in equal amounts in membrane fractions isolated by human hepatocytes.¹³ Moreover, hepatic expression of OATP1B3 depends on HNF-1a (Hepatic Nuclear Factor) and the bile acid nuclear receptor FXR/BAR (Farnesoid X Receptor/Bile Acid Receptor).^{12, 15} The latter statement implies that induction of SLCO1B3 that encoded OATP1B3 by bile acids could serve to maintain the hepatic extraction of xenobiotics and peptides under cholestatic conditions.¹⁵ OATP1B3 and OATP1B1 present a wide overlap in terms of substrates, sharing the same kind of endogenous and exogenous compounds. However, there are also some selective substrates for OATP1B3, which is considered the only hepatic OATP transporting digoxin^{7, 16, 21, 22}, paclitaxel^{7, 22}, doxetaxel^{7, 22} and CCK-8^{21, 22} (cholecystokinin octapeptide). Additionally, in comparison to

OATP1B1 and OATP2B1, OATP1B3 has been found capable of transporting amanitin, a natural toxin present in the mushrooms of *Amanita*.⁷

Subfamily OATP1C

Subfamily OATP1C contains only one human member: **OATP1C1**. OATP1C1 was first isolated from a cDNA brain library, consists of 712 amino acids and has a molecular mass of around 78 kDa.^{15, 16} It exhibits amino acid sequence identity of 85%/84% with the respective rat/mouse ortholog Oatp1c1.¹⁵ On mRNA level, it is expressed in the brain,^{11, 13, 15, 16} in glial cells throughout the hypothalamus,¹³ on the basolateral membrane of choroid plexus epithelial cells^{11, 13}, in Leydig cells of testes^{11, 13, 15, 16} and in the pars plana of ciliar epithelium.¹³ In contrast to multispecific OATP1A2, OATP1B1 and OATP1B3, OATP1C1 has a narrower range of substrates. It transports to some extent the common OATP substrates like bromosulphophthalein (BSP), estradiol-17 β -glucuronide, estrone- β -sulfate, and shows specificity towards the transport of thyroid hormones like T3 (triiodothyronine), rT3 (reverse triiodothyronine) and T4 (thyroxine).^{15, 16}

Family OATP2

Family OATP2 consists of two human subfamilies: OATP2A and OATP2B.^{15, 16}

Subfamily OATP2A

Subfamily OATP2A also contains only one human member: **OATP2A1**. OATP2A1 – alternatively known as prostaglandin transporter (PGT) - is a protein of 643 amino acids and a calculated molecular mass of around 70 kDa.^{15, 16} It shows 82%/83% amino acid sequence identity to its rat/mouse ortholog Oatp2a1.¹⁵ It was first cloned by a human kidney library.¹⁵ OATP2A1 is considered as one of the ubiquitously known OATPs, since it is expressed in almost every tissue tested.^{11, 13, 16} mRNA of OATP2B1 was found in brain, colon, heart, kidney, liver, lungs, ovary, pancreas, placenta, prostate, skeletal muscles, spleen and small intestine,^{11, 16} with higher mRNA levels found in heart, skeletal muscles and pancreas¹⁵. At the protein level, human OATP2A1 has been found to be expressed in retinal epithelial cells and in epithelial and endothelial cell layers of different eye tissues including the ciliary body, in the endometrium, in neurons, astrocytes, and microglia, as well as in the parietal cells of the gastric corpus

and the pyloric glands of the antrum.¹³ Recently, a study by Mandery et al. (2010) has found OATP2A1 protein expression in the upper gastrointestinal tract, localized in the pyloric glands of the antrum and parietal cells of the gastric corpus.¹¹ Among the endogenous substrates of OATP2A1 are prostaglandins and eicosanoids, but none of the typical OATP substrates, such as bromosulfophthalein (BSP), estradiol-17 β -glucuronide and estrone- β -sulfate.¹⁶ Currently no drugs are known to be substrates of OATP2A1⁷, apart from latanoprost free acid¹¹, which is technically a prostaglandin analogue.²³ OATP2A1 has been suggested to be involved in terminating prostaglandin signaling by transporting prostaglandins inside the cells.^{11, 16}

Subfamily OATP2B

OATP2B1 is the only human member of the OATP2B subfamily. It is a glycoprotein of 709 amino acids, presents 77% sequence identity with the respective rat orthologue,^{15, 16} and was first cloned by human brain cDNA.^{15, 24} It has a molecular mass of approximately 85 kDa in liver, placenta and heart, 95 kDa in ciliary body, while in the brain two bands of approximately 84 kDa and 95 kDa were detected, implying different ways of protein glycosylation.¹⁶ It is widely expressed throughout different organs and tissues of the body, such as liver placenta, brain, heart, lungs, kidney, spleen, testes, ovary and colon,^{11, 15, 16} but its highest levels were found in the liver.^{11, 15} At protein level, OATP2B1 can be detected in the sinusoidal membrane of hepatocytes, in the basolateral membrane of syncytiotrophoblasts, in the brush-border membrane in the small intestine, in keratinocytes, in the mammary gland, in the luminal membrane of endothelial cells of the blood–brain barrier, in the pars plicata and pars plana of the ciliary body, in endothelial cells in the heart, in human platelets, and in the skeletal muscles.¹³ Initially, OATP2B1 was believed to have a quite limited range of endogenous substrates. In studies on physiological pH of 7.4, it was able to transport only BSP, estrone-3-sulfate, and dehydroepiandrosterone-3-sulfate (DHEAS).^{15, 16} For other compounds, such as PGE2 and estradiol-17 β -glucuronide, controversial results were obtained.¹⁵ More recently, transport studies were conducted in acidic pH, where OATP2B1 was able to transport a wider range of compounds, including also taurocholate, fexofenadine, statins, glibenclamide, and the loop diuretic M17055.¹⁶ Taking the latter fact in mind, together with the ubiquitous expression of OATP2B1, it could constitute OATP2B1 responsible for the absorption and disposition of several endobiotics and xenobiotics.¹⁶

Family OATP3

Family OATP3 consists of one human subfamily: OATP3A.^{15, 16}

Subfamily OATP3A

Subfamily OATP3A has a single human member: OATP3A1. Depending on the tissue, the single gene *SLCO3A1* is encoded into two splice variants OATP3A1_v1 and OATP3A1_v2. The two proteins have a sequence of 710 and 692 amino acids, and a molecular mass of approximately 76 kDa and 74 kDa, respectively, with the only difference located in the C-terminal end.¹⁶ OATP3A1 shares a sequence identity of 97% with the rat and mouse respective orthologs *Oatp3a1*, which renders it the most conserved within the whole OATP superfamily.^{15, 16} OATP3A1_v1 was first cloned by a human kidney cDNA library, while OATP3A1_v2 was first cloned by a human brain cDNA library.¹⁶ OATP3A1 is ubiquitously expressed in several tissues throughout the body, with highest levels in testes, brain, heart, lungs, spleen, peripheral blood leucocytes and thyroid gland.^{13, 16} At the protein level, OATP3A1 is found in the ciliary body epithelium, in testes, in the choroid plexus, in neurons in the frontal cortex, at the plasma membrane of epithelial cells of the lactiferous ducts in normal breast tissue, and in the epidermal keratinocytes.^{11, 13} It has also been found that OATP3A1_v1 is the variant that is ubiquitously expressed, while OATP3A1_v2 is restricted in testes and brain.⁷ Additionally, in testes and in the brain two splice variants were shown to be expressed in a cell type-specific pattern. In testes, OATP3A1_v1 is expressed in germ cells while OATP3A1_v2 is expressed in Sertoli cells. In the choroid plexus variant 1 is expressed at the basolateral membrane while variant 2 is expressed at the apical and sub-apical membrane. In the frontal cortex, OATP3A1_v1 is localized in neuroglial cells of the grey matter and OATP3A1_v2 in cell bodies and axons of the neurons.^{11, 13, 16} Regarding their substrates, both splice variants transport prostaglandins, thyroid hormones, the cyclic peptide BQ-123, and vasopressin.¹⁶ Since now, transport of drugs by OATP3A1 has not been well described.⁵ Taking into account the high level of amino acid sequence conservation, the brain localization and the ability of transporting peptides, it can be deduced that OATP3A1 might be playing an important physiological role in the transport of neuro-active peptides and thyroid hormones, but further studies are required to enlighten more this aspect.¹⁶

Family OATP4

Family OATP4 consists of two human subfamilies: OATP4A and OATP4C.^{15, 16}

Subfamily OATP4A

Subfamily OATP4A has a single human member: **OATP4A1**. It is a protein of 722 amino acids, having a molecular mass of approximately 65 kDa in placenta and brain. It was first cloned by brain and kidney human cDNA libraries,^{15, 16} and shows 76% amino acid sequence identity with its rodent ortholog Oatp4a1.¹⁵ It has been detected in several tissues throughout the body, with highest levels in heart and placenta, followed by lungs, liver, skeletal muscles, kidney and pancreas.^{11, 13, 16} At protein level it has been detected in the ciliary body epithelium and in the apical membrane of syncytiotrophoblasts in placenta.^{11, 13, 16} In contrast to other multispecific OATPs, OATP4A1 has a rather narrow range of substrates. A first study by Tamai et al. (2000) reported the transport of oestrone-3-sulfate, oestradiol-17 β -glucuronide, benzylpenicillin and prostaglandin E2.^{16, 24} However, a second study by Fujiwara et al. (2001) reported the transport of thyroid hormones and taurocholate, but did not confirm the transport of prostaglandin E2.¹⁶ Analogously to OATP3A1 transport of drugs by OATP3A1 has not been well described up to now.⁵ Since it is abundantly expressed in placenta and it can transport thyroid hormones, OATP4A1 is believed to play an important role for transporting thyroid hormones from the mother to the fetus.¹⁶

Subfamily OATP4C

Subfamily OATP4C has a single human member: **OATP4C1**. It is a protein of 724 amino acids and a molecular mass of approximately 79 kDa. It was first cloned by a kidney human cDNA library, while it presents 80% sequence identity with its respective rat ortholog Oatp4c1.¹⁶ Based on northern blot analysis during the study by Mikkaichi et al. (2004), OATP4C1 was originally believed to be a kidney specific protein.^{11, 13, 16} Based on the localization of the rat Oatp4c1, human OATP4C1 is assumed that it is also localized in the basolateral membrane of proximal tubule cells.¹¹ Nevertheless, a more recent study in 2006 by Bleasby et al., suggests that OATP4C1 could also be expressed in the liver. However, this has not been confirmed by RT-PCR or protein analysis.^{11, 13} OATP4C1, similarly to the other member of the OATP4 family (OATP4A1) presents also a narrow range of substrates, transporting digoxin,

ouabain, triiodothyronine, thyroxine, methotrexate, cyclic AMP (cAMP) and the dipeptidyl peptidase-4 inhibitor sitagliptin. Due to its capability to transport thyroid hormones, it might possess an important role for transporting thyroid hormones to the kidney.¹⁶

Family OATP5

Family OATP5 consists of one human subfamily: OATP5A.^{15, 16}

Subfamily OATP5A

Subfamily OATP5A has a single human member: **OATP5A1**. OATP5A1 is a protein of 848 amino acids and a molecular mass of 79 kDa.¹⁶ On mRNA level it has been detected in fetal brain, prostate, skeletal muscles and thymus.^{11, 13} At protein level, it has been detected in the plasma membrane of epithelial cells of the lactiferous ducts in normal breast tissue.¹³ It needs further investigation regarding the localization of its expression and its substrates.

Family OATP6

Family OATP6 consists of one human subfamily: OATP6A.^{15, 16}

Subfamily OATP6A

Subfamily OATP6A has a single human member: **OATP6A1**. OATP6A1 is a protein of 719 amino acids and a molecular mass of 79 kDa. It was first cloned from a human testis cDNA library.¹⁶ On mRNA level it has been detected mainly in testes, and in lower levels in spleen, brain, fetal brain and placenta.^{11, 13, 16} Additional experiments are needed to confirm that OATP6A1 is indeed the functional ortholog of rat Oatp6b1 and Oatp6c1, which transport taurocholate, DHEAS, T3 and T4.¹⁶

OATPs and Genetic Diseases

Up to now, no severe human diseases concerning bile salt homeostasis, thyroid hormone biogenesis and metabolism, or steroid hormone biogenesis, have been found which are linked to mutations of the SLCO genes encoding OATPs. However, a few pathophysiological conditions that are associated to SLCO genes mutations have been identified.¹³

Mesolemia-synosteses syndrome (OMIM600383) is a rare genetic condition, which results in mesolemic limb shortening and acral synostoses. It is believed that the reason of the disease is unregulation in sulfate metabolism and/or homeostasis. A study on five patients from four different families showed a submicroscopic microdeletion on chromosome 8q13. The deletion spans two genes: SULF1 (heparin sulphate 6-O-endosulfatase 1) and SLCO5A1 (OATP5A1). As mentioned above, the function of OATP5A1 has not been well characterized so far. Since in all patients' cases the deletions spans both genes, the role of a missing or malfunctioning OATP5A1 needs to be cleared out, especially since a partial deletion of OATP5A1 has been reported for a healthy patient.¹³

Rotor syndrome is a rare, benign condition which is characterized by mild, mainly conjugated, but also unconjugated hyperbilirubinemia, which is characterized by an increase of total conjugated and unconjugated bilirubin in sinusoidal blood- and coproporphynuria.^{1, 13, 25-30} Histopathological examination of the liver does not reveal any architectural or cytomorphological abnormalities and there is no pigment present.²⁸ Patients with Rotor syndrome exhibit total deficiency in OATP1B1 and OATP1B3, due to lacking or impaired SLCO1B1 and SLCO1B3 genes, respectively.^{13, 25-30} There doesn't seem to be impairment of the MRP2 function that could result in conjugated hyperbilirubinemia, but the conjugated bilirubin is secreted back to sinusoidal blood by MRP3. Interestingly, total impairment of both OATP1B1 and OATP1B3 doesn't seem to radically influence the physiological function of the liver, since Rotor syndrome is considered a benign condition. However, the fact that lack of both of these transporters has an effect on the clearance of conjugated bilirubin, suggests the existence of one or more so far undetermined transporter(s) for unconjugated bilirubin.³¹

Moreover, elevated serum levels of rT3 have been found in carriers of the OATP1A2 p.172D variant. Additionally, a genome-wide association study in patients with progressive supranuclear palsy (PSP), a neurodegenerative condition that affects motor and cognitive functions and gradually leads to death³² revealed, among other genes, an association with SLCO1A2.¹³ Finally, a genome-wide association study

of Crohn's disease in an Ashkenazi Jewish population indicated an association with a variant of *SLCO6A1*.¹³

The above conditions are not curable and thus mainly symptomatically treated. Despite the genetic association of OATPs to those conditions, targeting OATPs as a mean of treatment seems not efficient.

OATPs and Cancer

The localization of OATPs under normal conditions has been described earlier. However, it was not mentioned that under pathological conditions such as cancer, the expression of OATPs in tissues changes, presenting either over-expression, or under-expression, depending on the transporter, tissue and type of cancer.³³⁻³⁶ A second characteristic of OATPs in association with cancer is the fact that several anticancer agents are substrates³⁴⁻³⁷ and/or inhibitors³⁸⁻⁴¹ of OATPs and they play an important role in the pharmacokinetics and pharmacodynamics of these drugs^{7, 34, 42-44}. Table 3^{11, 34, 36, 43} depicts a summary of endogenous and exogenous substrates of OATPs, as well as the localization of the transporters in healthy and cancer tissues. Moreover, among OATPs endogenous substrates are also several hormones and their conjugates, such as estrogens and androgens, which play a significant role for the development and progress of hormone-dependent cancers, such as breast cancer, ovarian cancer and prostate cancer.⁴⁵⁻⁵³

Because of the above characteristics of OATPs, they are believed to be able to contribute to cancer treatment with the following ways: i) regulate the OATP-mediated uptake of hormones, hormone conjugates and other tumor growing chemicals, by using selective OATP inhibitors, ii) develop novel anticancer agents as OATP substrates in order to increase the uptake of the drug in tumors where characterized by OATP over-expression, iii) enhance the uptake of anticancer agents by allosteric stimulators, iv) regulate the expression of OATPs in cytoplasmic membrane in order to increase or decrease the uptake of desired substrates into the cancer cells,⁴³ v) explain the reasons of chemoresistance for some anti-cancer agents and develop an OATP-targeted approach in order to reverse it^{54, 55}, vi) use of OATPs that are over-expressed in particular types of cancer as novel biomarkers for the response to chemotherapy and/or hormonal therapy³⁵.

Table 3: Summary of endogenous and exogenous substrates transported by human OATPs, highlighting the anticancer drugs (with red), and localization of OATPs in healthy and tumor tissues.^{11, 34, 36, 43, 51}

Transporter	Substrates	Localization in Healthy Tissues	Localization in Cancer (Change in Expression)*
OATP1A2	Hormones and conjugates Estradiol-17 β -glucuronide Estrone-3-sulfate DHEA-S Reverse triiodothyronine (rT3) Thyroxine (T4) Triiodothyronine (T3) Prostaglandins Prostaglandin E2 Bile acids Cholate Taurocholate Glycocholate Taurochenodeoxycholate Tauroursodeoxycholate Others DPDPE Drugs Acebutolol, Rosuvastatin, Atenolol, Pitavastatin, Sotalol, Ouabain, Labetalol, Deltorphan II, Nadolol, Ciprofloxacin, Talinolol, Fexofenadine, Saquinavir, Gatifloxacin, Darunavir, Levofloxacin Anticancer Drugs Imatinib, Methotrexate,	brain capillary endothelia; basolateral (abluminal) ^{a,b} liver; apical ^b kidney; apical ^b lung ^a small intestine; apical ^{a,b} eye pars plana ciliary body epithelium; basolateral ^b breast mammary epithelium ^a	colon polyp (decreased) ^a colon cancer (decreased) ^a brain glioma ^a breast cancer (increased) ^{a,b} non-small cell lung cancer (no change) ^a

	Paclitaxel, Doxorubicin, Docetaxel, Bemet-R2, Bemet-UD2		
OATP1B1	Hormones and conjugates Estradiol-17β-glucuronide Estrone-3-sulfate Thyroxine (T4) Triiodothyronine (T3) DHEA-S Prostaglandins Prostaglandin E2 Bile acids Cholate Taurocholate Tauroursodeoxycholate Imaging agents Gd-EOB-DTPA Drugs Atorvastatin, Olmesartan, Bosentan, Phalloidin, Caspofungin, Pitavastatin, Cefazolin, Pravastatin, Cerivastatin, Darunavir, Rifampicin, Enalapril, Rosuvastatin, Ezetimibe, Saquinavir, Fluvastatin, Temocapril, Gimatecan, Troglitazone, Lopinavir, Valsartan Anticancer Drugs Bamet-R2, Bemet-UD2, Methotrexate, BNP1350, Gimatecan, Doxorubicin,	liver; basolateral ^b small intestine ^a	colon polyp (increased) ^a colon cancer (increased) ^a hepatocellular carcinoma (no change) ^{a,b} hepatocellular carcinoma ^b

	Docetaxel, Flavopiridol, Rapamycin, Paclitaxel, CP- 724,714, Irinotecan (SN-38)		
OATP1B3	Hormones and conjugates Estradiol-17 β -glucuronide Estrone-3-sulfate DHEA-S Testosterone Imaging agents Gd-EOB-DTPA Drugs Atrasentan, Bosentan, Cefadroxil, Cefazolin, Cephalexin, Digoxin, Enalapril, Fexofenadine, Fluvastatin, Lopinavir, Demethylphalloin, Olmesartan, Phalloidin, Pitavastatin, Telmisartan, Rifampicin, Rosuvastatin, Valsartan Anticancer Drugs Paclitaxel, Docetaxel, Rapamycin, Methotrexate, Imatinib, Irinotecan (SN-38)	liver; basolateral ^{a,b} prostate ^a small intestine ^a	breast cancer ^b colon polyp (no change) ^a colon cancer (no change) ^a colon cancer (increased) ^{a,b} colon cancer ^{a,b} gastric cancer ^{a,b} hepatocellular carcinoma (decreased) ^{a,b} hepatocellular carcinoma ^b non-small cell lung cancer (increased) ^a pancreatic cancer ^{a,b} prostate cancer (no change) ^a prostate cancer metastasis (increased) ^a
OATP1C1	Hormones and conjugates Estradiol-17 β -glucuronide Estrone-3-sulfate Thyroxine (T4) Triiodothyronine (T3) Reverse triiodothyronine (rT3) Thyroxine sulfate (T4S) Others BSP	brain ^a eye pars plana ciliary body epithelium; basolateral ^b lung ^a testis; Leydig cells ^{a,b}	brain glioma ^a non-small cell lung cancer (no change) ^a

OATP2A1	Prostaglandins Prostaglandin E1 Prostaglandin E2 Prostaglandin F2 α Prostaglandin H2 Prostaglandin D2 8-iso-prostaglandin F2 α Others Thromboxane B2 Drugs Latanoprost	brain ^a breast ^a colon ^a eye ^a heart ^a kidney ^a liver ^a lung ^a prostate ^a skeletal muscle ^a small intestine ^a testis ^a	breast cancer (no change) ^a liver cancer (increased) ^a prostate (no change) ^a prostate cancer metastasis (increased) ^a non-small cell lung cancer (decreased) ^a
OATP2B1	Hormones and conjugates Estrone-3-sulfate DHEA-S Thyroxine (T4) Prostaglandins Prostaglandin E2 Drugs Atorvastatin Bosentan Ezetimibe Fluvastatin Glibenclamide Pitavastatin Pitavastatin Montelukast Rosuvastatin Talinolol	brain capillary endothelium; basolateral (abluminal) ^b breast lactiferous epithelium ^{a,b} colon ^a eye pars plana ciliary body epithelium; basolateral ^b heart ^a kidney ^a liver; basolateral ^{a,b} lung ^a ovary ^a prostate ^a skin ^{a,b} small intestine ^{a,b} spleen ^a	brain glioma ^a breast cancer (no change) ^a breast cancer (decreased) ^a breast cancer (no change) ^{a,b} non-small cell lung cancer (no change) ^a prostate (no change) ^a prostate cancer metastasis (increased) ^a
OATP3A1	Hormones and conjugates Thyroxine (T4) Estrone-3-sulfate Prostaglandins Prostaglandin E1 Prostaglandin E2	brain ^{a,b} breast lactiferous epithelium ^{a,b} choroid plexus ^{a,b} colon ^a heart ^a	breast cancer (decreased) ^a breast cancer (no change) ^{a,b} liver cancer (increased) ^a non-small cell lung cancer (no change) ^a

	Prostaglandin F2 α Drugs Deltorphan BQ-123 Benzylpenicillin Others Vasopressin Arachidonic acid	eye pars plana ciliary body epithelium; basolateral ^b kidney ^a leukocytes ^a liver ^a lung ^a ovary ^a pancreas ^a prostate ^a small intestine ^a testis ^{a,b}	prostate (no change) ^a prostate cancer metastasis (increased) ^a
OATP4A1	Hormones and conjugates Estradiol-17 β -glucuronide Estrone-3-sulfate Thyroxine (T4) Triiodothyronine (T3) Reverse triiodothyronine (rT3) Bile acids Taurocholate Prostaglandins Prostaglandin E2 Drugs Benzylpenicillin Unoprostone metabolite	brain ^{a,b} breast mammary epithelium ^a choroid plexus ^a colon ^a eye pars plana ciliary body epithelium; basolateral ^b heart ^a kidney ^a lung ^a ovary ^a pancreas ^a placenta ^{a,b} prostate ^a small intestine ^a testis ^a thymus ^a	breast cancer (decreased) ^a brain glioma ^a colon cancer (increased) ^a liver cancer (increased) ^a non-small cell lung cancer (no change) ^a prostate (no change) ^a prostate cancer metastasis (increased) ^a
OATP4C1	Hormones and conjugates Estrone-3-sulphate Thyroxine (T4) Triiodothyronine (T3) Others cAMP Drugs Digoxin , Ouabain, Sitagliptin	breast cancer (decreased) ^a brain glioma ^a	breast cancer (no change) ^a

	Anticancer Drugs		
	Methotrexate		
OATP5A1		breast lactiferous epithelium ^{a,b}	breast cancer (no change) ^{a,b} liver cancer (increased) ^a
OATP6A1		testis ^a	bladder cancer ^a oesophageal cancer ^a lung cancer ^a

Anticancer drugs are highlighted in red.

* Relative to paired healthy surrounding tissue

^a mRNA by qRT-PCR or Northern blot

^b protein by Western blot or immunohistochemistry

Abbreviations: BSP, bromosulphophthalein; DHEA-S, dehydroepiandrosterone sulfate; DPDPE, [D-penicillamine^{2,5}]enkephalin

Below follows the description of several types of cancer, the implication of OATPs and how they could be used as molecular and/or drug targets against these diseases.

