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Titel der Diplomarbeit

Expression of OATP Transporters
in Human Colorectal, Lung and Ovarial Cancer:
Impact on Therapy

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Verfasserin / Verfasser:	Angela Johanna Schöffmann
Matrikel-Nummer:	0400865
Studienrichtung /Studienzweig (lt. Studienblatt):	A449 Pharmazie
Betreuerin / Betreuer:	Ao. Univ.-Prof. Mag. Dr. Walter Jäger

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1 ABSTRACT

Colorectal cancer is one of the most frequent malign diseases worldwide, accounting for thousands of incidences every year. The investigation of the expression of transporters capable of influencing cancer treatment could be more than a small contribution to better therapy and lead to a better prognosis of colorectal cancer in the future.

In this respect, transporters of the organic anion transporting polypeptides (OATP) are interesting, as they mediate the transport of a broad spectrum of anionic substances, also including anticancer drugs to cancer cells.

Until now, 11 human OATPs have been detected. Their distribution in normal tissue as well as in neoplastic tissue has been the subject of many recent studies. Nevertheless little knowledge about their expression in colorectal cancer has been gained so far. However, the OATP superfamily is assumed to play an important role in efficacy of chemotherapy. Therefore, the subject of the present thesis was to investigate the expression of human OATPs on the mRNA level in three different sample types of tumor, namely human colorectal cancer (human colorectal cancer samples, with the adjacent healthy tissue, and colon cancer cell lines), human lung cancer, and human ovary carcinoma. Investigations are done by real time PCR and data are analyzed using Microsoft Excel®. The expression rates are expressed as MNE (mean normalized expression value), and consistently refer to the calibrators' mean MNE value.

In colorectal carcinoma samples six transporters show upregulation in cancerous tissue, i.e. 1B3, 2A1, 3A1, 4C1, 4A1 and 5A1. Most remarkably, OATP1B3 exceeds the second threshold in four samples, showing expression rates ranging from MNE 4.32 up to MNE 8.27. The most conspicuous expression level could be demonstrated for OATP4A1, reaching a MNE value of 9.85. Remarkably, in the adjacent normal tissue from the colon cancer patients, upregulation of these OATPs is not detectable.

Real time PCR studies in lung and ovary carcinoma cell lines show that OATPs 3A1, 1A2, 4A1, 4C1, and 5A1 are up regulated, but the expression rates are rather homogenous. The most surprising result is demonstrated for OATP3A1 in

DMS 456 cells (i.e. human small cell lung carcinoma cells). It shows a 14.7 fold upregulation.

Taken together, the data shows that OATPs are widely expressed in colon cancer and the OATP superfamily might be taken as a marker during anticancer therapy.

Das Kolorektalkarzinom stellt eine der häufigsten malignen Krankheiten weltweit dar, und zeichnet für tausende Krankheitsfälle jährlich verantwortlich. Die Expression von Transportern, die fähig sind die Effektivität von Chemotherapien zu beeinflussen, zu untersuchen könnte daher in Zukunft ein logischer und erforderlicher Beitrag zur Optimierung der Behandlung und Verbesserung der Prognose sein.

In dieser Hinsicht sind Transporter der organische Anionen transportierende Proteine („Organic anion transporting polypeptides“, OATPs) interessant, da sie die den Transport eines breiten Spektrums an oranischen, anionischen Substanzen vermitteln. Selbsterklärend zählen zu diesen auch die in der Krebstherapie verwendeten Arzneistoffe.

Bis zum jetzigen Zeitpunkt wurden 11 humane OATPs entdeckt. Ihre Expression, sowohl in gesundem, als auch in entartetem Gewebe, war und ist Gegenstand vieler rezenter Studien. Nichtsdestotrotz ist bisher nur wenig über ihr Auftreten in Tumorgeweben bekannt. Da den Transportern jedoch eine große Rolle im Rahmen der Wirksamkeit von Chemotherapien zugeschrieben wird, war es Ziel der vorliegenden Arbeit die Expression der einzelnen OATPs auf mRNA Level zu untersuchen.

Als Material fanden drei verschiedene Tumortypen Verwendung: humanes Kolonkarzinom und dessen umliegendes gesundes Gewebe, humanes Lungen- und Ovarialkarzinom. Die Forschungsergebnisse werden unter