Breast Cancer

Breast cancer is the most commonly diagnosed type of cancer and also the second main cause of cancer-associated death in women. Two thirds of the newly diagnosed breast cancers are hormone (estradiol)-related, which constitutes estrogens a main promoter of breast carcinogenesis.^{42, 46, 47} 75% of this hormone related cancers concern post-menopausal women. Interestingly, even though the plasma estradiol levels are 90% reduced in post menopause, there is no significant difference of estradiol levels in breast tissues between pre- and post-menopausal women, due to *in situ* synthesis of estradiol by exploitation of aromatase and sulfatase pathways. Estrone-3-sulfate is the primary source for tumor tissue estradiol.^{46, 47, 56} The concentration of estrone-3-sulfate in tumors is 2-20 times higher, than in normal plasma levels, while the tumor concentration of sulfatase, which converts estrone-3-sulfate into estrone (that is converted afterwards in estradiol), is 3-times higher, than in normal tissues. All this combined results in levels of estradiol being 2-3 times higher in tumor than in normal tissues.⁴⁶ Figure 1

provides the biosynthetic pathway of estrogens, including the conversion of estrone-3-sulphate to estrone.⁵⁷ Unlike estrone and estradiol, which are lipophilic, thus able to diffuse through the plasma membrane, estrone-3-sulphate is hydrophilic and has negative charge, so it needs an active transport mechanism to enter cells.⁴⁶ This active transport is provided through OATPs.^{45-47, 56, 58}

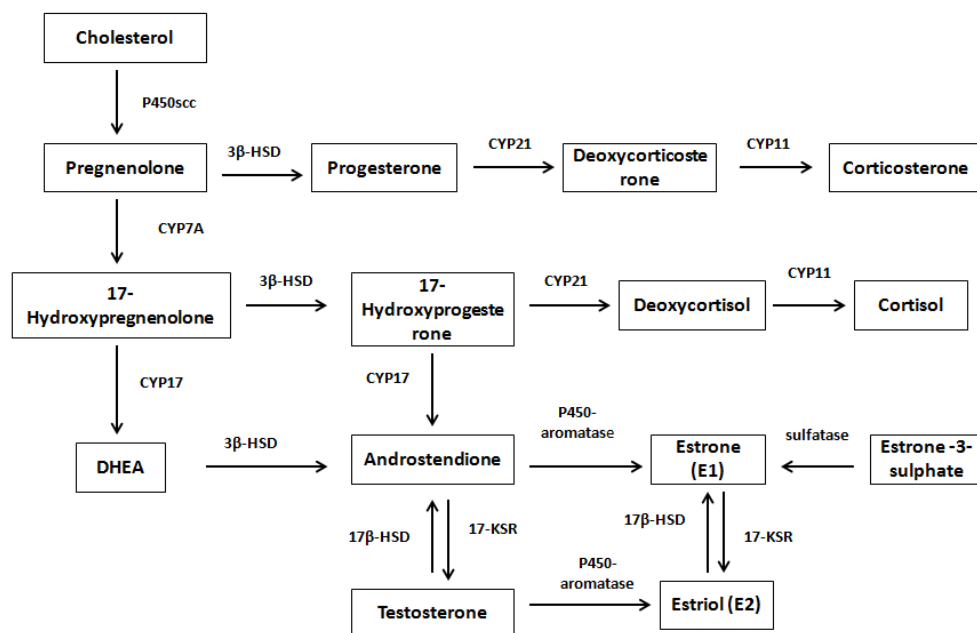


Figure 1: Biosynthetic pathway of estrogens from cholesterol.⁵⁷ DHEA, dehydroepiandrosterone-3-sulfate; E1, estrone; E2, estradiol; CYP, members of cytochrome P450; HSD, hydroxysteroid dehydrogenase, KSR, ketosteroid reductase.

Regarding the expression of OATPs in normal and malignant breast tissues, a study by Wlcek et al. in 2008 detected mRNA expression of OATP3A1 and OATP4A1⁵⁸ in breast tumor cells, also in concordance with the results of Pizzagali et al (2003)⁵⁹. However, in contrast to Miki et al (2006)⁶⁰, the levels of OATP2A1 were below detection limit for all four cell lines investigated.⁵⁸ In addition to OATP3A1 and 4A1, Wlcek et al. also identified for the first time the expression of OATP1B1, 1B3, 2A1, 4C1 and 5A1 in these cell lines by quantitative real-time RT-PCR.⁵⁸ An analogous study by Maeda et al. (2010) showed mRNA expression in malignant breast tissues of OATP1A2, OATP1B3, OATP3A1 and OATP4A1.⁵⁶ Another study by Kindla et al. (2011) demonstrated the mRNA expression of OATP2B1, OATP3A1, OATP5A1 in high levels in both malignant and normal tissues, some expression of OATP2A1, OATP4A1 and OATP4C1, whereas expression of OATP1A2, OATP1B1, OATP1B3, OATP1C1 and OATP6A1 was below the detection

limit.⁴⁸ A study by Banerjee et al. in 2012 revealed that the expression of 1A2, 1B1, 1B3, 2B1, and 3A1 is exclusive, similar, or significantly higher in cancer cells compared to MCF10A cells (normal breast tissue)⁴⁶, while a follow-up study by the same author in 2014 showed higher expression of these transporters in hormone-related breast cancer in contrast to non-hormone-related breast cancer⁴⁷. All the above studies confirm the expression or over-expression of several OATP members in malignant breast tissues, and since estrone-3-sulfate is a substrate for several of these OATPs, this proves their significance for the transport of estrone-3-sulfate in breast cancer.

In order to elucidate the role of estrone-3-sulfate in the proliferation of breast tumor cells, Nozawa et al. noticed significant cell growth for two different estrogen-dependent breast cancer cell lines (MCF-7 and T-47D) in presence of estrone-3-sulfate. During the studies, the proliferative activity was hindered by bromosulphophthalein (BSP), a universal OATP inhibitor.^{42, 61, 62} The particular findings^{42, 61, 62}, together with the findings of the studies by Banerjee^{46, 47}, Maeda⁵⁶ and Wlcek⁵⁸, suggest the potentiality of targeting OATPs in order to treat hormone-related breast cancer.

Ovarian Cancer

Ovarian cancer is currently the fifth most common type of cancer in women in industrialized countries.⁶³
⁶⁴ However, it is the deadliest of all gynecologic malignancies, presenting high death rates, heterogeneity, frequent metastasis and chemo-resistance.^{50, 63, 64} It is primarily a condition affecting post-menopausal women: at least 80% of women diagnosed with ovarian cancer are above the age of 50 years old.⁶⁴ There is substantial evidence of hormone-dependency, since from epidemiological data it becomes clear that estrogens affect the etiology, progression and prognosis of the ovarian cancer. While in premenopausal women the vast amount of active estrogens are synthesized in the ovaries, in post-menopausal women they are synthesized in other tissues locally, such as liver, brain and adipose tissue from androgen and estrogen precursors, and through blood they are transported to ovarian epithelia cells and taken up by transporters, such as OATPs.^{63, 64} Additionally, it has been proposed by Kirilovas et al. (2007) that estrone-3-sulfate is converted to estradiol inside the tumor⁶⁵, the same way it happens in case of breast cancer.

A study by Svoboda et al. (2013) revealed high expression of OATP1B1, OATP1B3 and OATP2B1 in ovarian cancer cells, while OATP2A1, OATP4A1 and OATP5A1 were primarily present. This is suggested to have an effect on the disposition of the anti-cancer agent paclitaxel, which is a substrate of OATP1B1 and OATP1B3. Exposing ovarian cancer cells overexpressing OATP1B1 and OATP1B3 to paclitaxel has lowered the IC_{50} of the drug in these cell lines, indicating that the sensitivity of ovarian cancer cells to paclitaxel can be modulated by altering the expression levels of OATP1B1/B3.⁵⁰

To our surprise, even though ovarian cancer is a hormone-related cancer where OATPs play a significant role in the uptake of hormones inside the malignant tissues, we found no particular reference in literature regarding directly targeting OATPs as an alternative therapy for ovarian cancer, like it was proposed for breast cancer.

Prostate Cancer

Prostate cancer is the most commonly diagnosed type of cancer in the USA and the second most common cancer-related cause of death for men. Prostate cancer accounts for 28% of cancer diagnosis and 10% cancer cause of death in men. Although local prostate cancer has quite good prognosis with radical prostatectomy or radiation therapy, advanced prostate cancer can only be treated with chemical or surgical castration. 90% of the men with castration resistant prostate cancer (CRPC) will develop bone metastasis. In case of progression to CRPC, the survival prognosis is less than two years.⁶⁶

Under normal conditions, the testes are responsible for the production of 90-95% of circulating androgens and the rest is synthesized by adrenal glands. Adrenal cortical cells and testicular Leydig cells convert cholesterol to pregnenolone, which is then converted into dehydroepiandrosterone (DHEA) by CYP17A enzyme. DHEA is the precursor of androstendione and testosterone.^{66, 67} Figure 2 shows the major biosynthetic pathways of androgens with the important enzymes in humans.

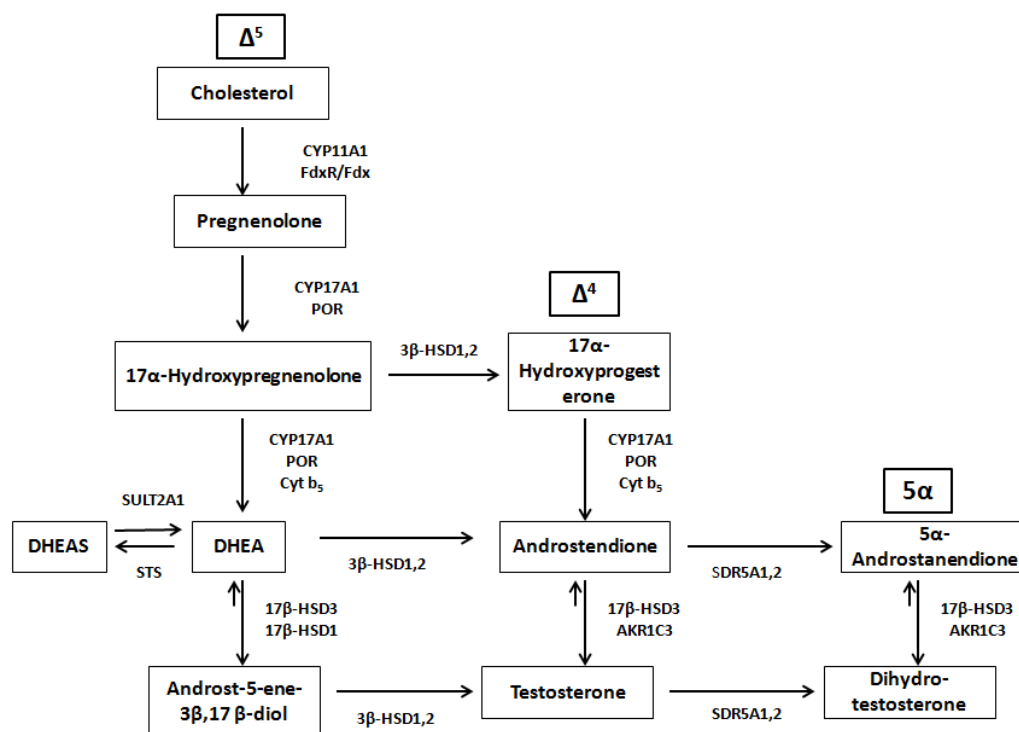


Figure 2: Major pathways of androgen biosynthesis with the important enzymes in humans. Smaller arrows indicate minor conversion. Steroid A/B ring structure is indicated as Δ^4 , Δ^5 , or 5α in boxes. Abbreviations of steroids and enzymes: DHEA, dehydroepiandrosterone; DHEAS, dehydroepiandrosterone-3-sulfate; CYP, members of cytochrome P450; Fdx, ferredoxin; FdxR, ferredoxin reductase; POR, P450-oxidoreductase; Cyt b₅, cytochrome b₅; SULT2A1, sulfotransferase 2A1; STS, steroid sulfatases; HSD, hydroxysteroid dehydrogenase; AKR, aldoketo reductase; SDR, short-chain dehydrogenase/aldoketo reductase.

Gonadal androgens play a vital role in the proliferation and progression of prostate cancer, which is considered a hormone-dependent type of cancer. A very common tactic for the treatment of advanced prostate cancer is androgen deprivation therapy (ADT), in order to remove gonadal testosterone or by antagonizing the androgen receptors.^{42, 52, 53, 66, 68} Unfortunately, very often the particular treatment is no longer effective due to the progression of the cancer in CRPC.^{52, 53, 68} Progression of the cancer to CRPC is considered to involve enhanced function of the androgen receptor (AR), mainly due to AR gene amplification/overexpression, stabilization of AR protein and increased sensitivity of AR to androgens, and finally ligand-independent activation.⁶⁸

A pivot role in prostate cancer is held by OATPs. A study by Wright et al. (2011) concerning all human OATPs showed that six of them are expressed in prostate cancer, including OATP1B1, OATP1B3, OATP2A1, OATP2B1, OATP3A1 and OATP4A1, while the rest of them are either not detected or the level of expression is not significant. Among them, OATP1B3 and OATP2B1 are considered to be linked with increased risk of CRPC.⁵² Into a similar conclusion for OATP1B3 and OATP2B1 ends up another study by Yang et al. (2011)⁵³, while a study by Arakawa et al. (2012) showed overexpression of OATP1A2 in androgen-dependent prostate cancer cells⁶⁸.

The role of OATPs in prostate cancer evolves very similar to their role in breast cancer, while in this case it is not through the uptake of estrone-3-sulfate, but by taking up dehydroepiandrosterone-3-sulfate (DHEAS). DHEAS is in much more higher concentration in serum than testosterone and it is practically not affected by ADT. DHEAS is hydrolyzed into DHEA by steroid sulfatase (STS), which can afterwards be converted into androstendione on prostate cancer, activating the AR function.^{42, 53, 66-68} Therefore, it has been suggested that OATPs can serve as novel biomarkers of response to ADT and as index of increased risk of prostate cancer mortality.^{52, 53} Additionally, and in order to suppress this particular function of OATPs, it has been suggested to combine the traditional hormone therapy with selective targeting of particular OATP members, such as OATP2A1, OATP2B1 and OATP1B3, through inhibition, to achieve enhanced therapeutic result.^{42, 53, 68}

Colorectal Cancer

Colorectal cancer (CRS) is a major health issue, worldwide. It is the third most frequent type of cancer in the western world and it affects equally men and women.^{54, 69-71} There are several factors that influence the development of colorectal cancer: It definitely has to do with lifestyle parameters: bad nutrition with high fats and poor in fibers, fruit and vegetables, as well as low folate uptake and smoking.⁷⁰ A second contributor is related to age: 90% of colorectal cancer cases concern people over 50 years old.^{54, 70} Finally, the person's ethnicity, previous history of colorectal cancer or family history of colorectal carcinomas play a significant role in the development and progression of the disease.⁷⁰

Colorectal cancer is believed to develop through accumulation of mutations, which may affect the apoptosis process. This could potentially lead to the selection of cells that are resistant to apoptosis and

increased rates of mutations.⁵⁴ Significant improvement in the prognosis of the disease can be achieved through early prognosis with colonoscopy and polypectomy.^{70, 71} The only curative therapy for colon cancer is curative resection, however, it is estimated that 2/3 of the patients having the operation, will have recurrence of the disease. The tumor recurrence can be somehow deteriorated by adjuvant chemotherapy.⁶⁹

Several studies attempting to elucidate the factors influencing the progression and outcome of colorectal cancer have revealed that OATPs play an important role, also for this type of cancer. In their study, Ballesterio et al. (2006) showed that in comparison to healthy colon tissue, the expression of OATP1B3 was approximately 50% and not substantially altered in cancer colon tissue. On the contrary the expression of OATP1A2 was remarkably impaired in colon cancer tissue and polyps. Finally, the expression of OATP1B1 was increased in colon cancer tissues, in comparison to healthy ones.⁷² In contrast to Ballesterio et al. study, two separate studies from Lee et al (2008)⁵⁴ and Lockhart et al (2008)⁶⁹ showed that actually OATP1B3 is highly overexpressed in tumor colorectal tissues. Finally, a study by Kleberg et al. (2012) revealed increased expression of OATP2B1 and OATP4A1 in patients with colorectal neoplasia.⁷³

In general, the overexpression of OATP1B3 in colorectal cancer has been associated to increased resistance of the tumor in chemotherapy^{54, 69}, while it has been suggested that OATP1B3 expression could serve as a prognostic factor for patient survival within a particular tumor grade group.⁶⁹ Finally, exploiting the transporter capacity of OATPs the use of cytostatic bile acid derivatives, such as Bimet-UD2, has been suggested. This family of anticancer agents combines the antitumor activity of cis-platin, with the substrate properties (for transporters) of bile acid derivatives. In case of OATP overexpression, the chemotherapeutic agents are actively imported into tumor cells, where they exert a cis-platin like effect. The particular strategy, apart from colon cancer, has been suggested to be used in other tumor types, such as liver cancer.⁷²

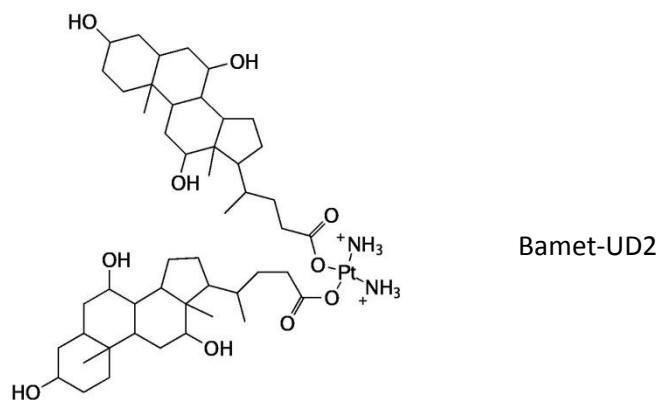


Figure 3: Bamet-UD2, the anticancer agent, product of conjugation of 2 molecules of ursodeoxycholic acid with one molecule of cis-platin.

Liver Cancer

Primary liver cancer (hepatocellular carcinoma, HCC; cholangiocellular carcinoma, CCC) is the 6th most common type of cancer world-wide and the 3rd most common cause of cancer-related death.^{74, 75} It has a very high mortality-to-incidence ratio and it has a very poor prognosis in advanced stages of the disease, mainly because it is resistant to conventional systematic therapies.^{75, 76} Over 80% of the deaths related to the disease occur in developing countries, where it is a major public health issue. The major risk factors for primary liver cancer are divided into three categories: a) the established factors, such as infection with hepatitis B or C, alcoholic cirrhosis, dietary aflatoxins and tobacco smoking, b) the likely factors, such as diabetes mellitus, inherited metabolic disorders, α -antitrypsin deficiency, hemochromatosis, porphyria cutanea tarda, cirrhosis of any etiology, and c) the possible factors, such as decreased consumption of vegetables, oral contraceptives, high parity, ionizing radiation and organic trichloroethylene solvent. Moreover, the differences in incidence and mortality rate among different countries and ethnic groups, might be an additional risk factor.⁷⁴

Surprisingly, in cases of liver cancer, the almost liver-specific transporters OATP1B1 and OATP1B3 are downregulated⁴², as supported by several studies. Particularly, Cui et al. (2003) showed down regulation of both OATP1B1 and OATP1B3 in hepatocellular carcinomas⁷⁷, while Zollner et al. showed the same for OATP1B1⁷⁸ (OATP1B3 was not tested). Wlcek et al. (2011), in their study investigated the expression of several OATPs in hepatocellular carcinomas, cholangiocellular carcinomas and liver metastases from

colon tumors and they showed down regulation of OATP1B1, OATP1B3, OATP1A2 and OATP2B1 in cancerous vs non-cancerous samples, while there was an increase in OATP2A1, OATP3A1, OATP4A1 and OATP5A1.⁵¹ Finally, a study by Ueno et al. (2014) pinpointed OATP1B3 as the most important transporter mediating the hepatocellular tumor enhancement in gadoxetic acid-enhanced magnetic resonance imaging (EOB-MRI).⁷⁵

Considering the particular patterns of expression of the various OATPs in liver cancer, OATP1B1 and OATP1B3 have been suggested as tumor biomarkers in liver cancer.⁷⁷ Furthermore, OATP1B3 might play a role in the diagnosis of liver cancer through EOB-MRI⁷⁵. Finally, the exploitation of high OATP2A1, OATP3A1, OATP4A1 and OATP5A1 expression in liver tumors has been proposed for the discovery of novel anticancer agents.⁵¹

Pancreatic Cancer

Pancreatic adenocarcinoma is one of the most aggressive and resistant forms of solid tumors, towards both classic chemotherapy and targeted agents. It has high mortality rate, being the fourth cause of cancer-related deaths in western world, with a number of deaths almost equal to the number of new incidences of the disease.^{79, 80} There is not an effective way to detect the disease at an early stage, where patients remain asymptomatic. It is diagnosed later, at an advanced stage, where radical pancreatic resection is impossible and there is no response to conventional chemotherapy.^{81, 82}

Regarding the role of OATPs in pancreatic cancer, Kounnis et al. (2011) found is over-expression of OATP1A2, OATP1B1 and OATP1B3 in pancreatic adenocarcinomas, which led to the proposal for novel therapeutics targeting OATPs.⁷⁹ Moreover, a study by Hays et al. (2012) has shown up regulation of OATP1B3, OATP2A1, OATP3A1 and OATP4A1 in pancreatic adenocarcinoma in comparison to healthy pancreas tissue. More particularly, OATP1B3 showed highest levels of expression in pancreatitis and stage one pancreatic adenocarcinoma, which finally leads to pancreatic cancer. Thus, OATP1B3 could be a novel biomarker for diagnosis in the early stages of the disease⁸², which is one of the vital needs for pancreatic cancer diagnosis.

Small Cell Lung Cancer (SCLC)

Lung cancer is the leading cause of cancer-related deaths in the western world, while small cell lung cancer (SCLC) accounts for 15-20% of the total cases of lung cancer.⁸³ SCLC is one of the most distinctive malignancies in the field of oncology, with characteristic clinical properties, responsiveness to particular chemotherapy, genetic features and highly reliable clinical diagnosis.⁸⁴ It is characterized by high tumor doubling time, high growth fraction, and development of extended metastases, especially in the brain, at quite early stage.⁸³

Similarly to other cancer types, also in SCLC OATPs show a particular pattern of expression. A study by Olszewski-Hamilton et al. (2011) shows that OATP5A1 is upregulated in SCLC and suggests possible correlation between this overexpression and chemoresistance to satraplatin, proposing the investigation of OATP5A1 as a marker for chemoresistance.⁸⁵ Another study by Brenner et al. (2015) investigating the expression of several OATPs in lung cancer, reveals overexpression of OATP1A2, OATP1B3, OATP4A1, OATP5A1 and OATP6A1 in SCLC cell lines compared to normal lung cell lines. On the other way round, it reveals downregulation of other OATPs in SCLC lines and other carcinoid tumor cells, including OATP2A1, OATP2B1 and OATP3A1. Based on the findings of the particular study, the authors propose the use of OATPs as novel biomarkers for tumor progression and the development of metastasis.⁸³

OATPs and other forms of cancer

There is evidence for altered OATP expression in some additional forms of cancer. A study by Liedauer et al. (2009) revealed that out of the 11 OATP members, 8 of them –particularly OATP1A2, 1C1, 2A1, 2B1, 3A1, 4A1, 4C1 and 5A1- are expressed in human bone tumors, with differences of expression for particular kind of tumors (osteosarcomas, bone metastases and benign bone tumors). This suggests that OATPs might be important regulators of bone homeostasis and affect tumor growth and progression. Additionally, the implementation of OATP-mediated uptake of anti-cancer drugs, which would increase the efficacy of chemotherapy, is proposed.⁸⁶

Moreover, a stage dependent overexpression of OATP1B3 in bladder tumors, in comparison to healthy respective tissues, has also been observed.^{42, 49}

Human gliomas show overexpression of OATP1A2, OATP1C1, OATP2B1 and OATP4A1, with OATP1A2 and OATP2B1 expressed in the canalicular membrane of the endothelial cells, forming the blood-tumor barrier. This information could help in the development of selective and efficient chemotherapeutics, depending of the substrate profile of these transporters.^{42, 87}

Finally, expression of OATP1B1, OATP1B3 and OATP4A1 in human melanoma cells has been detected. It has been suggested that OATPs promote a mechanism of resistance of the melanoma cells to apoptotic cell death, which is normally induced by cis-platin, contributing this way to the development of chemoresistance. This led to the development of OATP targeting strategies.⁵⁵

Concluding, it seems that there is high correlation between expression of various OATPs and several cancer types. Therefore, screening tumors for OATP expression before therapy could lead to an OATP-targeted therapy with higher efficacy and decreased side effects.³⁵ However, the simultaneous physiological role of these transporters and the consequences a potential inhibition of them should always be considered. For example, inhibition of OATP1B1 and OATP1B3, which are overexpressed in several cancer lines, might induce hyperbilirubinemia⁸⁸ or other forms of liver toxicity. On the other hand it might also lead to early removal of the drug from the circulation, leading to ineffective cancer treatment. Similarly, inhibition of OATP2B1 as breast cancer treatment, could potentially lead to toxic accumulation of the drug in the intestine. So, for sure, further investigation and clinical trials are necessary, before clinical implementation of these strategies.⁸⁹

OATPs as diagnostic markers

The usefulness of OATPs for diagnosing cancer has already been mentioned earlier in the cancer part. Here will be discussed a bit further some implementations of OATPs in the area of diagnostics.

A mechanistic study by Zhang et al (2013) pinpointed IR-780 dye as a potential tumor targeting and drug delivery agent, with possible implementation both in diagnostics and in therapy. More particularly, recently, near-infrared (NIR) dyes have emerged as potential tool for tumor imaging and tumor-targeted therapy. IR-780 iodide is a near-infrared fluorescent heptamine dye that has been found to selectively accumulate in the mitochondria of tumor cells. Zhang et al. showed that this selective accumulation of

IR-780 iodide depends on energy metabolism, the potential of the plasma membrane and OATP1B3, which is responsible for the uptake of the dye. They also showed that cellular endocytosis, the potential of the mitochondrial membrane, and ABC canalicular transporters play no role at all. Moreover, they constructed a new agent, IR-780NM, by introducing nitrogen mustard, an anticancer agent, to IR-780 iodide. IR-780NM also exhibits tumor targeting and tumor NIR imaging abilities and may be regarded as potential tumor targeted theranostic (i.e. diagnostic + therapeutic) agent. Of course its efficacy and toxicity need further investigation.⁹⁰

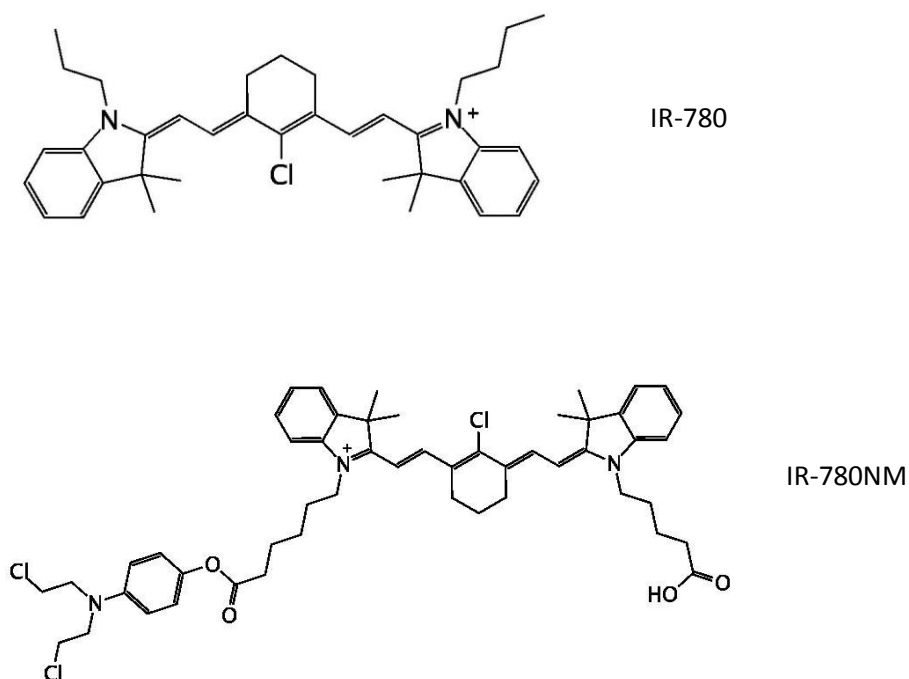


Figure 3: The near-infrared dye IR-780 and the new derivative IR-780NM, after combination of IR-780 iodide and nitrogen mustard.

Furthermore, since the expression and the transporting capacity of OATPs is altered in cases of liver disease, they have helped in diagnosis of the type and the stage of several liver pathological conditions. Particularly, liver imaging techniques with hepatobiliary contrast agents, traces and dyes that cross hepatocytes through the pathway of OATPs-MRPs were developed to detect and characterize focal lesions and to assess the severity of diffuse liver diseases and bile duct injury. The method of choice for

the detection of focal lesions is liver MRI following the injection of contrast agents, since conventional MRI (without contrast agent) in many cases is not sufficient to distinguish between benign conditions and malignant ones. Two hepatobiliary contrast agents for liver MRI are commercialized: gadobenate dimeglumine (BOPTA, MultiHance; Bracco Imaging SpA, Milan, Italy) and gadoxetate dimeglumine (EOB-DTPA, Primovist; Bayer Health-Care Pharmaceuticals, Berlin, Germany). The assessment of the liver function in diffuse liver disease is necessary in order to determine the prognosis of patients with liver cirrhosis, to define the optimal time-point for liver transplantation, and to assess whether patients with liver cirrhosis can undergo major extrahepatic surgery or tolerate partial hepatectomy.⁹¹

OATPs and Selective Delivery of Drugs

The effectiveness of oral drugs is highly associated with their physicochemical properties, which are influencing their ADMET profiles. Moreover, today we are aware of the influential role of the transporting systems of the body, i.e. the transmembrane transporters, in absorption, distribution, metabolism, excretion and toxicity of drugs.^{3-7, 20} Due to the distinctive expression patterns of OATPs in particular organs, they can be used as a useful therapeutic tool for selective delivery of drugs.⁸⁹ In the section for cancer, the potential exploitation of OATPs in drug delivery for treating cancer has already been analyzed. Here, the use of OATPs in drug delivery for other diseases will be discussed.

OATPs and intestinal drug absorption

OATP2B1 is believed to be responsible for the absorption of substrates from the gastrointestinal tract, because of its high expression in the small intestine. However, there is also some, but controversial, evidence for the expression of OATP1A2.^{5, 20, 89} As previously mentioned, the transport capacity of OATPs is often pH-dependent and this is the case also for OATP2B1 (it is increased at lower pH).^{18, 89} In healthy small bowel, the luminal pH is slightly acidic (5.5-7.0) increasing gradually to 7.5 in the terminal ileum. In healthy colon, luminal pH is also slightly acidic (5.5-7.5) increasing gradually (6.5-7.5) at the rectum. However, in most of the cases of intestinal diseases, such as Crohn's disease and ulcerative colitis, colonic luminal pH is reduced to 3 up to 6, as a result of several pathological factors. This could affect

the transport capacity of intestinal OATP2B1. It is suggested that optimizing a weakly acidic intraluminal environment could enhance the uptake of OATP substrates from the gut. Moreover, the presence of an intestinal disease could significantly impact an individual's systemic exposure to a drug due to increased uptake of the drug. Therefore, data assessing the pharmacokinetics of a drug in a healthy volunteer population should not be extrapolated to this patient population.⁸⁹

Additionally, there is evidence that genetic polymorphisms of OATP2B1 can affect the absorption of drugs. More particularly, two variants c.1457CNT and c.935GNA of the SLCO2B1 gene decrease the plasma concentration of drugs, such as fexofenadine and montelukast, which may cause a decreased efficacy of these drugs. Thus, special care should be taken for arranging the dosage regimens for patients with those genotypes.^{5, 20}

Furthermore, since the small intestine is exposed to various foods and drinks, intestinal transporters may also be affected by this material. Actually, the alteration of *in vivo* drug absorption by concomitantly administered drugs or food/juice provides further evidence for the contribution of transporters to the intestinal absorption of drugs.^{5, 20} Fruit juices proved to interact with intestinal transporters via inhibition of the transport activity of the transporter, rather than reduced expression of the protein.⁹² Both OATP2B1 and OATP2A1 are inhibited by fruit juices, with grapefruit juice being the most potent inhibitor, followed by orange juice and apple juice.^{20, 92} The substances that seem to be responsible for OATP inhibition are flavonoids (naringenin and hesperidin, among others), as well as furanocoumarins, which have been found in grapefruit and orange juice. However, the ingredients of apple juice that cause OATP inhibition remain unknown.⁹² Drugs whose absorption has been proven to be modified due to concomitant juice consumption, are, among others, fexofenadine, ciprofloxacin, montelukast, aliskiren and β -blockers, such as atenolol, celiprolol, and talinolol. All the drugs above are substrates of OATP2B1 and/or OATP2A1.^{5, 92}

OATPs and targeted liver drug delivery

Since some OATPs, like OATP1B1 and OATP1B3, are highly and almost specifically expressed in the liver (under normal conditions), the structural and physicochemical characteristics of their substrates could be implemented for the development of drugs that could be used for the treatment of liver diseases or other systemic conditions.^{93, 94} In general, in order to achieve selective liver distribution, it is necessary to maximize the presence of the drug in the liver and to minimize the presence of the drug in peripheral

tissues. High liver exposure can be achieved with substrates of OATP1B1 and B3, while low exposure in peripheral tissues can be achieved by limiting passive diffusion in these tissues. Tu et al. (2013) in their study suggest the following characteristics for a hepatoselective oral drug⁹³:

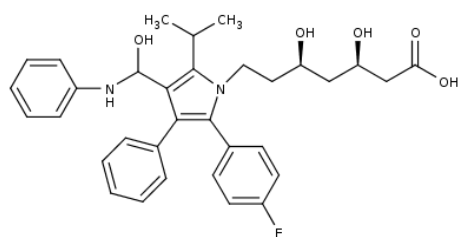
- Presence of an acidic moiety
- OATP1B1 and /or B3 substrate
- Passive permeability between 1.0 and $5.0 \times 10^{-6} \text{ cm.s}^{-1}$, as measured in a RRCK assay.
- Lipophilicity (logD at pH 7.4) between 0.5 and 2.0
- High solubility. Values are series specific and should be guided by the fraction absorbed.

Nevertheless, we must also point out that there is data in literature showing decrease in the mRNA levels of OATP1B1 and OATP1B3 in patients with hepatic diseases, such as hepatitis C and non-alcoholic steatohepatitis, which would alter the uptake of drugs and therefore also the effectiveness of this liver-targeted delivery approach.^{89, 95, 96} Below follow some examples of therapeutic agents that express their pharmacological action through high uptake by the liver.

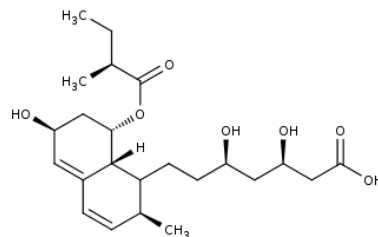
Statins:

Statins are a class of inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, an enzyme highly expressed in the liver that defines the rate of cholesterol biosynthesis.^{94, 97} Previously developed HMG-CoA inhibitors had cholesterol lowering effects, but also side effects, such as myopathy, which are due to the diffusion of the drugs into the muscle. The clinically used statins are liver-targeted molecules, having the feature of restricted tissue distribution, which is crucial for their tolerability. Statins are substrates of OATPs expressed in the liver and in the intestine. The beta-hydroxy heptanoic acid moiety that is shared across many statins is the core structural element for their reversible binding in the active site of HMG-CoA reductase.⁹⁴ It also serves as the moiety responsible for the specific liver-delivery through interaction with OATPs. This way, statins undergo enterohepatic circulation, which finally minimizes potential side effects to other tissues.⁹⁷

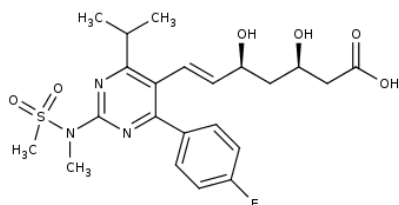
Figure 5 shows the chemical structures of several clinically important statins.



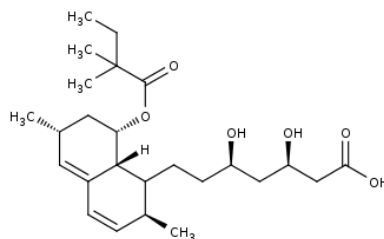
Atorvastatin



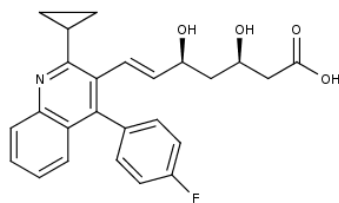
Pravastatin



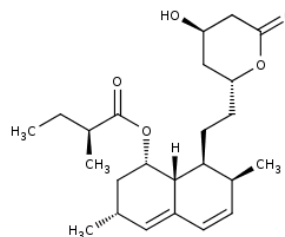
Rosuvastatin



Simvastatin



Pitavastatin



Lovastatin

Figure 5: The molecular structures of several statins. They all present the beta-hydroxy heptanoic acid moiety.

However, since not all statins present the beta-hydroxy heptanoic moiety and thus are not liver-selective, a different strategy has been adopted which exploits bile salt structural elements, in order to achieve liver-targeted delivery. The particular strategy offers two approaches: a) The development of hybrids between bile acids and HMG-CoA inhibitors combining structural characteristics of both parental substances, or b) development of conjugates between a bile acid and a HMG-CoA inhibitor in the C-3 position of the bile acid. In the later case, the bile salt moiety facilitates the uptake of the drug in the liver where the active agent can express its pharmacological effect. Figure 6 schematically depicts this approach with two examples: Compound A is a hybrid compound where the hexahydronaphthalene moiety of lovastatin was replaced by a modified bile acid and the 3,5-dihydroxy heptanoic acid side chain was conserved either in the open ring or in the lactone form. Compound B is the conjugate of bile

acid with an HMG-CoA inhibitor in C-3 position of the bile acid.⁹⁸

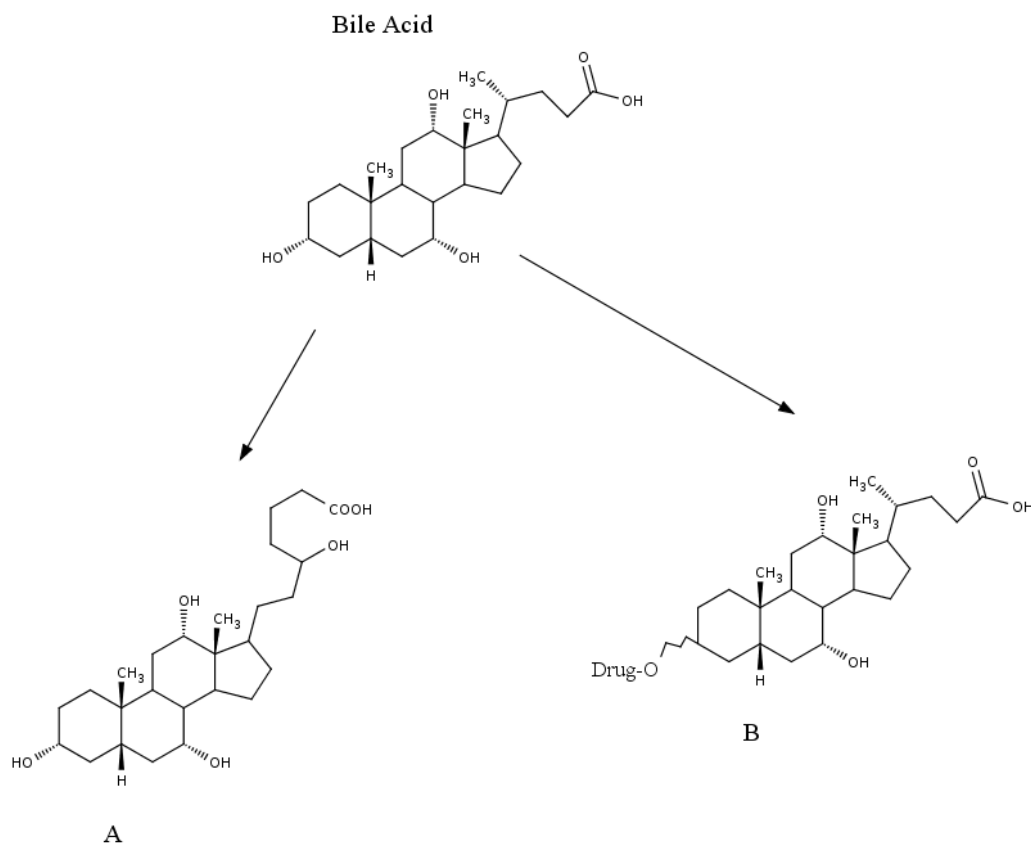


Figure 6: Strategy of combining the structural characteristics of bile salts with HMG-CoA inhibitors: A) Development of a hybrid compound containing structural characteristics of both parental compounds, B) Prodrug created by conjugation of a bile acid with an HMG-CoA inhibitor.

Glucokinase (GK) activators:

Glucokinase serves as an important regulator of glucose homeostasis by converting cellular glucose to 6-glucose-phosphate, which can further be used in metabolism. Allosteric activators of glucokinase (GKAs) in liver assist hepatic glucose uptake, reduce hyperglycemia and might present a promising concept for the treatment of diabetes mellitus type 2. However, earlier systemically distributed GK activators had the disadvantage of a dose-limited hypoglycemia effect due to excessive GK activation in pancreatic β -

cells, leading to overproduction of insulin. In order to deal with this systemic effect, liver-targeted GKAs containing carboxylic acid moiety, which are able to activate glucokinase inside the hepatocytes and not pancreatic β -cells, were designed. Apart from low passive permeability, which prevents the diffusion in peripheral tissues, these compounds were designed as OATP substrates to achieve selective uptake from the hepatocytes. Figure 7 shows a systemically distributed GK activator and the respective hepatoselective one, resulting from the replacement of a methyl group with a carboxylic group.^{94, 99}

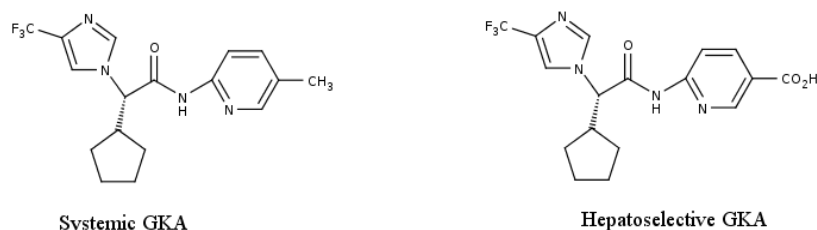


Figure 7: Example of a systemic and the respective novel hepatoselective GKA.

Stearoyl-CoA Desaturase-1 (SCD1) Inhibitors

One more example of liver-targeted molecules carrying structural characteristic of OATP substrates is the design of Stearoyl-CoA desaturase-1 (SCD1) inhibitors at Merck Frosst Laboratories. SCD1 is a long chain fatty acyl-CoA desaturase, which is highly expressed in the liver and is responsible for the *de novo* synthesis of oleic acid. Elevated activity of SCD1 is associated to obesity and several types of cancer. SCD1 inhibitors were considered promising agents for the treatment of type 2 diabetes mellitus, non-alcoholic steatohepatitis (NASH) and cancer. However, systemic inhibition of SCD1 causes dose-limiting adverse effects, such as dry skin, hair loss, as well as local lipid depletion. In order to overcome these side effects, liver-targeted SCD1 inhibitors, which are highly taken up by the hepatocytes while they also present low permeability over peripheral tissues, were designed. A series of structure-activity relationships (SAR) and cell assay studies ended up to the hepatoselective SCD1 inhibitor MK-8245 (Figure 8), which is a substrate of both OATP1B1 and B3.^{94, 100}

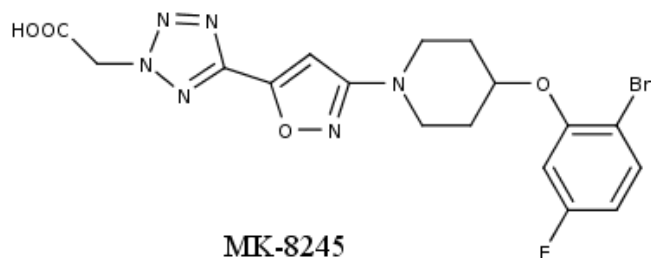


Figure 8. The hepatoselective SCDI inhibitor, MK-8245, substrate of OATP1B1 and B3.

OATPs and targeted pancreas drug delivery

In a study by Abe et al. (2010), the expression of the rat Oatp1a1/Slco1a1, Oatp1a4/Slco1a4 and Oatp1a5/slco1a5 in rat pancreas is presented.¹⁰¹ It is also known that pravastatin is a 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitor that contributes in reducing total LDL cholesterol and triglycerides.¹⁰² Apart from the lipid-lowering effects, the West of Scotland Coronary Prevention Study (WOSCOPS) demonstrated the use of pravastatin in inhibiting the new onset of diabetes.¹⁰³ Abe et al. showed in their study that rat Oatp1a1/Slco1a1, Oatp1a4/Slco1a4 and Oatp1a5/slco1a5 are responsible for the uptake of pravastatin in rat pancreas. There, according to the findings, pravastatin stimulated insulin secretion, as well as insulin sensitivity, which justifies its observed antidiabetic effect.¹⁰¹ Of course, this function needs further validation in humans.

OATPs and CNS drug delivery

The pharmacological treatment of CNS (central nervous system) diseases requires that the active agent reaches effective concentration in the brain. However, this is often hindered by the low permeability of the blood-brain barrier (BBB), as well as the efflux transporters. Therefore, targeting endogenous uptake transporters localized at the BBB might be an opportunity for effective CNS drug delivery. As previously documented, there are some OATPs expressed in the CNS, such as OATP1A2, OATP1C1, as well as in some extent the ubiquitous OATP2A1, OATP3A1 and OATP4A1. The function of these transporters might

be exploited in some particular CNS diseases, in order to achieve sufficient drug concentration in the CNS. Two potentially applicable cases are pain and cerebral hypoxia.^{89, 104-106}

Acute and chronic **pain** is often associated with inflammation, as a result of tissue destruction, nerve injury or abnormal immune activity. Pharmacological treatment of pain often evolves administration of opioids, which are considered the most potent analgesic drugs. They act through binding to opioid receptors that reside in brain, spinal cord and peripheral nerves.¹⁰⁵⁻¹⁰⁷ Despite the fact that opioids act also on peripheral receptors, providing analgesia, the analgesic result is more effective when there is accumulation of the drug in the CNS. Novel therapeutic approaches involve the development of opioid peptides that act as potent opioid receptor agonists. However, treatment with such peptides suffers from insufficient delivery into CNS due to the restricted permeability of the BBB.¹⁰⁶ Among OATPs, OATP1A2 is able to transport opioid peptides, such as DPDPE and deltorphin II.¹⁰⁴ In rodent brain there is expression of Oatp1a4, Oatp1c1 and Oatp2a1,^{105, 106, 108} while it has been proposed that Oatp1a4, an ortholog of the human OATP1A2, is the primary drug-transporting Oatp isoform expressed in the rat BBB.¹⁰⁵ In their study, Ronaldson et al. (2010) have shown that in rat brain microvessels, the expression of Oatp1a4 was increased during acute pain/inflammation. Moreover, the uptake of taurocholate and [D-Penicillamin^{2,5}]-enkephalin, two known Oatp substrates, was increased in cases where the animals were subjected to peripheral pain, suggesting increased Oatp1a4-mediated transport. Administration of the anti-inflammatory drug diclofenac for the inhibition of inflammatory pain resulted in the fading of these changes in Oatp1a4 expression, suggesting that peripheral inflammation can modulate BBB Oatp transporters, while the findings implicate also involvement of the cytokine transforming growth factor- β 1 (TGF- β 1) in the regulation of Oatp1a4 at the BBB. The authors suggest that BBB transporters can be targeted during drug development, in order to improve the CNS delivery of potent therapeutics.¹⁰⁶

Hypoxia and subsequent reoxygenation (H/R) is a characteristic of multiple diseases, such as traumatic brain injury, acute respiratory distress syndrome, obstructive sleep apnea, high-altitude cerebral edema and acute mountain sickness, cardiac arrest and ischemic stroke. H/R is associated with neuronal apoptosis, oxidative stress, which induces neuronal cell death, as well as depletion of the endogenous antioxidant glutathione (GSH) in the brain. Currently, there is an increasing interest for the neuroprotective/antioxidant properties of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (i.e. statins).¹⁰⁵ Recent studies suggest that statins, apart from their well-established lipid-lowering properties, can also act as free-radical scavengers, like in the case of the Barone et al. (2011) study in dogs with atorvastatin.^{105, 109} Statins are also substrates of OATPs in both humans and

rodents, thus they could be potentially useful by moderation of the transporting activity of OATPs in CNS. Of course, their use in cases of brain hypoxia needs further investigation.¹⁰⁵

Potential protective role of OATPs

OATP4C1 vs Chronic Kidney Disease (CKD)

Kidney is the primal organ for the regulation of water, nutrients and electrolytes in the body, through filtration, secretion and re-absorption, playing a significant role in homeostasis and pharmacokinetics.^{94, 110} Worldwide there is an increasing number of people suffering from chronic kidney disease (CKD), which imposes a huge burden on the health-care systems globally. CKD is recognized as the single biggest risk factor for cardiovascular disease and when it co-occurs with diabetes, it dramatically increases the risk of death.^{111, 112} In patients with CKD there is accumulation of uremic toxins, which cause further renal damage and increase the risk of cardiovascular diseases. It has been found that OATPs and particularly OATP4C1, which resides at the basolateral membrane of the proximal tubular cells in human kidney, takes up uremic acids, contributing this way in their elimination^{110, 113}. Statins can upregulate the expression of OATP4C1, which induces the elimination of uremic toxins. In their study, Suzuki et al. (2011) noticed that administration of statins in a rat renal failure model assisted the elimination of uremic toxins while preventing further renal damage. This could be a start for a new therapeutic strategy against CKD, where the regulation of the transporters responsible for the uptake of uremic acids will enhance the elimination of these toxins, while the levels of the candidate uremic toxins could serve as surrogate biomarkers.¹¹⁰

OATPs vs Amatoxins

Mushrooms are ubiquitous in nature. There are about 5000 mushroom species, out of which 50-100 are known to be poisonous for humans, while only 200-300 are known to be clearly safe.¹¹⁴ Worldwide there is an increase in deaths because of mushroom poisoning, with high mortality rates attributed to consumption of mushrooms containing amatoxins. Amatoxin poisoning is caused by mushroom species belonging to the genera *Amanita*, *Galerina* and *Lepiota* with the majority of lethal mushroom exposures

attributable to *Amanita phalloides* and its subspecies (*Amanita virosa*, *Amanita vernalis*). Amatoxins are heat-stable octapeptides. A wide variety of amatoxins have been isolated, but the primarily toxic one for humans is considered to be α -amanitin (α -AMA).¹¹⁵ Even though it occurs only sporadically, the clinical picture of amanitin poisoning is including severe gastroenteritis and extended liver damage, which requires liver transplantation if left untreated.^{89, 116, 117} After oral ingestion, amatoxins are absorbed through the walls of the gastrointestinal tract and they are transported to the liver. Amatoxin toxicity is promoted by inhibition of RNA polymerase II of the hepatocytes, which results in interruption of the protein synthesis and ends up in early cell death, since hepatocytes are depending on a high protein synthesis rate.^{89, 117} From the OATPs residing in the liver, i.e. mainly OATP1B1, OATP1B3 and OATP2B1, only OATP1B3 is responsible for the uptake of amanitin in the hepatocyte. As a therapeutic approach against amanitin poisoning, targeting OATP1B3 via inhibitors or substrates competitive with amanitin has been proposed. Among known amanitin antidotes, that are also OATP1B3 substrates and potentially inhibitors, are benzylpenicillin (penicillin G), silibinin and acetylcystein, rifampin, cyclosporine A, paclitaxel, the quinoline derivative MK571, montelukast, cholechystokinin octapeptide(CCK-8), bromosulfophthalein etc.^{89, 115-117}

OATPs and Drug-Drug Interactions

Nowadays, the implementation of polypharmacotherapy has raised the problem of drug-drug interactions (DDIs). The important role of transporters and metabolizing enzymes on pharmacokinetics, as well as the development of these drug-drug interactions, has also been recognized.^{118, 119} Drug-drug interactions can change the exposure of a drug and therefore potentially alter its efficacy and safety profile. They can be extremely complicated to predict since during the concomitant use of several drugs, multiple systems of transporters and enzymes are involved, with the participation of several substrates and potential inhibitors.¹²⁰ Since liver is the main organ of the body for metabolism and detoxification, hepatobiliary transporting systems receive a great amount of attention regarding their role in drug clearance as well as in pharmacokinetics and hepatic exposure.^{121, 122} Of course, also transporting systems residing on the intestine and kidney are of great importance.¹¹⁹ Apparently, the important role of OATPs as the primal uptake transporters of the liver, also in drug-drug interactions, is indisputable.¹¹⁸⁻

^{121, 123}

Several OATP transporters and/or inhibitors have been reported for participating in drug-drug, drug-herb or drug-food interactions. A main drug class with pivotal role in DDIs are the statins, which are OATP substrates. It seems that most of the statins used in pharmacotherapy also participate in DDIs, either by influencing the exposure of other drugs or by being influenced by other drugs..^{118, 119, 122, 124} The only doubt is raised for pitavastatin, since there are contradictory reports in literature. Particularly, a study by Hirano et al (2006) shows increased risk for DDIs when using pitavastatin concomitantly with other drugs (especially cyclosporin A, rifampicin, rifamycin, clarithromycin and indinavir)¹²⁵, while a study by Gosho et al. (2014) suggests that pitavastatin is associated with low risk of DDIs in polymedicated patients.¹²⁶

Another category of drugs with possibility for causing DDIs are antidiabetic drugs. There is evidence for repaglinide^{119, 127, 128}, rosiglitazone¹²⁷ and nateglinide (in smaller extent)^{119, 128, 129}. Sufficient evidence also exists for several antimicrobial drugs. Among those are macrolides such as clarithromycin and roxithromycin¹¹⁸, as well as cyclosporin A^{118, 119, 128, 130} and rifampicin^{118, 119, 130}. There are also DDI reports for gemfibrozil^{118, 119, 122}, bosentan^{118, 119, 130} and sildenafil¹³⁰.

Moreover, there are reports concerning drug-drug and herb-drug interactions for some natural products. Representative examples are:

- i. Gingolide B¹³¹, a strong selective antagonist of platelet-activating factor, extracted from *Gingo biloba* and used in CNS treatment
- ii. Ginsenosides¹³², bioactive saponins derived from *Panax notoginseng* roots (Sanqi) and ginseng, which are used in cardiovascular diseases, and
- iii. Icaria, a flavonol glycoside, a product of traditional Chinese Medicine, extracted from *Herba epimedii* (*Berberidaceae*), commonly known as Horny Goat Weed (or Yin Yang Huo) that is used for the treatment of osteoporosis and sexual male dysfunction¹³³.

Due to the crucial role of DDIs in therapeutics, there has been considerable effort directed towards assessing DDIs and generating models that can quantitatively predict pharmacokinetics and DDIs early in drug development.¹²³ Reviews by Soars et al. (2009)¹³⁴ and more recently by Li et al. (2015)¹²¹ summarize several published models for predicting pharmacokinetic profiles and DDIs. Recently, also two models have been published by Varma et al.^{120, 123} that are not included in the review. The first one is a net-effect model that can accurately predict 58 out of 62 clinical combinations for DDIs¹²³, while the most recent one tries to quantitatively predict gemfibrozil drug interactions¹²⁰. As an alternative approach, a recent study by Ebner et al. (2015) proposes the use of probe drug cocktails containing several influx

and efflux transporter substrates, in order to assess the transporter-based drug-drug interactions in a clinical setting. The drugs-transporter substrates they are proposing are: digoxin (P-glycoprotein, P-gp), rosuvastatin (breast cancer resistance protein, BCRP; organic anion transporting polypeptides, OATP), metformin (organic cation transporter, OCT; multidrug and toxin extrusion transporters, MATE), and furosemide (organic anion transporter, OAT).¹³⁵ Finally, the Bayesian statistical model by van de Steeg (2015) for OATP1B1, OATP1B3 and OATP1B1*15 inhibition is proposed to be used also for predicting drug adverse effects, since it is known that OATP inhibitors are highly correlated with DDIs.¹³⁶

Conclusions and Outlook

The Organic anion transporting polypeptide superfamily is a rather novel class of transporters. Only in the last decade there have been more thorough studies among different members, while some transporters have still not been fully described. However, it is undeniable that OATPs comprise an important group of transporters implicated in various physiological and pathological conditions in humans. Towards this direction leads additionally the fact that they can be ubiquitously and/or selectively expressed in several epithelia throughout the body, depending on the conditions –health or disease- as we showed above. Another important aspect is their wide range of transporters and inhibitors that can potentially lead to drug-drug interactions and affecting pharmacodynamics and pharmacokinetics.

Due to their complex profile, OATPs cannot be regarded as a “classical” pharmacological target. Their inhibition, when necessary –e.g. in cases of disease- should be done with precaution, in order to avoid potential side-effects because of the transporter inhibition in some other healthy tissue. In most of the cases, the use of OATP inhibitors as therapeutics is still in experimental stage. Nevertheless, there are some particular clinical cases, e.g. the use of OATP1B3 inhibitors against amatoxin poisoning, where targeting a selectively expressed OATP member can be of great benefit in minimal risk. Moreover, there are various case of using OATPs as biomarkers or auxiliary, in order to enhance the effect of the main drug by affecting its pharmacokinetics profile. Concluding, we could say that as the amount of knowledge for OATPs is steadily growing, and more light is shed on their pathophysiological function, the accumulative information may bring us closer to steady therapeutic schema that involve OATPs.

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Marvin was used for drawing, displaying and characterizing chemical structures, substructures and reactions, Marvin 6.1.3, 2013, ChemAxon (<http://www.chemaxon.com>)

2. Supplements to Chapter 3

Table A1. OATP1B1 models: Description of the used settings

Model Name	Descriptors	weka.classifier	Cost matrix
B1_6MOE_RF	6 MOE descriptors: a_acc, a_don, logP (o/w), mr, TPSA and weight	trees.RandomForest (numTrees: 10, default)	meta.MetaCost -cost-matrix "[0.0, 1.0; 8.0, 0.0]
B1_6MOE_SMO	6 MOE descriptors: a_acc, a_don, logP (o/w), mr, TPSA and weight	functions.SMO.Puk.kernel (buildLogisticModels:True, the rest settings at default)	meta.MetaCost -cost-matrix "[0.0, 1.0; 8.0, 0.0]
B1_6PaD_RF	6 PaDEL descriptors: CrippenLogP, CrippenMR, nHBAcc_Lipinski, nHBDOn_Lipinski, TopoPSA, MW	trees.RandomForest (numTrees: 10, default)	meta.MetaCost -cost-matrix "[0.0, 1.0; 8.0, 0.0]
B1_6PaD_SMO	6 PaDEL descriptors: CrippenLogP, CrippenMR, nHBAcc_Lipinski, nHBDOn_Lipinski, TopoPSA, MW	functions.SMO.Puk.kernel (buildLogisticModels:True, the rest settings at default)	meta.MetaCost -cost-matrix "[0.0, 1.0; 8.0, 0.0]
B1_11PaD_RF	11 PaDEL descriptors: CrippenLogP, CrippenMR, nHBAcc_Lipinski,	trees.RandomForest (numTrees: 10, default)	meta.MetaCost -cost-matrix "[0.0, 1.0; 8.0, 0.0]

	nHBDon_Lipinski, TopoPSA, MW, nRotB, topoRadius, topoDiameter, topoShape, globalTopoChargeIndex		
B1_11PaD_SMO	11 PaDEL descriptors: CrippenLogP, CrippenMR, nHBAcc_Lipinski, nHBDon_Lipinski, TopoPSA, MW, nRotB, topoRadius, topoDiameter, topoShape, globalTopoChargeIndex	functions.SMO.Puk.kernel (buildLogisticModels:True, the rest settings at default)	meta.MetaCost -cost-matrix "[0.0, 1.0; 8.0, 0.0]"

Table A2. OATP1B3 models: Description of the used settings

Model Name	Descriptors	weka.classifier	Cost matrix
B3_6MOE_RF	6 MOE descriptors: a_acc, a_don, logP (o/w), mr, TPSA and weight	trees.RandomForest (numTrees: 10, default)	meta.MetaCost -cost-matrix "[0.0, 1.0; 13.0, 0.0]"
B3_6MOE_SMO	6 MOE descriptors: a_acc, a_don, logP (o/w), mr, TPSA and weight	functions.SMO.Puk.kernel (buildLogisticModels:True, the rest settings at default)	meta.MetaCost -cost-matrix "[0.0, 1.0; 13.0, 0.0]"
B3_6PaD_RF	6 PaDEL descriptors: CrippenLogP, CrippenMR, nHBAcc_Lipinski, nHBDon_Lipinski, TopoPSA, MW	trees.RandomForest (numTrees: 10, default)	meta.MetaCost -cost-matrix "[0.0, 1.0; 13.0, 0.0]"
B3_6PaD_SMO	6 PaDEL descriptors: CrippenLogP, CrippenMR, nHBAcc_Lipinski, nHBDon_Lipinski, TopoPSA, MW	functions.SMO.Puk.kernel (buildLogisticModels:True, the rest settings at default)	meta.MetaCost -cost-matrix "[0.0, 1.0; 13.0, 0.0]"
B3_11PaD_RF	11 PaDEL descriptors: CrippenLogP, CrippenMR,	trees.RandomForest (numTrees: 10, default)	meta.MetaCost -cost-matrix "[0.0, 1.0; 13.0, 0.0]"

	nHBAcc_Lipinski, nHBDOn_Lipinski, TopoPSA, MW, nRotB, topoRadius, topoDiameter, topoShape, globalTopoChargeIndex		
B3_11PaD_SMO	11 PaDEL descriptors: CrippenLogP, CrippenMR, nHBAcc_Lipinski, nHBDOn_Lipinski, TopoPSA, MW, nRotB, topoRadius, topoDiameter, topoShape, globalTopoChargeIndex	functions.SMO.Puk.kernel (buildLogisticModels:True, the rest settings at default)	meta.MetaCost -cost-matrix "[0.0, 1.0; 8.0, 0.0]"

Scripts developed in R for plotting the ROC curves obtained from all 6 OATP1B1 and OATP1B3 models. If not defined otherwise, the script was written by the author of the Thesis.

#####

Script 1

R script for plotting the ROC curves for all 6 OATP1B1 models

```
library(ROCR)
```

```
sum_scoresB1 <- c(OATP1B1_total$B1_Sum_.0.1.Pred)
labelsB1 <- c(OATP1B1_total$Actual_Binary_Characterization)
```

Plotting the separate ROC curves for each model and add them to the original curve.

```
B1_6MOE_RF <- c(OATP1B1_total$OATP1B1_6MOEdscr_RF_.0.1.prediction)
pred_B1_6MOE_RF <- prediction(B1_6MOE_RF, labelsB1)
perf_B1_6MOE_RF <- performance(pred_B1_6MOE_RF, "tpr", "fpr")
plot(perf_B1_6MOE_RF, lwd=3, xlim= c(0.0,0.1), ylim= c(0.0,0.5), main = "OATP1B1 models ROC plots
zoom", cex.lab = 1.5, cex.axis= 1.5, cex.main = 1.8, col="red") ## red ROC curve for B1_6MOE_RF
```

```
B1_6MOE_SMO <- c(OATP1B1_total$OATP1B1_6MOEdscr_SMO_.0.1.prediction)
pred_B1_6MOE_SMO <- prediction(B1_6MOE_SMO, labelsB1)
perf_B1_6MOE_SMO <- performance(pred_B1_6MOE_SMO, "tpr", "fpr")
plot(perf_B1_6MOE_SMO, add=T, lwd=3, xlim= c(0.0,0.1), ylim= c(0.0,0.5), col="green") ## green ROC
curve for B1_6MOE_SMO
```

```
B1_6PaD_RF <- c(OATP1B1_total$OATP1B1_6PaDELdscr_RF_.0.1.prediction)
pred_B1_6PaD_RF <- prediction(B1_6PaD_RF, labelsB1)
```

```

perf_B1_6PaD_RF <- performance(pred_B1_6PaD_RF, "tpr", "fpr")
plot(perf_B1_6PaD_RF, add=T, lwd=3, xlim= c(0.0,0.1), ylim= c(0.0,0.5), col="blue") ## blue ROC curve
for B1_6PaD_RF

B1_6PaD_SMO <- c(OATP1B1_total$OATP1B1_6PaDELdscr_SMO_.0.1.prediction)
pred_B1_6PaD_SMO <- prediction(B1_6PaD_SMO, labelsB1)
perf_B1_6PaD_SMO <- performance(pred_B1_6PaD_SMO, "tpr", "fpr")
plot(perf_B1_6PaD_SMO, add=T, lwd=3, xlim= c(0.0,0.1), ylim= c(0.0,0.5), col="yellow") ## yellow ROC
curve for B1_6PaD_SMO

B1_11PaD_RF <- c(OATP1B1_total$OATP1B1_11PaDELdscr_RF_.0.1.prediction)
pred_B1_11PaD_RF <- prediction(B1_11PaD_RF, labelsB1)
perf_B1_11PaD_RF <- performance(pred_B1_11PaD_RF, "tpr", "fpr")
plot(perf_B1_11PaD_RF, add=T, lwd=3, xlim= c(0.0,0.1), ylim= c(0.0,0.5), col="cyan") ## cyan ROC curve
for B1_11_PaD_RF

B1_11PaD_SMO <- c(OATP1B1_total$OATP1B1_11PaDELdscr_SMO_.0.1.prediction)
pred_B1_11PaD_SMO <- prediction(B1_11PaD_SMO, labelsB1)
perf_B1_11PaD_SMO <- performance(pred_B1_11PaD_SMO, "tpr", "fpr")
plot(perf_B1_11PaD_SMO, add=T, lwd=3, xlim= c(0.0,0.1), ylim= c(0.0,0.5), col="violet") ## violet ROC
curve for B1_11PaD_SMO

## Plotting the consensus ROC curve
labelsB1 <- c(OATP1B1_total$Actual_Binary_Characterization)
B1_consensus_pred <- prediction(sum_scoresB1, labelsB1)
B1_consensus_perf <- performance(B1_consensus_pred, "tpr", "fpr")
plot(B1_consensus_perf, add=T, lwd=3, xlim= c(0.0,0.1), ylim= c(0.0,0.5), colorize=F) ## The consensus
ROC curve black

##Plotting the Random Performance line
abline(a=0, b=1, lwd=3, lty=5, xlim= c(0.0,0.1), ylim= c(0.0,0.5), col= "brown")

```

```
#####
```

Script 2

```
#### R script for plotting the ROC curves for all 6 OATP1B3 models
```

```
library(ROCR)
```

```

sum_scoresB3 <- c(OATP1B3_total$B3_Sum_.0.1.Pred_.0.6.)
labelsB3 <- c(OATP1B3_total$B3_Actual_Binary_Characterization)

## Plotting the separate ROC curves for each model and add them to the original curve.
B3_6MOE_RF <- c(OATP1B3_total$OATP1B3_6MOEdscr_RF_.0.1.prediction)
pred_B3_6MOE_RF <- prediction(B3_6MOE_RF, labelsB3)
perf_B3_6MOE_RF <- performance(pred_B3_6MOE_RF, "tpr", "fpr")
plot(perf_B3_6MOE_RF, lwd=3, xlim= c(0.0,0.1), ylim= c(0.0,0.5), main = "OATP1B3 models ROC plots
zoom", cex.lab = 1.5, cex.axis= 1.5, cex.main = 1.8, col="red") ## red ROC curve for B3_6MOE_RF

```



```

B3_6MOE_SMO <- c(OATP1B3_total$OATP1B3_6MOEdscr_SMO_.0.1.prediction)
pred_B3_6MOE_SMO <- prediction(B3_6MOE_SMO, labelsB3)
perf_B3_6MOE_SMO <- performance(pred_B3_6MOE_SMO, "tpr", "fpr")
plot(perf_B3_6MOE_SMO, add=T, lwd=3, xlim= c(0.0,0.1), ylim= c(0.0,0.5), col="green") ## green ROC
curve for B3_6MOE_SMO

B3_6PaD_RF <- c(OATP1B3_total$OATP1B3_6PaDELdscr_RF_.0.1.prediction)
pred_B3_6PaD_RF <- prediction(B3_6PaD_RF, labelsB3)
perf_B3_6PaD_RF <- performance(pred_B3_6PaD_RF, "tpr", "fpr")
plot(perf_B3_6PaD_RF, add=T, lwd=3, xlim= c(0.0,0.1), ylim= c(0.0,0.5), col="blue") ## blue ROC curve
for B3_6PaD_RF

B3_6PaD_SMO <- c(OATP1B3_total$OATP1B3_6PaDELdscr_SMO_.0.1.prediction)
pred_B3_6PaD_SMO <- prediction(B3_6PaD_SMO, labelsB3)
perf_B3_6PaD_SMO <- performance(pred_B3_6PaD_SMO, "tpr", "fpr")
plot(perf_B3_6PaD_SMO, add=T, lwd=3, xlim= c(0.0,0.1), ylim= c(0.0,0.5), col="yellow") ## yellow ROC
curve for B3_6PaD_SMO

B3_11PaD_RF <- c(OATP1B3_total$OATP1B3_11PaDELdscr_RF_.0.1.prediction)
pred_B3_11PaD_RF <- prediction(B3_11PaD_RF, labelsB3)
perf_B3_11PaD_RF <- performance(pred_B3_11PaD_RF, "tpr", "fpr")
plot(perf_B3_11PaD_RF, add=T, lwd=3, xlim= c(0.0,0.1), ylim= c(0.0,0.5), col="cyan") ## cyan ROC curve
for B3_11_PaD_RF

B3_11PaD_SMO <- c(OATP1B3_total$OATP1B3_11PaDELdscr_SMO_.0.1.prediction)
pred_B3_11PaD_SMO <- prediction(B3_11PaD_SMO, labelsB3)
perf_B3_11PaD_SMO <- performance(pred_B3_11PaD_SMO, "tpr", "fpr")
plot(perf_B3_11PaD_SMO, add=T, lwd=3, xlim= c(0.0,0.1), ylim= c(0.0,0.5), col="violet") ## violet ROC
curve for B3_11PaD_SMO

## Plotting the consensus ROC curve
B3_consensus_pred <- prediction(sum_scoresB3, labelsB3)
B3_consensus_perf <- performance(B3_consensus_pred, "tpr", "fpr")
plot(B3_consensus_perf, add=T, lwd=3, xlim= c(0.0,0.1), ylim= c(0.0,0.5), colorize=F) ## The consensus
ROC curve black

##Plotting the Random Performance line
abline(a=0, b=1, lwd=3, lty=5, xlim= c(0.0,0.1), ylim= c(0.0,0.5), col= "brown")
#####

```

3. Supplements to Chapter 4 (and 6)

Table A3: List of the 93 molecular 2D MOE descriptors used for the DILI classification model for human data.

	MOE Descriptor	Description
1	apol	Sum of the atomic polarizabilities (including implicit hydrogens) with polarizabilities taken from [CRC 1994]
2	a_acc	Number of hydrogen bond acceptor atoms (not counting acidic atoms but counting atoms that are both hydrogen bond donors and acceptors such as -OH).
3	a_acid	Number of acidic atoms.
4	a_aro	Number of aromatic atoms.
5	a_count	Number of atoms (including implicit hydrogens). This is calculated as the sum of $(1 + h_i)$ over all non-trivial atoms i .
6	a_don	Number of hydrogen bond donor atoms (not counting basic atoms but counting atoms that are both hydrogen bond donors and acceptors such as -OH).
7	a_donacc	Number of hydrogen bond donor and hydrogen bond acceptor atoms.
8	a_heavy	Number of heavy atoms $\#\{Z_i \mid Z_i > 1\}$.
9	a_hyd	Number of hydrophobic atoms.
10	a_IC	Atom information content (total). This is calculated to be a_ICM times n .
11	a_ICM	Atom information content (mean). This is the entropy of the element distribution in the molecule (including implicit hydrogens but not lone pair pseudo-atoms). Let n_i be the number of occurrences of atomic number i in the molecule. Let $p_i = n_i / n$ where n is the sum of the n_i . The value of a_ICM is the negative of the sum over all i of $p_i \log p_i$.
12	a_nBr	Number of bromine atoms: $\#\{Z_i \mid Z_i = 35\}$.
13	a_nC	Number of carbon atoms: $\#\{Z_i \mid Z_i = 6\}$.
14	a_nCl	Number of chlorine atoms: $\#\{Z_i \mid Z_i = 17\}$.
15	a_nF	Number of fluorine atoms: $\#\{Z_i \mid Z_i = 9\}$.
16	a_nH	Number of hydrogen atoms (including implicit hydrogens). This is calculated as the sum of h_i over all non-trivial atoms i plus the number of non-trivial hydrogen atoms.
17	A_nI	Number of iodine atoms: $\#\{Z_i \mid Z_i = 53\}$
18	a_nN	Number of nitrogen atoms: $\#\{Z_i \mid Z_i = 7\}$.
19	a_nO	Number of oxygen atoms: $\#\{Z_i \mid Z_i = 8\}$.
20	a_nP	Number of phosphorus atoms: $\#\{Z_i \mid Z_i = 15\}$.
21	a_nS	Number of sulfur atoms: $\#\{Z_i \mid Z_i = 16\}$.
22	bpol	Sum of the absolute value of the difference between atomic

		polarizabilities of all bonded atoms in the molecule (including implicit hydrogens) with polarizabilities taken from [CRC 1994].
23	b_1rotN	Number of rotatable single bonds. Conjugated single bonds are not included (e.g. ester and peptide bonds).
24	b_1rotR	Fraction of rotatable single bonds: b_1rotN divided by b_heavy.
25	b_ar	Number of aromatic bonds.
26	b_count	Number of bonds (including implicit hydrogens). This is calculated as the sum of $(d_i/2 + h_i)$ over all non-trivial atoms i .
27	b_double	Number of double bonds. Aromatic bonds are not considered to be double bonds.
28	b_heavy	Number of bonds between heavy atoms.
29	b_max1len	Maximum single bond chain length.
30	b_rotN	Number of rotatable bonds. A bond is rotatable if it has order 1, is not in a ring, and has at least two heavy neighbors.
31	b_rotR	Fraction of rotatable bonds: b_rotN divided by b_heavy.
32	b_single	Number of single bonds (including implicit hydrogens). Aromatic bonds are not considered to be single bonds.
33	b_triple	Number of triple bonds. Aromatic bonds are not considered to be triple bonds.
34	chiral_u	The number of unconstrained chiral centers.
35	density	Molecular mass density: Weight divided by vdw_vol (amu/Å ³).
36	diameter	Largest value in the distance matrix [Petitjean 1992]
37	lip_acc	The number of O and N atoms.
38	lip_don	The number of OH and NH atoms.
39	logP(o/w)	Log of the octanol/water partition coefficient (including implicit hydrogens). This property is calculated from a linear atom type model [LOGP 1998] with $r^2 = 0.931$, RMSE=0.393 on 1,827 molecules.
40	logS	Log of the aqueous solubility (mol/L). This property is calculated from an atom contribution linear atom type model [Hou 2004] with $r^2 = 0.90$, ~1,200 molecules.
41	mr	Molecular refractivity (including implicit hydrogens). This property is calculated from an 11 descriptor linear model [MREF 1998] with $r^2 = 0.997$, RMSE = 0.168 on 1,947 small molecules.
42	PC+	Total positive partial charge: the sum of the positive q_i . Q_PC+ is identical to PC+ which has been retained for compatibility.
43	PC-	Total negative partial charge: the sum of the negative q_i . Q_PC- is identical to PC- which has been retained for compatibility.
44	PEOE_PC+	Total positive partial charge: the sum of the positive q_i .
45	Q_PC+	
46	PEOE_PC-	Total negative partial charge: the sum of the negative q_i .
47	Q_PC-	
48	PEOE_RPC+	Relative positive partial charge: the largest positive q_i divided by the sum of the positive q_i .
49	Q_RPC+	
50	PEOE_RPC-	Relative negative partial charge: the smallest negative q_i divided

51	Q_RPC-	by the sum of the negative q_i .
52 53	PEOE_VSA_FHYD Q_VSA_FHYD	Fractional hydrophobic van der Waals surface area. This is the sum of the v_i such that $ q_i $ is less than or equal to 0.2 divided by the total surface area. The v_i are calculated using a connection table approximation.
54 55	PEOE_VSA_FNEG Q_VSA_FNEG	Fractional negative van der Waals surface area. This is the sum of the v_i such that q_i is negative divided by the total surface area. The v_i are calculated using a connection table approximation.
56 57	PEOE_VSA_FPNEG Q_VSA_FPNEG	Fractional negative polar van der Waals surface area. This is the sum of the v_i such that q_i is less than -0.2 divided by the total surface area. The v_i are calculated using a connection table approximation.
58 59	PEOE_VSA_FPOL Q_VSA_FPOL	Fractional polar van der Waals surface area. This is the sum of the v_i such that $ q_i $ is greater than 0.2 divided by the total surface area. The v_i are calculated using a connection table approximation.
60 61	PEOE_VSA_FPOS Q_VSA_FPOS	Fractional positive van der Waals surface area. This is the sum of the v_i such that q_i is non-negative divided by the total surface area. The v_i are calculated using a connection table approximation.
62 63	PEOE_VSA_FPPOS Q_VSA_FPPOS	Fractional positive polar van der Waals surface area. This is the sum of the v_i such that q_i is greater than 0.2 divided by the total surface area. The v_i are calculated using a connection table approximation.
64 65	PEOE_VSA_HYD Q_VSA_HYD	Total hydrophobic van der Waals surface area. This is the sum of the v_i such that $ q_i $ is less than or equal to 0.2. The v_i are calculated using a connection table approximation.
66 67	PEOE_VSA_NEG Q_VSA_NEG	Total negative van der Waals surface area. This is the sum of the v_i such that q_i is negative. The v_i are calculated using a connection table approximation.
68 69	PEOE_VSA_PNEG Q_VSA_PNEG	Total negative polar van der Waals surface area. This is the sum of the v_i such that q_i is less than -0.2. The v_i are calculated using a connection table approximation.
70 71	PEOE_VSA_POL Q_VSA_POL	Total polar van der Waals surface area. This is the sum of the v_i such that $ q_i $ is greater than 0.2. The v_i are calculated using a connection table approximation.
72 73	PEOE_VSA_POS Q_VSA_POS	Total positive van der Waals surface area. This is the sum of the v_i such that q_i is non-negative. The v_i are calculated using a connection table approximation.
74 75	PEOE_VSA_PPOS Q_VSA_PPOS	Total positive polar van der Waals surface area. This is the sum of the v_i such that q_i is greater than 0.2. The v_i are calculated using a connection table approximation.
76	radius	If r_i is the largest matrix entry in row i of the distance matrix D , then the radius is defined as the smallest of the r_i [Petitjean 1992].
77	reactive	Indicator of the presence of reactive groups. A non-zero value

		indicates that the molecule contains a reactive group. The table of reactive groups is based on the Oprea set [Oprea 2000] and includes metals, phospho-, N/O/S-N/O/S single bonds, thiols, acyl halides, Michael Acceptors, azides, esters, etc.
78	rings	The number of rings.
79	RPC+	Relative positive partial charge.
80	RPC-	Relative negative partial charge.
81	SlogP	Log of the octanol/water partition coefficient (including implicit hydrogens). This property is an atomic contribution model [Crippen 1999] that calculates logP from the given structure; i.e. the correct protonation state (washed structures). Results may vary from the logP(o/w) descriptor. The training set for SlogP was ~7000 structures.
82	SMR	Molecular refractivity (including implicit hydrogens). This property is an atomic contribution model [Crippen 1999] that assumes the correct protonation state (washed structures). The model was trained on ~7000 structures and results may vary from the mr descriptor.
83	TPSA	Polar surface area ($\text{\AA}^2$) calculated using group contributions to approximate the polar surface area from connection table information only. The parameterization is that of Ertl <i>et al.</i> [Ertl 2000].
84	vdw_area	Area of van der Waals surface ($\text{\AA}^2$) calculated using a connection table approximation.
85	vdw_vol	van der Waals volume ($\text{\AA}^3$) calculated using a connection table approximation.
86	vsa_acc	Approximation to the sum of VDW surface areas ($\text{\AA}^2$) of pure hydrogen bond acceptors (not counting acidic atoms and atoms that are both hydrogen bond donors and acceptors such as -OH).
87	vsa_acid	Approximation to the sum of VDW surface areas of acidic atoms ($\text{\AA}^2$).
88	Vsa_base	Approximation to the sum of VDW surface areas of basic atoms.
89	vsa_don	Approximation to the sum of VDW surface areas of pure hydrogen bond donors (not counting basic atoms and atoms that are both hydrogen bond donors and acceptors such as -OH) ($\text{\AA}^2$).
90	vsa_hyd	Approximation to the sum of VDW surface areas of hydrophobic atoms ($\text{\AA}^2$).
91	vsa_other	Approximation to the sum of VDW surface areas ($\text{\AA}^2$) of atoms typed as "other".
92	vsa_pol	Approximation to the sum of VDW surface areas ($\text{\AA}^2$) of polar atoms (atoms that are both hydrogen bond donors and acceptors), such as -OH.
93	Weight	Molecular weight (including implicit hydrogens) in atomic mass units with atomic weights taken from [CRC 1994].

In Table A4 is presented the average statistical performance for 10-fold cross validation of the 8 developed models for all possible combinations of settings:

- 1) all 2D MOE descriptors trained with Random Forest (DILI_all_2D_MOE_dscrs_RF)
- 2) all 2D MOE descriptors and transporters predictions included, trained with Random Forest (DILI_all_2D_MOE_dscrs_transp_pred_RF)
- 3) all 2D MOE descriptors trained with combination of RealAdaBoost and Random Forest (DILI_all_2D_MOE_dscrs_RealAdaBoost_RF)
- 4) all 2D MOE descriptors and transporters predictions included, trained with combination of RealAdaBoost and Random Forest (DILI_all_2D_MOE_dscrs_transp_pred_RealAdaBoost_RF)
- 5) 93 2D MOE descriptors trained with Random Forest (DILI_93_2D_MOE_dscrs_RF)
- 6) 93 2D MOE descriptors and transporters predictions included, trained with Random Forest (DILI_93_2D_MOE_dscrs_transp_pred_RF)
- 7) 93 2D MOE descriptors trained with combination of RealAdaBoost and Random Forest (DILI_93_2D_MOE_dscrs_RealAdaBoost_RF)
- 8) 93 2D MOE descriptors and transporters predictions included, trained with combination of RealAdaBoost and Random Forest (DILI_all_93_MOE_dscrs_transp_pred_RealAdaBoost_RF)

The statistic metrics presented are accuracy, sensitivity, specificity, Matthews Correlation Coefficient (MCC), area under the curve (AUC) and precision. For each statistics metric is presented the average value for 10-fold cross validation out of 50 iterations and the standard deviation.

Table A4. The average performance for 10-fold cross validation out of 50 iterations and the standard deviation for accuracy, sensitivity, specificity, MCC, AUC and precision for the best obtained models

Average of 50 iterations						
	Accuracy	Sensitivity	Specificity	MCC	AUC	Precision
1. DILI_all_2D_MOE_dscrs_RF	0.643	0.677	0.607	0.285	0.692	0.649
sd	0.009	0.012	0.015	0.018	0.008	0.009
2. DILI_all_2D_MOE_dscrs_ transp_pred_RF	0.646	0.680	0.609	0.290	0.692	0.651
sd	0.009	0.013	0.014	0.019	0.008	0.009
3. DILI_all_2D_MOE_dscrs_ transp_pred_RealAdaBoost_RF	0.656	0.700	0.609	0.308	0.702	0.658

RealAdaBoost_RF						
sd	0.008	0.011	0.011	0.018	0.007	0.008
4. DILI_all_2D_MOE_dscrs_	0.654	0.700	0.607	0.307	0.702	0.656
transp_pred_RealAdaBoost_RF						
sd	0.008	0.010	0.012	0.017	0.007	0.008
5. DILI_93_2D_MOE_dscrs_RF	0.630	0.660	0.599	0.266	0.664	0.637
sd	0.008	0.010	0.017	0.061	0.060	0.008
6. DILI_93_2D_MOE_dscrs_	0.625	0.657	0.591	0.249	0.671	0.633
transp_pred_RF						
sd	0.009	0.013	0.012	0.018	0.008	0.008
7. DILI_93_2D_MOE_dscrs_	0.637	0.675	0.597	0.273	0.676	0.642
RealAdaBoost_RF						
sd	0.008	0.012	0.013	0.017	0.007	0.008
8. DILI_93_2D_MOE_dscrs_	0.635	0.675	0.593	0.269	0.674	0.641
transp_pred_RealAdaBoost_RF						
sd	0.008	0.011	0.012	0.017	0.007	0.008

Table A5 presents the statistical performance of the same models on the external validation sets by Liew et al.⁴⁸ of 910 compounds (527 positives and 383 negatives) and Mulliner et al.³⁰ of 1586 compounds (980 positives and 606 negatives).

Table A5. The statistical performance on external validation for accuracy, sensitivity, specificity, MCC, AUC and precision for the best obtained models. The external test validated are the datasets by Liew et al.⁴⁸ of 910 compounds (527 positives and 383 negatives) and Mulliner et al.³⁰ of 1586 compounds (980 positives and 606 negatives)

Model	Test Set	Accuracy	Sensitivity	Specificity	MCC	AUC	Precision
1. DILI_all_2D_MOE_dscrs_RF	Liew 910 cpds	0.709	0.710	0.708	0.413	0.782	0.770
2. DILI_all_2D_MOE_dscrs_transp_pred_RF	Mulliner 1586 cpds	0.605	0.609	0.598	0.194	0.641	0.756
	Liew 910 cpds	0.697	0.685	0.713	0.393	0.782	0.766
3. DILI_all_2D_MOE_dscrs_RealAdaBoost_RF	Mulliner 1586 cpds	0.599	0.598	0.602	0.188	0.639	0.754
	Liew 910cpds	0.718	0.704	0.736	0.435	0.777	0.786
4. DILI_all_2D_MOE_dscrs_transp_pred_RealAdaBoost_RF	Mulliner 1586 cpds	0.603	0.614	0.580	0.183	0.642	0.749
	Liew 910 cpds	0.714	0.698	0.736	0.429	0.778	0.785
	Mulliner 1586 cpds	0.601	0.609	0.585	0.182	0.642	0.750
	Liew 910 cpds	0.714	0.700	0.734	0.429	0.783	0.783
5. DILI_93_2D_MOE_dscrs_RF							
6. DILI_93_2D_MOE_dscrs_transp_pred_RF	Mulliner 1586 cpds	0.575	0.584	0.561	0.141	0.592	0.683
	Liew 910 cpds	0.712	0.704	0.723	0.422	0.784	0.778
7. DILI_93_2D_MOE_dscrs_RealAdaBoost_RF	Mulliner 1586 cpds	0.575	0.597	0.540	0.133	0.587	0.677
	Liew 910 cpds	0.716	0.710	0.726	0.431	0.776	0.781
	Mulliner 1586 cpds	0.569	0.599	0.521	0.118	0.593	0.669

8. DILI_93_2D_MOE_dscrs_	Liew 910 cpds	0.713	0.704	0.723	0.422	0.784	0.778
transp_pred_RealAdaBoost_RF							
	Mulliner	0.574	0.611	0.513	0.122	0.595	0.670
	1586 cpds						

Script 1

Script for P-gp inhibition classification model. Developed by **Floriane Montanari**:

"""

This python script allows building, cross-validating and using the P-glycoprotein inhibition model reported in the publication. "Subtle Structural Differences Trigger Inhibitory Activity of Propafenone Analogues at the Two Polyspecific ABC Transporters: P-Glycoprotein (P-gp) and Breast Cancer Resistance Protein (BCRP), Schwarz, T., Montanari, F., Cseke, A.a, Wlcek, K., Visvader, L., Palme, S., Chiba, P., Kuchler, K., Urban, E., Ecker, G.F."

"""

It requires:

- python 2.7 or higher, but not 3.x
- the scikit-learn machine learning library
- the rdkit machine learning library
- the numpy library
- the sdf file of the training set

NOTE: When training the model, you may obtain at the cross-validation step results that slightly differ from what is reported in the paper. This is due to different random number generators used for the cross-validation.

If this script is useful to your work, please cite the corresponding paper.

"""

```
import numpy as np
import os.path as op
import cPickle as pickle
from copy import copy
from sklearn import svm
from sklearn.grid_search import GridSearchCV
from sklearn.cross_validation import StratifiedKFold, cross_val_score
from sklearn.metrics import confusion_matrix
from sklearn.utils import shuffle
from rdkit.Chem import AllChem
```

TO CUSTOMIZE

#####

TRAINING =

'/media/eleni/Helios/Classification_Models_Floriane/MDR1.class.FM.2/Cruciani_pgp_inhib_training.sdf'
path to the training set

TRAINED_MODEL =

'/media/eleni/Helios/Classification_Models_Floriane/MDR1.class.FM.2/pgp_inhibition.pkl' # where the
trained model will be stored

TEST_SET =

'/media/eleni/Helios/eTOX_Hackathon/Hackathon_2015/Paper_Chem_Res_Toxicol_and_Support_Info/
Datasets/Data_Standardization/Human_data/H_HT_class_2089cpds_to_predict.sdf' # path to the
data to predict

MOLID_TEST = 'Index' # name of the property in the sdf file that corresponds to the unique identifier of the molecules

PREDICTIONS =

'/media/eleni/Helios/eTOX_Hackathon/Hackathon_2015/Paper_Chem_Res_Toxicol_and_Support_Info/
 Datasets/Data_Standardization/Human_data/predictions_Pgp_inhibition.csv' # path to the file where
 the predictions are stored

#####

def compute_fpts(mols, radius=4, folding_size=1024):

"""

For ECFP8, insert radius=4

Given a list of rdkit molecules, returns an array of Morgan fingerprints folded to the required folding
 size

and having the required radius.

"""

X = []

for mol in mols:

ecfp = AllChem.GetMorganFingerprintAsBitVect(mol, radius, nBits=folding_size)

ecfp_bits = [int(ecfp.GetBit(i)) for i in range(ecfp.GetNumBits())]

X.append(ecfp_bits)

return np.array(X)

def xys(sdf, label_name, radius=4, folding_size=1024, class1='1'):

"""

Given an sdf file with at least one property (of name label_name) containing the label. The class value
 that should

be considered as positive is explicitly defined in class1.

returns the fingerprints matrix X (num_molecules x num_bits) and the y vector containing the actual
 label

"""

mols = []

labels = []

for mol in AllChem.SDMolSupplier(sdf):

if mol is not None:

mols.append(mol)

labels.append(1 if mol.GetProp(label_name) == class1 else 0)

y = np.array(labels).astype(float)

x = compute_fpts(mols, radius=radius, folding_size=folding_size)

return x, y

def build_model(training_set, label_name, model_pickle_file):

model = svm.SVC(probability=True)

params = [{'C': [0.5, 1, 5, 10, 100], 'kernel': ['rbf'], 'gamma': [1e-4, 1e-3, 0.01, 0.1, 0], 'probability':
 [True]}]

SVM = GridSearchCV(model, params, cv=5, scoring='roc_auc', n_jobs=1)

model = copy(SVM)

X, y = xys(training_set, label_name)

```

# train the model
model.fit(X, y)
print 'Best parameters: '
print model.best_params_
# save the model
with open(model_pickle_file, 'w') as writer:
    pickle.dump(model, writer)
return model

def cross_validate_model(training_set, label_name):
    model = svm.SVC(probability=True)
    params = [{'C': [0.5, 1, 5, 10, 100], 'kernel': ['rbf'], 'gamma': [1e-4, 1e-3, 0.01, 0.1, 0], 'probability':
[True]}]
    SVM = GridSearchCV(model, params, cv=5, scoring='roc_auc', n_jobs=1)
    model = copy(SVM)

    X, y = xys(training_set, label_name)
    num_cvfolds = 10
    X, y = shuffle(X, y, random_state=0)
    skf = StratifiedKFold(y, n_folds=num_cvfolds, random_state=0)
    all_scores = []
    ys = []
    for train, test in skf:
        Xtrain = X[train]
        ytrain = y[train]
        Xtest = X[test]
        ytest = y[test]
        scores = model.fit(Xtrain, ytrain).predict_proba(Xtest)[:, 1]
        all_scores.append(scores)
        ys.append(ytest)
    all_scores = np.hstack(all_scores) >= 0.5
    ys = np.hstack(ys)
    aucs = cross_val_score(model, X, y=y, scoring='roc_auc', cv=skf)
    return confusion_matrix(np.array(ys), np.array(all_scores)), np.mean(np.array(aucs))

def predict_proba(dataset, model_file, preds_file=None, save_preds=False, col_name=None):
    """
    dataset is a cleaned sdf. It has to contain a property (col_name) with an identifier.
    model_file is a pickled file containing the trained model
    preds_file: optional, path to the csv file where the predictions will be stored
    """

    # 1. Read and compute descriptors
    mols = []
    molids = []
    for i, mol in enumerate(AllChem.SDMolSupplier(dataset)):
        if mol is not None:
            mols.append(mol)
            if col_name is None:

```

```

        try:
            molid = mol.GetProp('_Name')
        except:
            molid = i
    else:
        molid = mol.GetProp(col_name)
    molids.append(molid)
else:
    print 'Could not read molecule: %i' % i
X = compute_fpts(mols)

# 2. Load the model
with open(model_file, 'r') as reader:
    model = pickle.load(reader)

# 3. Predict the probability of being a BCRP inhibitor
scores = model.predict_proba(X)[:, 1]
if save_preds:
    with open(preds_file, 'w') as writer:
        for i, score in enumerate(scores):
            writer.write(str(molids[i]))
            writer.write(',')
            writer.write(str(scores[i]))
            writer.write('\n')
    return zip(molids, scores)

if __name__ == '__main__':

    # check whether the model exists
    if op.exists(TRAINED_MODEL) and op.isfile(TRAINED_MODEL):
        try:
            # predict P-glycoprotein inhibition for the given TEST_SET, save the predictions into PREDICTIONS
            predict_proba(TEST_SET, TRAINED_MODEL, col_name=MOLID_TEST, save_preds=True,
preds_file=PREDICTIONS)
        except:
            print 'Could not predict the test set. Check that the model is trained, and the paths are properly
customized.'
    # if the model does not exist, build it and evaluate it
    else:
        print 'The model does not seem to exist yet. Building it now...'
        # 1. Build the model
        try:
            build_model(TRAINING, 'Activity', TRAINED_MODEL)
        except:
            print 'Could not train the model. Check that the paths are properly customized.'
        # 2. Evaluate by 10-fold CV
        try:
            confusion_mat, auc = cross_validate_model(TRAINING, 'Activity')

```

```

    print 'AUC: %.3f' % auc
    print 'TP: %i' % confusion_mat[1][1]
    print 'TN: %i' % confusion_mat[0][0]
    print 'FP: %i' % confusion_mat[0][1]
    print 'FN: %i' % confusion_mat[1][0]
except:
    print 'Could not cross-validate the model. Check that the paths are properly customized'
# 3. Predict the TEST_SET
try:
    predict_proba(TEST_SET, TRAINED_MODEL, col_name=MOLID_TEST, save_preds=True,
preds_file=PREDICTIONS)
except:
    print 'Could not predict the test set. Check that the model is trained, and the paths are properly
customized.'
```

Script 2

Script for BCRP inhibition classification model. Developed by **Floriane Montanari**:

''''''

This python script allows building, cross-validating and using the BCRP inhibition model reported in the publication.

"Virtual screening of DrugBank reveals two drugs as new BCRP inhibitors", Montanari F., Wlcek K., Cseke A., Ecker G.F.

It requires:

- python 2.7 or higher, but not 2.3
- the scikit-learn machine learning library
- the rdkit machine learning library
- the numpy library
- the sdf file of the training set

NOTE: When training the model, you may obtain at the cross-validation step results that slightly differ from Table 1

in the paper. This is due to different random number generators used for the cross-validation.

If this script is useful to your work, please cite the corresponding paper.

''''''

```

import numpy as np
import os.path as op
import cPickle as pickle
from copy import copy
from sklearn.linear_model import LogisticRegression
from sklearn.grid_search import GridSearchCV
from sklearn.cross_validation import StratifiedKFold, cross_val_score
from sklearn.metrics import confusion_matrix
from sklearn.utils import shuffle
from rdkit.Chem import AllChem
```

```

##### TO CUSTOMIZE
#####
TRAINING = '/media/eleni/Helios/Classification_Models_Floriane/BCRP.class.FM.1/BCRP_training.sdf' #
path to the training set, available in Supplementary Information
TRAINED_MODEL =
'/media/eleni/Helios/Classification_Models_Floriane/BCRP.class.FM.1/bcrp_inhibition.pkl' # where the
trained model will be stored
TEST_SET =
'/media/eleni/Helios/eTOX_Hackathon/Hackathon_2015/Paper_Chem_Res_Toxicol_and_Support_Info/
Datasets/Data_Standardization/Human_data/H_HT_class_2089cpds_for_BCRP_predict.sdf' # path to the
data to predict
MOLID_TEST = 'Index' # name of the property in the sdf file that corresponds to the unique identifier of
the molecules
PREDICTIONS =
'/media/eleni/Helios/eTOX_Hackathon/Hackathon_2015/Paper_Chem_Res_Toxicol_and_Support_Info/
Datasets/Data_Standardization/Human_data/predictions_bcrp_inhibition.csv' # path to the file where
the predictions are stored
#####
#####

def compute_fpts(mols, radius=4, folding_size=1024):
    """
    For ECFP8, insert radius=4
    Given a list of rdkit molecules, returns an array of Morgan fingerprints folded to the required folding
    size
    and having the required radius.
    """
    X = []
    for mol in mols:
        ecfp = AllChem.GetMorganFingerprintAsBitVect(mol, radius, nBits=folding_size)
        ecfp_bits = [int(ecfp.GetBit(i)) for i in range(ecfp.GetNumBits())]
        X.append(ecfp_bits)
    return np.array(X)

def xys(sdf, label_name, radius=4, folding_size=1024, class1='INHIBITOR'):
    """
    Given an sdf file with at least one property (of name label_name) containing the label. The class value
    that should
    be considered as positive is explicitly defined in class1.
    returns the fingerprints matrix X (num_molecules, num_bits) and the y vector containing the actual
    label
    """
    mols = []
    labels = []
    for mol in AllChem.SDMolSupplier(sdf):
        if mol is not None:
            mols.append(mol)
            labels.append(1 if mol.GetProp(label_name) == class1 else 0)
    y = np.array(labels).astype(float)

```

```

x = compute_fps(mols, radius=radius, folding_size=folding_size)
return x, y

def build_model(training_set, label_name, model_pickle_file):
    log = LogisticRegression()
    params = [{'penalty': ['l1', 'l2'], 'C': [0.001, 0.01, 0.1, 0.5, 1, 2, 5, 10]}]
    logreg = GridSearchCV(log, params, cv=5, scoring='roc_auc', n_jobs=1)
    model = copy(logreg)
    X, y = xys(training_set, label_name)
    # train the model
    model.fit(X, y)
    print 'Best parameters: '
    print model.best_params_
    # save the model
    with open(model_pickle_file, 'w') as writer:
        pickle.dump(model, writer)
    return model

def cross_validate_model(training_set, label_name):
    log = LogisticRegression()
    params = [{'penalty': ['l1', 'l2'], 'C': [0.001, 0.01, 0.1, 0.5, 1, 2, 5, 10]}]
    logreg = GridSearchCV(log, params, cv=5, scoring='roc_auc', n_jobs=1)
    model = copy(logreg)

    X, y = xys(training_set, label_name)
    num_cvfolds = 10
    X, y = shuffle(X, y, random_state=0)
    skf = StratifiedKFold(y, n_folds=num_cvfolds, random_state=0)
    all_scores = []
    ys = []
    for train, test in skf:
        Xtrain = X[train]
        ytrain = y[train]
        Xtest = X[test]
        ytest = y[test]
        scores = model.fit(Xtrain, ytrain).predict_proba(Xtest)[: , 1]
        all_scores.append(scores)
        ys.append(ytest)
    all_scores = np.hstack(all_scores) >= 0.5
    ys = np.hstack(ys)
    aucs = cross_val_score(model, X, y=y, scoring='roc_auc', cv=skf)
    return confusion_matrix(np.array(ys), np.array(all_scores)), np.mean(np.array(aucs))

def predict_proba(dataset, model_file, preds_file=None, save_preds=False, col_name=None):
    """
    dataset is a cleaned sdf. It has to contain a property (col_name) with an identifier.
    model_file is a pickled file containing the trained model
    preds_file: optional, path to the csv file where the predictions will be stored

```



```

"""
# 1. Read and compute descriptors
mols = []
molids = []
for i, mol in enumerate(AllChem.SDMolSupplier(dataset)):
    if mol is not None:
        mols.append(mol)
        if col_name is None:
            try:
                molid = mol.GetProp('_Name')
            except:
                molid = i
        else:
            molid = mol.GetProp(col_name)
        molids.append(molid)
    else:
        print 'Could not read molecule: %i' % i
X = compute_fps(mols)

# 2. Load the model
with open(model_file, 'r') as reader:
    model = pickle.load(reader)

# 3. Predict the probability of being a BCRP inhibitor
scores = model.predict_proba(X)[: , 1]
if save_preds:
    with open(preds_file, 'w') as writer:
        for i, score in enumerate(scores):
            writer.write(str(molids[i]))
            writer.write(',')
            writer.write(str(scores[i]))
            writer.write('\n')
    return zip(molids, scores)
if __name__ == '__main__':

    # check whether the model exists
    if op.exists(TRAINED_MODEL) and op.isfile(TRAINED_MODEL):
        try:
            # predict BCRP inhibition for the given TEST_SET, save the predictions into PREDICTIONS
            predict_proba(TEST_SET, TRAINED_MODEL, col_name=MOLID_TEST, save_preds=True,
preds_file=PREDICTIONS)
        except:
            print 'Could not predict the test set. Check that the model is trained, and the paths are properly
customized.'
    # if the model does not exist, build it and evaluate it
    else:
        print 'The model does not seem to exist yet. Building it now...'
        # 1. Build the model

```

```

try:
    build_model(TRAINING, 'Activity', TRAINED_MODEL)
except:
    print 'Could not train the model. Check that the paths are properly customized.'
# 2. Evaluate by 10-fold CV
try:
    confusion_mat, auc = cross_validate_model(TRAINING, 'Activity')
    print 'AUC: %.3f' % auc
    print 'TP: %i' % confusion_mat[1][1]
    print 'TN: %i' % confusion_mat[0][0]
    print 'FP: %i' % confusion_mat[0][1]
    print 'FN: %i' % confusion_mat[1][0]
except:
    print 'Could not cross-validate the model. Check that the paths are properly customized'
# 3. Predict the TEST_SET
try:
    predict_proba(TEST_SET, TRAINED_MODEL, col_name=MOLID_TEST, save_preds=True,
preds_file=PREDICTIONS)
except:
    print 'Could not predict the test set. Check that the model is trained, and the paths are properly
customized.'

```

Script 3

Classification model for DILI for all 2D MOE descriptors (without including the transporters predictions), as implemented in R.

#####

Random Forest Classification Model for DILI without including the transporters predictions

The performance of the models with and without transporters predictions is very similar.

They give almost same results

Only difference: one more TN correct in confusion matrix of test set when no transporters prediction is used

#Make sure you have imported in your environment the training set for the model generation and the test set in order to make the prediction.

#If we don't have two different datasets, we might split the initial dataset.

#However for this case there are two separate datasets: one training and one test set.

DILI_Train = DILI_968cmps_all_2DMOE

DILI_Test = Liew_910cmps_2DMOEdscrs

#DILI_Test2 = H_HT_class_unique_1586cpds_all_2DMOE_dscrs

DILI_Train\$Binary_Characterization = as.factor(DILI_Train\$Binary_Characterization)

DILI_Test\$Binary_Characterization = as.factor(DILI_Test\$Binary_Characterization)

#DILI_Test2\$Binary_Characterization = as.factor(DILI_Test2\$Binary_Characterization)

#str(DILI_Train)

#Build RF model

```

library(randomForest) #prerequisite package to run Random Forest in R

set.seed(1) # The seed should be set in order to have repetitive results
# Still with R even when you set the seed the result might sbe slightly different across machines
# or even on the same machine
# Basic code for generating the RF model
# Index is exclude from the set of descriptors
# ntree=100 is the number of trees; I keep it 100 like the model in WEKA
# I don't set mtry (number of features used in each split), I let R take the default number of
cvclassification sqrt(),
# which is the square root of the total number of features
#I also do not restrict the depth of the trees, since it is also unlimited in WEKA
DILI_RF = randomForest(Binary_Characterization ~ . -Index, data = DILI_Train, ntree=100)

#Code for doing the prediction of the RF (DILI_RF) on the test set (newdata)
# type = 'class' indicates that it is a classification problem and will give back 0 or 1
Predict_DILI_RF2 = predict(DILI_RF, newdata = DILI_Test2, type='class')
#Predict_DILI_RF = predict(DILI_RF, newdata = DILI_Test, type='class')
#Code for calculating the confusion matrix
#Rows of table give the true class anf the columns the predicted class
table(DILI_Test$Binary_Characterization, Predict_DILI_RF)
#These two rows of code are to be used when the true class of the data is known
#Obviously, if you don't know the true class of the test data, you cannot use the table function

#From the confusion matrix accuracy, sensitivity and specificity can be calculated
#accuracy= (TP+TN)/(TP+FP+TN+FN)
#accuracy
#sensitivity= TP/(TP+FN)
#sensitivity
#specificity= TN/(TN+FP)
#specificity
#MCC= ((TP*TN)-(FP*FN))/sqrt((TP+FP)*(TP+FN)*(TN+FP)*(TN+FN))
#MCC
#precision = TP/(TP+FP)
#precision

#Even if you don't know the true class of data, still you can calculate probability
# R is not going to complain

#Write the predictions in form of probability
Predict_DILI_RF_prob = predict(DILI_RF, newdata = DILI_Test, type='prob')[,2]

#Write the predictions in the probability form in a csv file
#First create a dataframe containing the probabilities and the index number of each prediction
Predictions_DILI_RF_no_transp = data.frame(Prediction_no_transp=Predict_DILI_RF_prob, Index=
DILI_Test$Index)
#Write the dataframe into a csv file
#The csv file is going to be written in the working directory of R

```

```

#Otherwise you should define the path according to your wishes
write.csv(Predictions_DILI_RF_no_transp, "Predictions_DILI_RF_without_transp.csv",
row.names=FALSE)

#Calculate the ROC curve
library(ROCR)
#Calculate the probabilities, like above
Predict_DILI_prob = predict(DILI_RF, newdata = DILI_Test, type='prob')[,2]
#Code for calculating and plotting the ROC area
ROC_RF_DILI_prob = prediction(Predict_DILI_prob, DILI_Test2$Binary_Characterization)
perf_DILI = performance(ROC_RF_DILI_prob2, "tpr", "fpr")
plot(perf_DILI)
abline(a=0,b=1,lwd=2,lty=2,col="red")
#Calculate the area under the curve
AUC = as.numeric(performance(ROC_RF_DILI_prob, "auc")@y.values)
AUC #gives back AUC

#Several pieces of code for evaluating the importance of variables
#Also in form of plots for depiction
vu = varUsed(DILI_RF, count=TRUE)
vusorted = sort(vu, decreasing = FALSE, index.return = TRUE)
dotchart(vusorted$x, names(DILI_RF$forest$xlevels[vusorted$ix]))
var_imp= importance(DILI_RF, class='Characterization')
sort(var_imp)
order(var_imp, decreasing=TRUE)
varImpPlot(DILI_RF)
#####

```

4. Supplements to Chapter 5

Table A6: List of the 92 molecular 2D MOE descriptors and the 2 descriptors for OATP1B1/1B3 inhibition used for the hyperbilirubinemia classification model for animal data.

	MOE Descriptor	Description
1	apol	Sum of the atomic polarizabilities (including implicit hydrogens) with polarizabilities taken from [CRC 1994]
2	a_acc	Number of hydrogen bond acceptor atoms (not counting acidic atoms but counting atoms that are both hydrogen bond donors and acceptors such as -OH).
3	a_acid	Number of acidic atoms.
4	a_aro	Number of aromatic atoms.
5	a_count	Number of atoms (including implicit hydrogens). This is calculated as the sum of $(1 + h_i)$ over all non-trivial atoms i .
6	a_don	Number of hydrogen bond donor atoms (not counting basic atoms but counting atoms that are both hydrogen bond donors and acceptors such

		as -OH).
7	a_donacc	Number of hydrogen bond donor and hydrogen bond acceptor atoms.
8	a_heavy	Number of heavy atoms $\#\{Z_i \mid Z_i > 1\}$.
9	a_hyd	Number of hydrophobic atoms.
10	a_IC	Atom information content (total). This is calculated to be a_ICM times n .
11	a_ICM	Atom information content (mean). This is the entropy of the element distribution in the molecule (including implicit hydrogens but not lone pair pseudo-atoms). Let n_i be the number of occurrences of atomic number i in the molecule. Let $p_i = n_i / n$ where n is the sum of the n_i . The value of a_ICM is the negative of the sum over all i of $p_i \log p_i$.
12	a_nBr	Number of bromine atoms: $\#\{Z_i \mid Z_i = 35\}$.
13	a_nC	Number of carbon atoms: $\#\{Z_i \mid Z_i = 6\}$.
14	a_nCl	Number of chlorine atoms: $\#\{Z_i \mid Z_i = 17\}$.
15	a_nF	Number of fluorine atoms: $\#\{Z_i \mid Z_i = 9\}$.
16	a_nH	Number of hydrogen atoms (including implicit hydrogens). This is calculated as the sum of h_i over all non-trivial atoms i plus the number of non-trivial hydrogen atoms.
17	a_nN	Number of nitrogen atoms: $\#\{Z_i \mid Z_i = 7\}$.
18	a_nO	Number of oxygen atoms: $\#\{Z_i \mid Z_i = 8\}$.
19	a_nP	Number of phosphorus atoms: $\#\{Z_i \mid Z_i = 15\}$.
20	a_nS	Number of sulfur atoms: $\#\{Z_i \mid Z_i = 16\}$.
21	bpol	Sum of the absolute value of the difference between atomic polarizabilities of all bonded atoms in the molecule (including implicit hydrogens) with polarizabilities taken from [CRC 1994].
22	b_1rotN	Number of rotatable single bonds. Conjugated single bonds are not included (e.g. ester and peptide bonds).
23	b_1rotR	Fraction of rotatable single bonds: b_1rotN divided by b_heavy.
24	b_ar	Number of aromatic bonds.
25	b_count	Number of bonds (including implicit hydrogens). This is calculated as the sum of $(d_i/2 + h_i)$ over all non-trivial atoms i .
26	b_double	Number of double bonds. Aromatic bonds are not considered to be double bonds.
27	b_heavy	Number of bonds between heavy atoms.
28	b_max1len	Maximum single bond chain length.
29	b_rotN	Number of rotatable bonds. A bond is rotatable if it has order 1, is not in a ring, and has at least two heavy neighbors.
30	b_rotR	Fraction of rotatable bonds: b_rotN divided by b_heavy.
31	b_single	Number of single bonds (including implicit hydrogens). Aromatic bonds are not considered to be single bonds.
32	b_triple	Number of triple bonds. Aromatic bonds are not considered to be triple bonds.
33	chiral_u	The number of unconstrained chiral centers.
34	density	Molecular mass density: Weight divided by vdw_vol ($\text{amu}/\text{\AA}^3$).
35	diameter	Largest value in the distance matrix [Petitjean 1992]

36	lip_acc	The number of O and N atoms.
37	lip_don	The number of OH and NH atoms.
38	logP(o/w)	Log of the octanol/water partition coefficient (including implicit hydrogens). This property is calculated from a linear atom type model [LOGP 1998] with $r^2 = 0.931$, RMSE=0.393 on 1,827 molecules.
39	logS	Log of the aqueous solubility (mol/L). This property is calculated from an atom contribution linear atom type model [Hou 2004] with $r^2 = 0.90$, ~1,200 molecules.
40	mr	Molecular refractivity (including implicit hydrogens). This property is calculated from an 11 descriptor linear model [MREF 1998] with $r^2 = 0.997$, RMSE = 0.168 on 1,947 small molecules.
41	PC+	Total positive partial charge: the sum of the positive q_i . Q_PC+ is identical to PC+ which has been retained for compatibility.
42	PC-	Total negative partial charge: the sum of the negative q_i . Q_PC- is identical to PC- which has been retained for compatibility.
43 44	PEOE_PC+ Q_PC+	Total positive partial charge: the sum of the positive q_i .
45 46	PEOE_PC- Q_PC-	Total negative partial charge: the sum of the negative q_i .
47 48	PEOE_RPC+ Q_RPC+	Relative positive partial charge: the largest positive q_i divided by the sum of the positive q_i .
49 50	PEOE_RPC- Q_RPC-	Relative negative partial charge: the smallest negative q_i divided by the sum of the negative q_i .
51 52	PEOE_VSA_FHYD Q_VSA_FHYD	Fractional hydrophobic van der Waals surface area. This is the sum of the v_i such that $ q_i $ is less than or equal to 0.2 divided by the total surface area. The v_i are calculated using a connection table approximation.
53 54	PEOE_VSA_FNEG Q_VSA_FNEG	Fractional negative van der Waals surface area. This is the sum of the v_i such that q_i is negative divided by the total surface area. The v_i are calculated using a connection table approximation.
55 56	PEOE_VSA_FPNEG Q_VSA_FPNEG	Fractional negative polar van der Waals surface area. This is the sum of the v_i such that q_i is less than -0.2 divided by the total surface area. The v_i are calculated using a connection table approximation.
57 58	PEOE_VSA_FPOL Q_VSA_FPOL	Fractional polar van der Waals surface area. This is the sum of the v_i such that $ q_i $ is greater than 0.2 divided by the total surface area. The v_i are calculated using a connection table approximation.
59 60	PEOE_VSA_FPOS Q_VSA_FPOS	Fractional positive van der Waals surface area. This is the sum of the v_i such that q_i is non-negative divided by the total surface area. The v_i are calculated using a connection table approximation.
61 62	PEOE_VSA_FPPOS Q_VSA_FPPOS	Fractional positive polar van der Waals surface area. This is the sum of the v_i such that q_i is greater than 0.2 divided by the total surface area. The v_i are calculated using a connection table approximation.
63 64	PEOE_VSA_HYD Q_VSA_HYD	Total hydrophobic van der Waals surface area. This is the sum of the v_i such that $ q_i $ is less than or equal to 0.2. The v_i are calculated using a

		connection table approximation.
65 66	PEOE_VSA_NEG Q_VSA_NEG	Total negative van der Waals surface area. This is the sum of the v_i such that q_i is negative. The v_i are calculated using a connection table approximation.
67 68	PEOE_VSA_PNEG Q_VSA_PNEG	Total negative polar van der Waals surface area. This is the sum of the v_i such that q_i is less than -0.2. The v_i are calculated using a connection table approximation.
69 70	PEOE_VSA_POL Q_VSA_POL	Total polar van der Waals surface area. This is the sum of the v_i such that $ q_i $ is greater than 0.2. The v_i are calculated using a connection table approximation.
71 72	PEOE_VSA_POS Q_VSA_POS	Total positive van der Waals surface area. This is the sum of the v_i such that q_i is non-negative. The v_i are calculated using a connection table approximation.
73 74	PEOE_VSA_PPOS Q_VSA_PPOS	Total positive polar van der Waals surface area. This is the sum of the v_i such that q_i is greater than 0.2. The v_i are calculated using a connection table approximation.
75	radius	If r_i is the largest matrix entry in row i of the distance matrix D , then the radius is defined as the smallest of the r_i [Petitjean 1992].
76	reactive	Indicator of the presence of reactive groups. A non-zero value indicates that the molecule contains a reactive group. The table of reactive groups is based on the Oprea set [Oprea 2000] and includes metals, phospho-, N/O/S-N/O/S single bonds, thiols, acyl halides, Michael Acceptors, azides, esters, etc.
77	rings	The number of rings.
78	RPC+	Relative positive partial charge.
79	RPC-	Relative negative partial charge.
80	SlogP	Log of the octanol/water partition coefficient (including implicit hydrogens). This property is an atomic contribution model [Crippen 1999] that calculates logP from the given structure; i.e. the correct protonation state (washed structures). Results may vary from the logP(o/w) descriptor. The training set for SlogP was ~7000 structures.
81	SMR	Molecular refractivity (including implicit hydrogens). This property is an atomic contribution model [Crippen 1999] that assumes the correct protonation state (washed structures). The model was trained on ~7000 structures and results may vary from the mr descriptor.
82	TPSA	Polar surface area ($\text{\AA}^2$) calculated using group contributions to approximate the polar surface area from connection table information only. The parameterization is that of Ertl <i>et al.</i> [Ertl 2000].
83	vdw_area	Area of van der Waals surface ($\text{\AA}^2$) calculated using a connection table approximation.
84	vdw_vol	van der Waals volume ($\text{\AA}^3$) calculated using a connection table approximation.
85	vsa_acc	Approximation to the sum of VDW surface areas ($\text{\AA}^2$) of pure hydrogen bond acceptors (not counting acidic atoms and atoms that are both

		hydrogen bond donors and acceptors such as -OH).
86	vsa_acid	Approximation to the sum of VDW surface areas of acidic atoms ($\text{\AA}^2$).
87	vsa_don	Approximation to the sum of VDW surface areas of pure hydrogen bond donors (not counting basic atoms and atoms that are both hydrogen bond donors and acceptors such as -OH) ($\text{\AA}^2$).
88	vsa_hyd	Approximation to the sum of VDW surface areas of hydrophobic atoms ($\text{\AA}^2$).
89	vsa_other	Approximation to the sum of VDW surface areas ($\text{\AA}^2$) of atoms typed as "other".
90	vsa_pol	Approximation to the sum of VDW surface areas ($\text{\AA}^2$) of polar atoms (atoms that are both hydrogen bond donors and acceptors), such as -OH.
91	Weight	Molecular weight (including implicit hydrogens) in atomic mass units with atomic weights taken from [CRC 1994].
92	zagreb	Zagreb index: the sum of d_i^2 over all heavy atoms i .
93	B1_Sum_[0 1]Pred	Sum of the float scores of the 6 classification models for OATP1B1 inhibition
94	B3_Sum_[0 1]Pred	Sum of the float scores of the 6 classification models for OATP1B3 inhibition

5. Supplements to Chapter 6

Table A7: List of the 93 molecular 2D MOE descriptors and the 5 descriptors for BSEP, BCRP, P-gp, OATP1B1 and 1B3 inhibition prediction used for the cholestasis classification model for human data.

	MOE Descriptor	Description
1	apol	Sum of the atomic polarizabilities (including implicit hydrogens) with polarizabilities taken from [CRC 1994]
2	a_acc	Number of hydrogen bond acceptor atoms (not counting acidic atoms but counting atoms that are both hydrogen bond donors and acceptors such as -OH).
3	a_acid	Number of acidic atoms.
4	a_aro	Number of aromatic atoms.
5	a_count	Number of atoms (including implicit hydrogens). This is calculated as the sum of $(1 + h_i)$ over all non-trivial atoms i .
6	a_don	Number of hydrogen bond donor atoms (not counting basic atoms but counting atoms that are both hydrogen bond donors and acceptors such as -OH).
7	a_donacc	Number of hydrogen bond donor and hydrogen bond acceptor atoms.
8	a_heavy	Number of heavy atoms $\#\{Z_i \mid Z_i > 1\}$.
9	a_hyd	Number of hydrophobic atoms.
10	a_IC	Atom information content (total). This is calculated to be a_ICM

		times n .
11	a_ICM	Atom information content (mean). This is the entropy of the element distribution in the molecule (including implicit hydrogens but not lone pair pseudo-atoms). Let n_i be the number of occurrences of atomic number i in the molecule. Let $p_i = n_i / n$ where n is the sum of the n_i . The value of a_ICM is the negative of the sum over all i of $p_i \log p_i$.
12	a_nBr	Number of bromine atoms: $\#\{Z_i \mid Z_i = 35\}$.
13	a_nC	Number of carbon atoms: $\#\{Z_i \mid Z_i = 6\}$.
14	a_nCl	Number of chlorine atoms: $\#\{Z_i \mid Z_i = 17\}$.
15	a_nF	Number of fluorine atoms: $\#\{Z_i \mid Z_i = 9\}$.
16	a_nH	Number of hydrogen atoms (including implicit hydrogens). This is calculated as the sum of h_i over all non-trivial atoms i plus the number of non-trivial hydrogen atoms.
17	A_nI	Number of iodine atoms: $\#\{Z_i \mid Z_i = 53\}$.
18	a_nN	Number of nitrogen atoms: $\#\{Z_i \mid Z_i = 7\}$.
19	a_nO	Number of oxygen atoms: $\#\{Z_i \mid Z_i = 8\}$.
20	a_nP	Number of phosphorus atoms: $\#\{Z_i \mid Z_i = 15\}$.
21	a_nS	Number of sulfur atoms: $\#\{Z_i \mid Z_i = 16\}$.
22	bpol	Sum of the absolute value of the difference between atomic polarizabilities of all bonded atoms in the molecule (including implicit hydrogens) with polarizabilities taken from [CRC 1994].
23	b_1rotN	Number of rotatable single bonds. Conjugated single bonds are not included (e.g. ester and peptide bonds).
24	b_1rotR	Fraction of rotatable single bonds: b_1rotN divided by b_heavy.
25	b_ar	Number of aromatic bonds.
26	b_count	Number of bonds (including implicit hydrogens). This is calculated as the sum of $(d_i/2 + h_i)$ over all non-trivial atoms i .
27	b_double	Number of double bonds. Aromatic bonds are not considered to be double bonds.
28	b_heavy	Number of bonds between heavy atoms.
29	b_max1len	Maximum single bond chain length.
30	b_rotN	Number of rotatable bonds. A bond is rotatable if it has order 1, is not in a ring, and has at least two heavy neighbors.
31	b_rotR	Fraction of rotatable bonds: b_rotN divided by b_heavy.
32	b_single	Number of single bonds (including implicit hydrogens). Aromatic bonds are not considered to be single bonds.
33	b_triple	Number of triple bonds. Aromatic bonds are not considered to be triple bonds.
34	chiral_u	The number of unconstrained chiral centers.
35	density	Molecular mass density: Weight divided by vdw_vol (amu/Å ³).
36	diameter	Largest value in the distance matrix [Petitjean 1992]
37	lip_acc	The number of O and N atoms.
38	lip_don	The number of OH and NH atoms.

39	logP(o/w)	Log of the octanol/water partition coefficient (including implicit hydrogens). This property is calculated from a linear atom type model [LOGP 1998] with $r^2 = 0.931$, RMSE=0.393 on 1,827 molecules.
40	logS	Log of the aqueous solubility (mol/L). This property is calculated from an atom contribution linear atom type model [Hou 2004] with $r^2 = 0.90$, ~1,200 molecules.
41	mr	Molecular refractivity (including implicit hydrogens). This property is calculated from an 11 descriptor linear model [MREF 1998] with $r^2 = 0.997$, RMSE = 0.168 on 1,947 small molecules.
42	PC+	Total positive partial charge: the sum of the positive q_i . Q_PC+ is identical to PC+ which has been retained for compatibility.
43	PC-	Total negative partial charge: the sum of the negative q_i . Q_PC- is identical to PC- which has been retained for compatibility.
44 45	PEOE_PC+ Q_PC+	Total positive partial charge: the sum of the positive q_i .
46 47	PEOE_PC- Q_PC-	Total negative partial charge: the sum of the negative q_i .
48 49	PEOE_RPC+ Q_RPC+	Relative positive partial charge: the largest positive q_i divided by the sum of the positive q_i .
50 51	PEOE_RPC- Q_RPC-	Relative negative partial charge: the smallest negative q_i divided by the sum of the negative q_i .
52 53	PEOE_VSA_FHYD Q_VSA_FHYD	Fractional hydrophobic van der Waals surface area. This is the sum of the v_i such that $ q_i $ is less than or equal to 0.2 divided by the total surface area. The v_i are calculated using a connection table approximation.
54 55	PEOE_VSA_FNEG Q_VSA_FNEG	Fractional negative van der Waals surface area. This is the sum of the v_i such that q_i is negative divided by the total surface area. The v_i are calculated using a connection table approximation.
56 57	PEOE_VSA_FPNEG Q_VSA_FPNEG	Fractional negative polar van der Waals surface area. This is the sum of the v_i such that q_i is less than -0.2 divided by the total surface area. The v_i are calculated using a connection table approximation.
58 59	PEOE_VSA_FPOL Q_VSA_FPOL	Fractional polar van der Waals surface area. This is the sum of the v_i such that $ q_i $ is greater than 0.2 divided by the total surface area. The v_i are calculated using a connection table approximation.
60 61	PEOE_VSA_FPOS Q_VSA_FPOS	Fractional positive van der Waals surface area. This is the sum of the v_i such that q_i is non-negative divided by the total surface area. The v_i are calculated using a connection table approximation.
62 63	PEOE_VSA_FPPOS Q_VSA_FPPOS	Fractional positive polar van der Waals surface area. This is the sum of the v_i such that q_i is greater than 0.2 divided by the total surface area. The v_i are calculated using a connection table approximation.

64 65	PEOE_VSA_HYD Q_VSA_HYD	Total hydrophobic van der Waals surface area. This is the sum of the v_i such that $ q_i $ is less than or equal to 0.2. The v_i are calculated using a connection table approximation.
66 67	PEOE_VSA_NEG Q_VSA_NEG	Total negative van der Waals surface area. This is the sum of the v_i such that q_i is negative. The v_i are calculated using a connection table approximation.
68 69	PEOE_VSA_PNEG Q_VSA_PNEG	Total negative polar van der Waals surface area. This is the sum of the v_i such that q_i is less than -0.2. The v_i are calculated using a connection table approximation.
70 71	PEOE_VSA_POL Q_VSA_POL	Total polar van der Waals surface area. This is the sum of the v_i such that $ q_i $ is greater than 0.2. The v_i are calculated using a connection table approximation.
72 73	PEOE_VSA_POS Q_VSA_POS	Total positive van der Waals surface area. This is the sum of the v_i such that q_i is non-negative. The v_i are calculated using a connection table approximation.
74 75	PEOE_VSA_PPOS Q_VSA_PPOS	Total positive polar van der Waals surface area. This is the sum of the v_i such that q_i is greater than 0.2. The v_i are calculated using a connection table approximation.
76	radius	If r_i is the largest matrix entry in row i of the distance matrix D , then the radius is defined as the smallest of the r_i [Petitjean 1992].
77	reactive	Indicator of the presence of reactive groups. A non-zero value indicates that the molecule contains a reactive group. The table of reactive groups is based on the Oprea set [Oprea 2000] and includes metals, phospho-, N/O/S-N/O/S single bonds, thiols, acyl halides, Michael Acceptors, azides, esters, etc.
78	rings	The number of rings.
79	RPC+	Relative positive partial charge.
80	RPC-	Relative negative partial charge.
81	SlogP	Log of the octanol/water partition coefficient (including implicit hydrogens). This property is an atomic contribution model [Crippen 1999] that calculates logP from the given structure; i.e. the correct protonation state (washed structures). Results may vary from the logP(o/w) descriptor. The training set for SlogP was ~7000 structures.
82	SMR	Molecular refractivity (including implicit hydrogens). This property is an atomic contribution model [Crippen 1999] that assumes the correct protonation state (washed structures). The model was trained on ~7000 structures and results may vary from the mr descriptor.
83	TPSA	Polar surface area ($\text{\AA}^2$) calculated using group contributions to approximate the polar surface area from connection table information only. The parameterization is that of Ertl <i>et al.</i> [Ertl 2000].
84	vdw_area	Area of van der Waals surface ($\text{\AA}^2$) calculated using a connection table approximation.

85	vdw_vol	an der Waals volume ($\text{\AA}^3$) calculated using a connection table approximation.
86	vsa_acc	Approximation to the sum of VDW surface areas ($\text{\AA}^2$) of pure hydrogen bond acceptors (not counting acidic atoms and atoms that are both hydrogen bond donors and acceptors such as -OH).
87	vsa_acid	Approximation to the sum of VDW surface areas of acidic atoms ($\text{\AA}^2$).
88	vsa_don	Approximation to the sum of VDW surface areas of pure hydrogen bond donors (not counting basic atoms and atoms that are both hydrogen bond donors and acceptors such as -OH) ($\text{\AA}^2$).
89	vsa_hyd	Approximation to the sum of VDW surface areas of hydrophobic atoms ($\text{\AA}^2$).
90	vsa_other	Approximation to the sum of VDW surface areas ($\text{\AA}^2$) of atoms typed as "other".
91	vsa_pol	Approximation to the sum of VDW surface areas ($\text{\AA}^2$) of polar atoms (atoms that are both hydrogen bond donors and acceptors), such as -OH.
92	Weight	Molecular weight (including implicit hydrogens) in atomic mass units with atomic weights taken from [CRC 1994].
93	zagreb	Zagreb index: the sum of d_i^2 over all heavy atoms i .
94	ABCB1 Inhib	P-gP inhibition prediction (float number score)
95	ABCG2 Inhib	BCRP inhibition prediction (float number score)
96	BSEP Inhib	BSEP inhibition prediction (float number score)
97	OATPB1_Inhib_Sum_binary	Sum of the binary scores of the 4 classification models for OATPB1 inhibition (integer score between 0 and 4)
98	OATPB3_Inhib_Sum_binary	Sum of the binary scores of the 4 classification models for OATPB3 inhibition (integer score between 0 and 4)

Table A8. p-values from the respective two-sample paired t-tests comparing several model-pairs .

Comparisons	Accuracy	Sensitivity	Specificity	MCC	AUC	Precision	Weighted Precision	Conclusions
p-values:	$<2.2*10^{-16}$	$<2.2*10^{-16}$	$1.913*10^{-3}$	$<2.2*10^{-16}$	$<2.2*10^{-16}$	$<2.2*10^{-16}$	$<2.2*10^{-16}$	For all statistics metrics, using 93 2D dscrs + transporters performs better
i)comparison 93 2D dscrs + transp vs 93 2D dscrs								
p-values:	$1.025*10^{-9}$	0.02194	$1.09*10^{-11}$	0.01397	0.4546	$5.143*10^{-6}$	0.07158	In terms of <u>AUC</u> and <u>weighted precision</u> , the two models perform equally. For the <u>rest of the statistics metrics</u> , including BSEP to the 93 2D dscrs yields better performance.
ii)comparison 93 2D dscrs + BSEP vs 93 2D dscrs								
p-values:	$3.574*10^{-12}$	$<2.2*10^{-16}$	$4*10^{-5}$	$<2.2*10^{-16}$	$<2.2*10^{-16}$	$<2.2*10^{-16}$	$<2.2*10^{-16}$	For <u>all statistics metrics</u> , <u>apart from specificity</u> , using 93 2D dscrs + transporters performs better. For <u>specificity</u> using 93 2D dscrs + BSEP (only) performs better.
iii)comparison 93 2D dscrs + transp vs 93 2D dscrs + BSEP								
p-values:	$8.72*10^{-7}$	0.01042	$1.566*10^{-11}$	0.2483	$1.614*10^{-6}$	$6.253*10^{-3}$	0.7796	For <u>accuracy</u> , <u>specificity</u> , <u>AUC</u> and <u>precision</u> the performance of the model is better when all transporters are used. For <u>MCC</u> and <u>weighted precision</u> the two models perform equally. In terms of <u>sensitivity</u> the performance is better when BSEP is not included.
iv)comparison 93 2D dscrs + transp vs 93 2D dscrs + transporters without BSEP								

6. Supplements to Chapter 7

Supplement for 7.1

Table A9. a) Histopathological terms organized into 7 clusters. The same main and secondary clusters are reported with the same color code. The number of positives for each cluster is also reported.

Standardized term: histopathology	No. of positives	Cluster (main)	Cluster (secondary)
hypertrophy	111	hypertrophy	
intracellular vacuolation	101	steatosis	bile duct abnormalities
hypertrophy, hepatocyte	100	hypertrophy	
intracellular increase of lipids	94	steatosis	
necrosis	88	necrosis	
vacuolation, lipidic (fatty change)	70	steatosis	
necrosis, hepatocellular	51	necrosis	
decreased, glycogen	47	glycogen decrease	
hyperplasia	47	preneoplastic effect	
inflammatory cell infiltration	42	inflammation-2 nd effect	
inflammation	40	inflammation-2 nd effect	
bile duct hyperplasia	35	bile duct abnormalities	
single cell necrosis	33	necrosis	
vacuolation, lipidic	32	steatosis	
increased mitoses	31	preneoplastic	
necrosis, focal/multifocal	19	necrosis	
lymphohistioplasmacytic inflammatory cell infiltration	17	inflammation-2 nd effect	
increased, glycogen	16		
increased hematopoiesis	11		
macrophage aggregates	10	inflammation-2 nd effect	
aggregation, alveolar macrophages: terminal bronchioles/alveoli	9		

foamy macrophages	9		
intracellular increase of fluids	9		
vacuolation, biliary epithelium	9	bile duct abnormalities	
mixed cell infiltration	8	inflammation-2 nd effect	
mixed-cell inflammation	8	inflammation-2 nd effect	
necrosis, zonal	8	necrosis	
congestion	7		
hyperplasia, reticulo-endothelial cell	6	preneoplastic	
hypertrophy, epithelial	6	bile duct abnormalities	
hypertrophy/hyperplasia, kupffer cells	6	inflammation-2 nd effect	
lymphohistioplasmacytic inflammation	6	inflammation-2 nd effect	

Table A10: Statistics of global hepatotoxicity and consensus modeling approach predictions on the external test by Mulliner et al.

Model	Accuracy	Sensitivity	Specificity	MCC	AUC	Precision
Global hepatotoxicity-Single model	0.567	0.321	0.745	0.073	0.565	0.478
Consensus modeling approach-threshold: positives$\geq$1	0.541	0.607	0.492	0.099	0.550	0.465
Consensus modeling approach-threshold: positives$\geq$1, except for hypertrophy: positive$>$1	0.560	0.475	0.621	0.096	0.548	0.477
Consensus modeling approach-threshold: Positives$>$1	0.582	0.317	0.776	0.103	0.546	0.506

Table A11. Contradictory compounds between training and test set. The class for each compound is reported for both datasets.

N	Contradictory compounds	Training set class	External class label
1	amodiaquine;amodiaquine hydrochloride	positive	negative
2	cyclophosphamide	negative	positive
3	aripiprazole	negative	positive
4	glyburide	negative	positive
5	rimonabant	negative	positive
6	furosemide	negative	positive
7	clomipramine hydrochloride;clomipramine	negative	positive
8	carprofen	negative	positive
9	indomethacin;indomethacin sodium	negative	positive
10	diclofenac potassium;diclofenac sodium;diclofenac	negative	positive
11	leflunomide	negative	positive
12	metformin hydrochloride;metformin	negative	positive
13	maprotiline hydrochloride;maprotiline	negative	positive
14	imipramine hydrochloride;imipramine	negative	positive
15	aspirin;acetylsalicylic acid	negative	positive
16	agomelatine	negative	positive
17	erlotinib hydrochloride;erlotinib	negative	positive
18	alfuzosin hydrochloride;alfuzosin	negative	positive
19	pentoxifylline	negative	positive
20	ciclopirox	negative	positive
21	milrinone lactate;milrinone	negative	positive
22	divalproex sodium;valproate sodium;valproic acid	negative	positive
23	metronidazole;metronidazole hydrochloride	negative	positive
24	zafirlukast	negative	positive
25	troglitazone	negative	positive
26	rosiglitazone maleate;rosiglitazone	negative	positive
27	anastrozole;anastrozone	negative	positive
28	olanzapine	negative	positive

Script for generating the heatmaps and clustering in R

#####

Script for heatmaps and hierarchical clustering

#Process the full file

Vitic_MDS = Vitic_764cmps_7endpoints_MDS_for_R

Vitic_MDS\$Index = NULL

Make the database_substance_id the row names

rownames(Vitic_MDS) = Vitic_MDS\$database_substance_id

Vitic_MDS\$database_substance_id = NULL # I need then to remove the field

head(Vitic_MDS)

#Create heatmaps

library(heatmaply)

library(gplots)

Vitic_matrix = as.matrix(Vitic_MDS) #convert Vitic_MDS to marix

Vitic_heatmap= heatmap(Vitic_matrix, Colv= NA, scale="column")

#Finally saved the plot obtained by heatmap.2()

Vitic_heatmap2= heatmap.2(Vitic_matrix, col= c("green", "red"), srtCol= 20, margins = c(5.5,6),
tracecol=NA)

#Heatmap for the whole dataset without dendrogram

Vitic_heatmap2= heatmap.2(Vitic_matrix, col= c("green", "red"), srtCol= 20, margins = c(5,1),
tracecol=NA, dendrogram = "none")

#####

#Process only the positives' file

Vitic_positives= Vitic_764cmps_7endpoints_MDS_for_R_only_positives

Vitic_positives\$Index = NULL

Make the database_substance_id the row names

rownames(Vitic_positives) = Vitic_positives\$database_substance_id

Vitic_positives\$database_substance_id = NULL # I need then to remove the field

#Create heatmaps

Vitic_pos_matrix = as.matrix(Vitic_positives)

Vitic_pos_heatmap= heatmap(Vitic_pos_matrix, Colv= NA, scale="column")

Vitic_pos_heatmap2= heatmap.2(Vitic_pos_matrix, col= c("green", "red"), srtCol= 20, margins = c(5.5,6),
tracecol=NA)

#Heatmap for positives without dendrogram

Vitic_pos_heatmap2= heatmap.2(Vitic_pos_matrix, col= c("green", "red"), srtCol= 20, margins = c(5,1),
tracecol=NA, dendrogram = "none")

library(heatmaply)

heatmaply(Vitic_pos_matrix)

#####

Perform Clustering

```

#This part of code was repeated previously for the heatmaps
Vitic_MDS = Vitic_764cmps_7endpoints_MDS_for_R
Vitic_MDS$Index = NULL
# Make the database_substance_id the row names
rownames(Vitic_MDS) = Vitic_MDS$database_substance_id
Vitic_MDS$database_substance_id = NULL # I need then to remove the field
head(Vitic_MDS)

# Create dissimilarity object with daisy() function
library(cluster)
##First I need to reverse the dataframe Vitic MDS
Vitic_MDS_transp = t(Vitic_MDS)
# Use daisy() function
Vitic_diss_matrix = daisy(Vitic_MDS_transp, metric = "euclidean")

# Perform hierarchical clustering using the hclust() function and method "complete"
Vitic_clusters = hclust(Vitic_diss_matrix, method="complete")
plot(Vitic_clusters)

# Perform hierarchical clustering using the hclust() function and method "ward.D"
Vitic_clusters_2 = hclust(Vitic_diss_matrix, method="ward.D")
plot(Vitic_clusters_2)

# Perform hierarchical clustering using the hclust() function and method "ward.D2"
Vitic_clusters_3 = hclust(Vitic_diss_matrix, method="ward.D2")
plot(Vitic_clusters_3)

# Perform hierarchical clustering using the hclust() function and method "single"
Vitic_clusters_4 = hclust(Vitic_diss_matrix, method="single")
plot(Vitic_clusters_4)

# Perform hierarchical clustering using the hclust() function and method "centroid"
Vitic_clusters_5 = hclust(Vitic_diss_matrix, method="centroid")
plot(Vitic_clusters_5)

# Perform hierarchical clustering using the hclust() function and method "average"
Vitic_clusters_6 = hclust(Vitic_diss_matrix, method="average")
plot(Vitic_clusters_6)

```

Supplement for 7.2

Table A12. Tuned settings of the best performing models for each meta-classifier/method

a. OATP1B1 dataset

Method	2D MOE descriptors	ECFP6 fingerprints	MACCS fingerprints
Stratified Bagging	-	-	-
CostSensitiveClassifier	cost 30:1 matrix: [0.0, 1.0; 450.0, 0.0]	cost 100:1 matrix: [0.0, 1.0; 100.0, 0.0]	cost 100:1 matrix: [0.0, 1.0; 100.0, 0.0]
MetaCost	cost 10:1 matrix: [0.0, 1.0; 10.0, 0.0]	cost 30:1 matrix: [0.0, 1.0; 30.0, 0.0]	cost 25:1 matrix: [0.0, 1.0; 25.0, 0.0]
SMOTE	1500% synthetic instances	2000% synthetic instances	1500% synthetic instances

b. OATP1B3 dataset

Method	2D MOE descriptors	ECFP6 fingerprints	MACCS fingerprints
Stratified Bagging	-	-	-
CostSensitiveClassifier	cost 70:1 matrix: [0.0, 1.0; 70.0, 0.0]	cost 280:1 matrix: [0.0, 1.0; 280.0, 0.0]	cost 200:1 matrix: [0.0, 1.0; 200.0, 0.0]
MetaCost	cost 13:1 matrix: [0.0, 1.0; 13.0, 0.0]	cost 50:1 matrix: [0.0, 1.0; 50.0, 0.0]	cost 40:1 matrix: [0.0, 1.0; 40.0, 0.0]
SMOTE	1500% synthetic instances	2000% synthetic instances	1300% synthetic instances

c. Cholestasis human dataset

Method	2D MOE descriptors	ECFP6 fingerprints	MACCS fingerprints
Stratified Bagging	cost 2:1	cost 2:1	cost 2:1
CostSensitiveClassifier	cost 14:1 matrix: [0.0, 1.0; 14.0, 0.0]	cost 12:1 matrix: [0.0, 1.0; 12.0, 0.0]	cost 12:1 matrix: [0.0, 1.0; 12.0, 0.0]
MetaCost	cost 8:1 matrix: [0.0, 1.0; 8.0, 0.0]	cost 8:1 matrix: [0.0, 1.0; 8.0, 0.0]	cost 8:1 matrix: [0.0, 1.0; 8.0, 0.0]
SMOTE	1300% synthetic instances	3000% synthetic instances	1300% synthetic instances

d. Cholestasis animal dataset

Method	2D MOE descriptors	ECFP6 fingerprints	MACCS fingerprints
Stratified Bagging	cost 2:1	cost 2:1	cost 2:1
CostSensitiveClassifier	cost 450:1 matrix: [0.0, 1.0; 450.0, 0.0]	cost 500:1 matrix: [0.0, 1.0; 500.0, 0.0]	cost 500:1 matrix: [0.0, 1.0; 500.0, 0.0]
MetaCost	cost 45:1 matrix: [0.0, 1.0; 45.0, 0.0]	cost 45:1 matrix: [0.0, 1.0; 45.0, 0.0]	cost 50:1 matrix: [0.0, 1.0; 50.0, 0.0]
SMOTE	3000% synthetic instances	3000% synthetic instances	3000% synthetic instances

Table A13. Results on OATP1B1 inhibition dataset for all calculated statistics metrics : Accuracy, Balanced Accuracy, Sensitivity, Specificity, MCC, AUC, Precision, Weighted Precision. The performance is given for both 10-fold cross-validation and on the external test set. With bold font are depicted those models that gave a satisfactory result of >0.5 sensitivity and they were further investigated by performing 20 iterations.

Model Settings	Descriptors	Validation	Accuracy					Precision			
			Accuracy	Balanced	Sensitivity	Specificity	MCC	AUC	Precision	Weighted	Precision
RF100	MOE	10 CV	0.893	0.614	0.253	0.974	0.322	0.809	0.545	0.872	0.872
		Test set	0.711	0.568	0.172	0.964	0.233	0.837	0.688	0.705	0.705
	ECFP6	10 CV	0.892	0.573	0.163	0.983	0.256	0.798	0.544	0.864	0.864
		Test set	0.701	0.548	0.125	0.971	0.188	0.804	0.667	0.692	0.692
Bagging	MACCS	10 CV	0.899	0.623	0.268	0.978	0.356	0.778	0.6	0.879	0.879
		Test set	0.711	0.560	0.141	0.978	0.233	0.768	0.75	0.722	0.722
	MOE	Training set	0.897	0.616	0.254	0.978	0.340	0.74	0.585	-	-
		Test set	0.701	0.535	0.078	0.993	0.194	0.724	0.833	-	-
	ECFP6	Training set	0.892	0.585	0.190	0.979	0.272	0.694	0.529	-	-
		Test set	0.701	0.544	0.109	0.978	0.211	0.572	0.700	-	-
	MACCS	Training set	0.904	0.636	0.291	0.981	0.394	0.701	0.655	-	-
		Test set	0.706	0.552	0.125	0.978	0.187	0.572	0.727	-	-
Stratified Bagging	MOE	Training set	0.809	0.768	0.714	0.821	0.395	0.819	0.333	-	-
		Test set	0.831	0.830	0.828	0.832	0.634	0.887	0.697	-	-
	ECFP6	Training set	0.807	0.736	0.646	0.827	0.354	0.790	0.317	-	-
		Test set	0.736	0.653	0.422	0.883	0.347	0.774	0.628	-	-
CostSensitive Classifier	MACCS	Training set	0.783	0.757	0.725	0.790	0.365	0.798	0.300	-	-
		Test set	0.741	0.689	0.547	0.832	0.390	0.809	0.603	-	-
	MOE	10 CV	0.843	0.719	0.621	0.817	0.399	0.822	0.376	0.885	0.885
		Test set	0.841	0.804	0.703	0.905	0.625	0.856	0.776	0.838	0.838
	ECFP6	10 CV	0.653	0.711	0.784	0.637	0.269	0.791	0.213	0.876	0.876
		Test set	0.721	0.670	0.625	0.766	0.38	0.789	0.556	0.732	0.732
	MACCS	10 CV	0.645	0.701	0.774	0.628	0.257	0.79	0.207	0.873	0.873
		Test set	0.751	0.739	0.703	0.774	0.458	0.779	0.592	0.767	0.767

MetaCost	MOE	10 CV	0.819	0.746	0.653	0.839	0.376	0.826	0.337	0.882
		Test set	0.841	0.825	0.781	0.869	0.64	0.87	0.735	0.844
	ECFP6	10 CV	0.622	0.693	0.784	0.602	0.245	0.769	0.198	0.873
		Test set	0.657	0.677	0.734	0.62	0.331	0.758	0.475	0.719
	MACCS	10 CV	0.673	0.703	0.742	0.664	0.263	0.767	0.217	0.872
		Test set	0.756	0.772	0.813	0.73	0.509	0.769	0.584	0.795
Threshold Selector	MOE	10 CV	0.881	0.721	0.516	0.926	0.423	0.806	0.467	0.886
		Test set	0.816	0.740	0.531	0.949	0.555	0.837	0.829	0.818
	ECFP6	10 CV	0.868	0.712	0.511	0.912	0.390	0.797	0.422	0.880
		Test set	0.761	0.679	0.453	0.905	0.41	0.804	0.69	0.751
	MACCS	10 CV	0.880	0.656	0.368	0.944	0.342	0.775	0.452	0.870
		Test set	0.711	0.584	0.234	0.934	0.242	0.768	0.625	0.692
SMOTE	MOE	10 CV	0.869	0.710	0.505	0.914	0.389	0.807	0.425	0.880
		Test set	0.816	0.749	0.563	0.934	0.555	0.823	0.800	0.814
	ECFP6	10 CV	0.896	0.620	0.263	0.976	0.341	0.791	0.575	0.876
		Test set	0.716	0.572	0.172	0.971	0.253	0.767	0.733	0.721
	MACCS	10 CV	0.898	0.657	0.347	0.966	0.391	0.777	0.564	0.882
		Test set	0.711	0.560	0.141	0.978	0.233	0.787	0.75	0.722

Table A14. Results on OATP1B3 inhibition dataset for all calculated statistics metrics : Accuracy, Balanced Accuracy, Sensitivity, Specificity, MCC, AUC, Precision, Weighted Precision. The performance is given for both 10-fold cross-validation and on the external test set. With bold font are depicted those models that gave a satisfactory result of >0.5 sensitivity and they were further investigated by performing 20 iterations.

Model Settings	Descriptors	Validation	Accuracy				Specificity	MCC	AUC	Precision	Weighted Precision
			Accuracy	Balanced	Sensitivity						
RF100	MOE	10 CV	0.926	0.593	0.202	0.983	0.276	0.868	0.472	0.907	0.907
		Test set	0.818	0.573	0.175	0.97	0.246	0.912	0.583	0.785	0.785
	ECFP6	10 CV	0.926	0.540	0.089	0.991	0.168	0.841	0.423	0.897	0.897
		Test set	0.804	0.526	0.075	0.976	0.112	0.795	0.429	0.743	0.743
Bagging	MOE	10 CV	0.926	0.596	0.210	0.981	0.278	0.813	0.464	0.907	0.907
		Test set	0.804	0.526	0.075	0.976	0.112	0.821	0.429	0.743	0.743
	ECFP6	Training set	0.879	0.531	0.075	0.986	0.137	0.708	0.421	-	-
		Test set	0.818	0.554	0.125	0.982	0.220	0.645	0.625	-	-
Stratified Bagging	MOE	Training set	0.930	0.568	0.145	0.991	0.261	0.632	0.563	-	-
		Test set	0.797	0.520	0.075	0.965	0.078	0.609	0.333	-	-
	ECFP6	Training set	0.929	0.571	0.153	0.989	0.253	0.646	0.514	-	-
		Test set	0.813	0.551	0.125	0.976	0.196	0.547	0.556	-	-
CostSensitive Classifier	MOE	Training set	0.842	0.800	0.750	0.849	0.392	0.814	0.278	-	-
		Test set	0.813	0.856	0.925	0.787	0.588	0.915	0.507	-	-
	ECFP6	Training set	0.882	0.747	0.589	0.905	0.379	0.789	0.324	-	-
		Test set	0.818	0.611	0.275	0.947	0.297	0.772	0.550	-	-
CostSensitive Classifier	MOE	Training set	0.798	0.724	0.637	0.811	0.278	0.800	0.207	-	-
		Test set	0.789	0.679	0.500	0.858	0.345	0.817	0.455	-	-
	ECFP6	10 CV	0.874	0.802	0.718	0.886	0.428	0.873	0.327	0.929	0.929
		Test set	0.852	0.842	0.825	0.858	0.603	0.9	0.579	0.882	0.882
CostSensitive Classifier	ECFP6	10 CV	0.647	0.725	0.815	0.634	0.237	0.814	0.147	0.918	0.918
		Test set	0.727	0.698	0.650	0.746	0.331	0.766	0.377	0.800	0.800
	MACCS	10 CV	0.733	0.737	0.742	0.732	0.267	0.819	0.177	0.916	0.916
		Test set	0.761	0.728	0.675	0.781	0.389	0.818	0.422	0.817	0.817

MetaCost	MOE	10 CV	0.863	0.796	0.718	0.874	0.409	0.872	0.307	0.928
		Test set	0.837	0.842	0.850	0.834	0.589	0.894	0.548	0.881
	ECFP6	10 CV	0.683	0.736	0.798	0.674	0.254	0.796	0.159	0.919
		Test set	0.670	0.634	0.575	0.692	0.219	0.742	0.307	0.765
	MACCS	10 CV	0.717	0.751	0.790	0.711	0.277	0.816	0.175	0.920
		Test set	0.718	0.721	0.725	0.716	0.36	0.767	0.377	0.813
Threshold Selector	MOE	10 CV	0.908	0.754	0.573	0.934	0.433	0.868	0.403	0.908
		Test set	0.847	0.791	0.700	0.882	0.544	0.912	0.583	0.860
	ECFP6	10 CV	0.912	0.722	0.500	0.944	0.406	0.838	0.411	0.921
		Test set	0.804	0.583	0.225	0.941	0.227	0.795	0.474	0.767
	MACCS	10 CV	0.915	0.676	0.395	0.956	0.356	0.814	0.408	0.914
		Test set	0.813	0.627	0.325	0.929	0.308	0.821	0.52	0.789
SMOTE	MOE	10 CV	0.886	0.686	0.452	0.92	0.311	0.742	0.304	0.909
		Test set	0.837	0.728	0.55	0.905	0.464	0.886	0.579	0.834
	ECFP6	10 CV	0.926	0.585	0.185	0.984	0.263	0.829	0.469	0.906
		Test set	0.804	0.526	0.075	0.976	0.112	0.823	0.429	0.743
	MACCS	10 CV	0.922	0.638	0.306	0.97	0.328	0.831	0.442	0.911
		Test set	0.809	0.548	0.125	0.97	0.176	0.852	0.5	0.762

Table A15. Results on Cholestasis human dataset for all calculated statistics metrics : Accuracy, Balanced Accuracy, Sensitivity, Specificity, MCC, AUC, Precision, Weighted Precision. The performance is given for both 10-fold cross-validation and on the external test set. With bold font are depicted those models that gave a satisfactory result of >0.5 sensitivity and they were further investigated by performing 20 iterations.

Model Settings	Descriptors	Validation	Accuracy	Accuracy				MCC	AUC	Precision	Weighted Precision
				Accuracy	Balanced	Sensitivity	Specificity				
RF100	MOE	10 CV	0.839	0.622	0.622	0.265	0.979	0.382	0.772	0.754	0.827
		Test set	0.835	0.728	0.728	0.528	0.927	0.501	0.81	0.683	0.826
	ECFP6	10 CV	0.833	0.606	0.606	0.231	0.98	0.35	0.773	0.741	0.820
		Test set	0.823	0.719	0.719	0.528	0.91	0.469	0.835	0.635	0.814
Bagging	MACCS	10 CV	0.831	0.635	0.635	0.311	0.958	0.364	0.774	0.643	0.810
		Test set	0.861	0.778	0.778	0.623	0.933	0.589	0.844	0.733	0.856
	MOE	Training set	0.837	0.617	0.617	0.254	0.980	0.375	0.691	0.759	-
		Test set	0.835	0.723	0.723	0.519	0.927	0.492	0.73	0.675	-
	ECFP6	Training set	0.835	0.613	0.613	0.248	0.979	0.364	0.701	0.741	-
		Test set	0.826	0.717	0.717	0.519	0.916	0.734	0.471	0.643	-
	MACCS	Training set	0.838	0.634	0.634	0.297	0.970	0.387	0.685	0.710	-
		Test set	0.857	0.764	0.764	0.596	0.933	0.567	0.763	0.721	-
Stratified Bagging +cost2:1	MOE	Training set	0.781	0.719	0.719	0.617	0.821	0.394	0.768	0.457	-
		Test set	0.761	0.716	0.716	0.635	0.798	0.395	0.747	0.478	-
	ECFP6	Training set	0.804	0.717	0.717	0.573	0.860	0.413	0.773	0.501	-
		Test set	0.791	0.736	0.736	0.635	0.837	0.445	0.761	0.532	-
CostSensitive Classifier	MACCS	Training set	0.785	0.728	0.728	0.634	0.822	0.410	0.775	0.466	-
		Test set	0.774	0.752	0.752	0.712	0.792	0.451	0.807	0.500	-
	MOE	10 CV	0.724	0.701	0.701	0.663	0.739	0.337	0.78	0.383	0.798
		Test set	0.797	0.769	0.769	0.717	0.82	0.492	0.795	0.543	0.823
	ECFP6	10 CV	0.773	0.714	0.714	0.614	0.813	0.381	0.789	0.445	0.807
		Test set	0.810	0.751	0.751	0.642	0.860	0.483	0.825	0.576	0.818
	MACCS	10 CV	0.751	0.710	0.710	0.643	0.777	0.362	0.78	0.414	0.804
		Test set	0.751	0.710	0.710	0.643	0.777	0.362	0.78	0.414	0.804

MetaCost	MOE	Test set 10 CV	0.775	0.741	0.679	0.803	0.44	0.823	0.507	0.805
		Test set	0.697	0.678	0.642	0.713	0.310	0.724	0.400	0.762
	ECP6	10 CV	0.750	0.697	0.608	0.785	0.343	0.762	0.409	0.796
		Test set	0.684	0.682	0.679	0.685	0.313	0.746	0.391	0.766
	MACCS	10 CV	0.694	0.696	0.700	0.692	0.32	0.771	0.357	0.797
		Test set	0.701	0.707	0.717	0.697	0.355	0.773	0.413	0.782
Threshold Selector	MOE	10 CV	0.798	0.670	0.536	0.863	0.385	0.771	0.488	0.806
		Test set	0.831	0.771	0.660	0.882	0.532	0.81	0.625	0.835
	ECP6	10 CV	0.816	0.683	0.464	0.902	0.387	0.77	0.537	0.807
		Test set	0.827	0.762	0.642	0.882	0.517	0.835	0.618	0.829
	MACCS	10 CV	0.775	0.702	0.582	0.822	0.368	0.774	0.445	0.802
		Test set	0.805	0.761	0.679	0.843	0.490	0.844	0.563	0.821
SMOTE	MOE	10 CV	0.780	0.697	0.559	0.834	0.364	0.785	0.451	0.800
		Test set	0.810	0.744	0.623	0.865	0.476	0.825	0.579	0.815
	ECP6	10 CV	0.835	0.748	0.308	0.965	0.381	0.777	0.682	0.818
		Test set	0.836	0.637	0.585	0.910	0.517	0.849	0.660	0.830
	MACCS	10 CV	0.818	0.651	0.375	0.927	0.353	0.774	0.556	0.799
		Test set	0.848	0.776	0.642	0.91	0.563	0.849	0.68	0.846
ClassBalancer	MOE	10 CV	0.824	0.697	0.438	0.919	0.396	0.776	0.569	0.811
		Test set	0.844	0.773	0.642	0.904	0.554	0.788	0.667	0.842
	ECP6	10 CV	0.840	0.687	0.435	0.939	0.437	0.78	0.637	0.826
		Test set	0.827	0.749	0.604	0.893	0.504	0.833	0.627	0.825
	MACCS	10 CV	0.809	0.678	0.464	0.893	0.371	0.776	0.514	0.802
		Test set	0.835	0.774	0.660	0.888	0.541	0.835	0.636	0.838

Table A16. Results on Cholestasis animal dataset for all calculated statistics metrics : Accuracy, Balanced Accuracy, Sensitivity, Specificity, MCC, AUC, Precision, Weighted Precision. The performance is given for both 10-fold cross-validation and on the external test set. With bold font are depicted those models that gave a satisfactory result of >0.5 sensitivity and they were further investigated by performing 20 iterations.

Model Settings	Descriptor	Validation	Accuracy	Balanced	Sensitivity	Specificity	MCC	AUC	Precision	Weighted Precision
s										
RF100	MOE	10 CV	0.953	0.500	0.000	1.000	0.000	0.703	0.000	0.908
	ECFP6	10 CV	0.953	0.500	0.000	1.000	0.000	0.629	0.000	0.908
	MACCS	10 CV	0.951	0.511	0.027	0.997	0.083	0.700	0.333	0.925
Bagging	MOE	Training set	0.952	0.500	0.000	0.999	-0.006	0.503	0.000	-
	ECFP6	Training set	0.953	0.500	0.000	1.000	0.000	0.498	0.000	-
	MACCS	Training set	0.952	0.512	0.027	0.998	0.093	0.521	0.400	-
Stratified Bagging +cost2:1	MOE	Training set	0.636	0.594	0.547	0.641	0.083	0.715	0.070	-
	ECFP6	Training set	0.722	0.639	0.547	0.731	0.131	0.686	0.092	-
CostSensitive Classifier	MACCS	Training set	0.623	0.637	0.653	0.621	0.119	0.732	0.079	-
	MOE	10 CV	0.632	0.623	0.613	0.633	0.108	0.665	0.077	0.928
MetaCost	ECFP6	10 CV	0.532	0.527	0.520	0.533	0.023	0.531	0.052	0.914
	MACCS	10 CV	0.579	0.633	0.693	0.573	0.114	0.690	0.075	0.932
	MOE	10 CV	0.582	0.597	0.613	0.580	0.083	0.644	0.068	0.925
Threshold Selector	ECFP6	10 CV	0.599	0.587	0.573	0.600	0.075	0.600	0.066	0.923
	MACCS	10 CV	0.588	0.645	0.707	0.582	0.124	0.674	0.077	0.933
	MOE	10 CV	0.875	0.580	0.253	0.906	0.112	0.686	0.118	0.921
SMOTE	ECFP6	10 CV	0.874	0.567	0.227	0.906	0.094	0.624	0.107	0.919
	MACCS	10 CV	0.848	0.635	0.4	0.87	0.163	0.687	0.132	0.927
	MOE	10 CV	0.943	0.533	0.080	0.985	0.105	0.728	0.214	0.921
	ECFP6	10 CV	0.953	0.500	0.000	1.000	0.000	0.638	0.000	0.908
	MACCS	10 CV	0.949	0.511	0.027	0.995	0.057	0.708	0.2	0.918

Table A17. Results on OATP1B1 inhibition dataset only for the best performing methods on the appropriate set of descriptors (Sensitivity ≥ 0.5) for all calculated statistics metrics : Accuracy, Balanced Accuracy, Sensitivity, Specificity, MCC, AUC, Precision, Weighted Precision. The mean performance out of 20 iterations and the standard deviation values are provided.

Model Settings	Descriptors	Statistical	Value	Accuracy	Balanced	Sensitivity	Specificity	MCC	AUC	Precision	Weighted
Accuracy											
-Stratified Bagging	MOE	mean	0.769	0.817	0.823	0.810	0.334	0.715	0.334	-	-
		sd	0.002	0.005	0.010	0.002	0.007	0.005	0.004	-	-
	ECFP6	mean	0.805	0.734	0.642	0.826	0.351	0.795	0.315	-	-
		sd	0.002	0.005	0.009	0.003	0.007	0.006	0.004	-	-
	MACCS	mean	0.721	0.724	0.728	0.721	0.299	0.803	0.245	-	-
		sd	0.003	0.004	0.007	0.003	0.006	0.004	0.003	-	-
CostSensitive Classifier	MOE	mean	0.847	0.754	0.634	0.873	0.413	0.804	0.385	0.887	0.887
		sd	0.003	0.011	0.020	0.003	0.016	0.067	0.008	0.003	0.003
		mean	0.641	0.701	0.778	0.624	0.256	0.785	0.206	0.873	0.873
	ECFP6	sd	0.009	0.014	0.018	0.010	0.013	0.007	0.006	0.004	0.004
		mean	0.646	0.707	0.784	0.629	0.264	0.798	0.212	0.872	0.872
	MACCS	sd	0.005	0.011	0.017	0.005	0.011	0.006	0.013	0.017	0.017
		mean	0.817	0.747	0.656	0.837	0.376	0.822	0.335	0.883	0.883
MetaCost	MOE	sd	0.006	0.009	0.011	0.006	0.011	0.005	0.009	0.002	0.002
		mean	0.625	0.694	0.782	0.605	0.245	0.770	0.201	0.867	0.867
	ECFP6	sd	0.008	0.013	0.017	0.008	0.012	0.005	0.012	0.023	0.023
		mean	0.666	0.705	0.755	0.655	0.264	0.772	0.215	0.873	0.873
	MACCS	sd	0.007	0.011	0.014	0.008	0.011	0.005	0.005	0.003	0.003
		mean	0.879	0.721	0.519	0.924	0.420	0.813	0.460	0.886	0.886
Threshold Selector	MOE	sd	0.005	0.017	0.027	0.006	0.018	0.007	0.018	0.004	0.004
		mean	0.875	0.703	0.483	0.924	0.391	0.794	0.442	0.880	0.880
	ECFP6	sd	0.005	0.014	0.021	0.007	0.015	0.007	0.017	0.003	0.003
		mean	0.870	0.715	0.517	0.914	0.398	0.811	0.430	0.881	0.881
	MACCS	sd	0.005	0.014	0.021	0.007	0.015	0.007	0.017	0.003	0.003
		mean	0.870	0.715	0.517	0.914	0.398	0.811	0.430	0.881	0.881

sd	0.005	0.011	0.017	0.005	0.019	0.003	0.018	0.004

Table A18. Results on OATP1B3 inhibition dataset only for the best performing methods on the appropriate set of descriptors (Sensitivity ≥ 0.5) for all calculated statistics metrics : Accuracy, Balanced Accuracy, Sensitivity, Specificity, MCC, AUC, Precision, Weighted Precision. The mean performance out of 20 iterations and the standard deviation values are provided.

Model Settings	Descriptors	Statistical Value	Accuracy	Balanced Accuracy	Sensitivity	Specificity	MCC	AUC	Precision	Weighted Precision
Stratified Bagging	MOE	mean	0.841	0.804	0.761	0.847	0.395	0.819	0.278	-
		sd	0.002	0.005	0.044	0.004	0.060	0.031	0.004	-
	ECFP6	mean	0.882	0.755	0.606	0.904	0.388	0.789	0.328	-
		sd	0.002	0.005	0.010	0.002	0.008	0.009	0.006	-
	MACCS	mean	0.799	0.729	0.647	0.811	0.285	0.800	0.210	-
		sd	0.003	0.010	0.019	0.003	0.012	0.008	0.006	-
CostSensitive Classifier	MOE	mean	0.871	0.792	0.695	0.890	0.420	0.874	0.328	0.928
		sd	0.024	0.009	0.015	0.003	0.010	0.0037	0.007	0.002
	ECFP6	mean	0.651	0.725	0.811	0.639	0.238	0.809	0.148	0.918
		sd	0.009	0.014	0.018	0.010	0.010	0.008	0.004	0.002
	MACCS	mean	0.737	0.729	0.720	0.739	0.260	0.820	0.176	0.914
		sd	0.006	0.013	0.021	0.006	0.013	0.007	0.005	0.002
MetaCost	MOE	mean	0.864	0.7964	0.7174	0.8754	0.410	0.870	0.308	0.926
		sd	0.003	0.012	0.021	0.003	0.013	0.004	0.008	0.002
	ECFP6	mean	0.688	0.732	0.783	0.681	0.251	0.797	0.160	0.917
		sd	0.007	0.012	0.019	0.006	0.012	0.005	0.004	0.003
	MACCS	mean	0.711	0.738	0.769	0.706	0.262	0.802	0.169	0.917
		sd	0.007	0.016	0.024	0.007	0.014	0.009	0.006	0.003
Threshold Selector	MOE	mean	0.907	0.748	0.562	0.934	0.423	0.872	0.397	0.924
		sd	0.004	0.024	0.041	0.006	0.022	0.006	0.017	0.003

ECFP6	mean	0.903	0.725	0.518	0.937	0.388	0.820	0.374	0.919
	sd	0.006	0.018	0.030	0.007	0.022	0.012	0.021	0.003

Table A19. Results on human cholestasis dataset only for the best performing methods on the appropriate set of descriptors (Sensitivity ≥ 0.5) for all calculated statistics metrics : Accuracy, Balanced Accuracy, Sensitivity, Specificity, MCC, AUC, Precision, Weighted Precision. The mean performance out of 20 iterations and the standard deviation values are provided.

Model Settings	Descriptors	Statistical Value	Accuracy	Balanced Accuracy	Sensitivity	Specificity	MCC	AUC	Precision	Weighted Precision
Stratified Bagging +cost2:1	MOE	mean	0.777	0.713	0.607	0.819	0.384	0.768	0.450	-
		sd	0.002	0.005	0.011	0.003	0.008	0.004	0.005	-
	ECFP6	mean	0.806	0.722	0.583	0.860	0.421	0.773	0.505	-
		sd	0.003	0.003	0.006	0.004	0.006	0.004	0.006	-
	MACCS	mean	0.782	0.723	0.625	0.820	0.400	0.772	0.460	-
CostSensitive Classifier		sd	0.005	0.004	0.007	0.005	0.009	0.005	0.008	-
	MOE	mean	0.731	0.707	0.667	0.74685	0.346	0.786	0.392	0.802
		sd	0.005	0.009	0.013	0.005	0.011	0.005	0.006	0.004
	ECFP6	mean	0.771	0.704	0.596	0.812	0.369	0.782	0.440	0.803
		sd	0.006	0.014	0.017	0.012	0.015	0.006	0.011	0.005
MetaCost	MACCS	mean	0.753	0.705	0.629	0.782	0.343	0.776	0.415	0.802
		sd	0.006	0.010	0.011	0.010	0.073	0.005	0.008	0.004
	MOE	mean	0.671	0.681	0.698	0.664	0.293	0.755	0.337	0.767
		sd	0.008	0.014	0.019	0.010	0.015	0.007	0.007	0.103
	ECFP6	mean	0.750	0.699	0.614	0.783	0.346	0.768	0.409	0.798
Threshold		sd	0.006	0.011	0.015	0.007	0.013	0.006	0.009	0.004
	MACCS	mean	0.690	0.692	0.695	0.689	0.313	0.764	0.353	0.794
		sd	0.005	0.006	0.013	0.007	0.010	0.004	0.006	0.004
	MOE	mean	0.801	0.694	0.518	0.870	0.382	0.775	0.495	0.805
		sd	0.005	0.006	0.013	0.007	0.010	0.004	0.006	0.004

Script for generating the plot representing balanced accuracy and sensitivity for each model. **Developed by Sankalp Jain.**

```
#####
```

```
### Script for generating the plot representing the sensitivity and balanced accuracy of the models.
```

```
#On axis x is represented balanced accuracy and on axis y sensitivity
```

```
#Function for the dimension and location to the image(on computer)
```

```
antialias_png<-function(filename, width, height, pointsize, units="px", res=res)
```

```
{
```

```
  png(filename=filename, width=width, height=height, pointsize=pointsize, units=units, res=res)
```

```
#initialize the plot that will be written directly to a file using .png
```

```
}
```

```
#function to generate the plot
```

```
plot_pca <-function()
```

```
{
```

```
  antialias_png(filename="LOCATION ON COMPUTER WHERE TO SAVE THE IMAGE/IMAGE.png",  
width=900, height=700, pointsize=12, res=100); # Location on computer where to save the image. image  
dimensions
```

```
  #data to represent on the plot
```

```
    model_names <- c("Sbagging MOE", "costSensitive ECFP6", "metaCost MACCS", "metaCost  
MOE", "costSensitive MOE", "costSensitive ECFP6", "Sbagging ECFP6")
```

```
    model_balanced_acc <- c(0.8, 0.9, 0.7, 0.6, 0.4, 0.7, 0.2)
```

```
    model_sensitivity <- c(6, 3, 5, 4, 7, 6, 2)
```

```
    model_color <- c("red","green","blue","blue","green","green","red")
```

```
    model_shape <- c(15, 16, 17,15,15,16,16)
```

```
    # x-axis
```

```
xmin = min(model_balanced_acc)
```

```
xmax = max(model_balanced_acc)
```

```
xarea = xmax-xmin
```



```

# Y-axis

ymin = min(model_sensitivity)

ymax = max(model_sensitivity)

yarea = ymax-ymin

# plot margin

par(mar=c(4, 4.3, 3, 16))# mar (Bottom,left,top,right)

plot(1, xlim=c(xmin-0.1*xarea,xmax+0.1*xarea), ylim=c(ymin-0.1*yarea,ymax+0.1*yarea), type='n',
xlab="Balanced Accuracy", ylab="Sensitivity",xaxs="i",yaxs="i",
xaxt="n",yaxt="n",cex.lab=1.2,cex.main=1.5,main="MODEL NAME-TITLE") #cex.lab = axis labels size,
cex.main = title size, main = title)

axis(1,cex.axis=1.2)# x-axis size

axis(2,cex.axis=1.2)# y-axis size

points(x=model_balanced_acc, y=model_sensitivity, pch=model_shape, col=model_color, cex=2)
#Points size on the plot

legend(model_names, xpd=NA, pch=model_shape, col=model_color, x=xmax+0.15*xarea,
y=ymax+0.1*yarea, y.intersp = 1.19) #size and dimension of the legend block

dev.off() #close the plot/file

```

6. Abbreviation List

ABCG5/G8: ATP-binding cassette subfamily G members 5 and 8

ABC-transporter: ATP-binding cassette transporter

ATP7B: copper-transporting P-type ATP-ase

ATP8B1: ATPase class I type 8B member 1

AUC: area under the curve

BCRP: breast cancer resistance protein

BSEP: bile salt export pump

CFTR: cystic fibrosis transmembrane conductance regulator

CV: cross-validation
DILI: drug-induced liver injury
ECFP: extended connectivity fingerprints
EV: external validation
FDA: Food and Drugs Administration
G6PT: glucose-6-phosphate transporter
MATE: Multidrug and toxin extrusion transporter
MCC: Matthews correlation coefficient
MDR: multi-drug resistance
MRP: multidrug resistance-related protein
NTCP: Na⁺ + -taurocholate cotransporting polypeptide
OAT: organic anion transporter
OATP: organic anion transporter polypeptide
OCT: organic cation transporter
OST α -OST β : organic solute transporter alpha-beta
PDB: Protein Data Bank
PFIC: progressive familial intrahepatic cholestasis
P-gp: P-glycoprotein
QSAR: quantitative structure-activity relationship
RF: random forest
ROC: receiver operating characteristics
SLC: solute carrier
SMO: sequential minimal optimization
SVM: support vector machine

Abstract

Drug-induced liver injury (DILI) is currently a major challenge for drug development in pharmaceutical industry: it is one of the main causes for attrition during clinical and pre-clinical studies and the primary reason for drug withdrawal from the market. Subsequently, there is great need for recognizing or foreseeing potential hepatotoxicity issues as early as possible. Unfortunately, predicting hepatotoxicity is not an easy task, due to the complexity of the endpoint and potential idiosyncratic phenomena. In recent years, liver transporters attracted lots of attention regarding their role in development of drug induced hepatotoxicity. There are many reports in literature for several transporters, including among others bile salt export pump (BSEP), breast cancer resistance protein (BCRP), P-glycoprotein (P-gp) and organic anion transporting polypeptide 1B1 and 1B3 (OATP1B1 and OATP1B3).

Main topic of the current thesis is modeling liver toxicity endpoints, as well as general drug-induced hepatotoxicity, by combining information of the liver transporters' inhibition aforementioned and molecular descriptors. Due to lack of *in vitro* data, predictions of transporters' inhibition were used instead. For this cause, classification models for OATP1B1 and OATP1B3 inhibition were initially developed, while for the rest of BSEP, BCRP and P-gp *in silico* models already available in-house were used. The studied endpoints were drug-induced liver injury, hyperbilirubinemia and cholestasis. Apart from modeling, also the role of hepatic transporters' inhibition was investigated for the cases of the toxicity endpoint. Mainly human, - and in some cases also animal - data were used. They come primarily from public sources – thus, extended careful curation was provided - while some of the animal *in vivo* data were provided from the eTOX consortium.

Several models were developed, both for transporters and toxicity endpoints, with some of them yielding very satisfactory performance. In general, the modeling of the transporters was a comparably easier task and gave better results with simpler classification schema. For toxicity endpoints with a more straightforward mechanistic basis, like cholestasis, association between transporter inhibition and toxicity was also shown. For more general forms of toxicity, like DILI, there was no clear trend. Of course, there are more hepatic transporters, as well as enzymes, playing an important role and their inclusion in a further study would be interesting.

Zusammenfassung

Arzneimittelinduzierte Leberschädigung (DILI) ist eine der großen Herausforderungen für die Pharmaindustrie und stellt einer der Hauptgründe für das Scheiterns neuer Substanzen während klinischer und präklinischer Phasen dar. Weiters ist DILI der primäre Grund für den Rückruf vom Markt. Es besteht daher die dringende Notwendigkeit, eine potenzielle Hepatotoxizität möglichst früh zu erkennen. Allerdings ist die Vorhersage aufgrund der Komplexität des klinischen Endpunkts und möglicher idiosynkratischer Lebertoxizität eine schwierige Aufgabe. In den letzten Jahren rückten Transporter in der Leber durch ihre Rolle bei der Entwicklung von arzneimittelinduzierter Hepatotoxizität ins Zentrum der Aufmerksamkeit. Die Literatur berichtet über mehrere Transporter, unter anderem die Gallensalz-Export-Pumpe (BSEP), das Brustkrebs-Resistenzprotein (BCRP), P-Glykoprotein (P-gp), und die Organo-Anion-Transporter 1B1 und 1B3 (OATP1B1 und OATP1B3).

Die Hauptthemen der vorliegenden Arbeit beinhalten die Modellierung klinischer Endpunkte von Lebertoxizität und allgemeiner arzneimittelinduzierter Hepatotoxizität durch Kombination von Informationen über die Hemmung der zuvor genannten Leber-Transporter mit molekularen Deskriptoren. Anstelle der fehlenden *in vitro* Daten wurden Vorhersagen der Transporterhemmung verwendet. Nachdem für BSEP, BCRP und P-gp lagen bereits *in silico* Modelle in der Arbeitsgruppe vorhanden waren, wurden zuerst Klassifikationsmodelle für die Hemmung von OATP1B1 und OATP1B3 entwickelt. Die untersuchten Endpunkte waren medikamenteninduzierte Leberschädigung, Hyperbilirubinämie und Cholestase. Für den Einfluss der Hemmung von Transportern wurden hauptsächlich menschliche, in einigen Fällen auch tierische Daten aus vordergründig öffentlichen Quellen verwendet, was sorgfältige Kuration erforderte. Ein Teil der tierischen *in vivo* Daten wurde aus dem eTOX Konsortium zur Verfügung gestellt.

Aufbauend auf diesen Daten wurden mehrere Modelle sowohl für Transporter als auch für toxische Endpunkte entwickelt. Die Modellierung der Transporter war die vergleichsweise leichtere Aufgabe und führte mit einem einfachen Klassifikationsschema zu guten Ergebnissen. Für toxische Endpunkte mit einer klaren mechanistischen Basis, wie beispielsweise Cholestase, konnte ebenfalls eine Assoziation zwischen Transporter-Hemmung und Toxizität gezeigt werden. Komplexere Formen der Toxizität, wie DILI, ergaben keinen klaren Trend. Die Berücksichtigung weiterer hepatischer Transporter und auch Enzyme, die wichtige Rollen in diesem Zusammenhang spielen, wird daher für zukünftige Studien von Interesse sein.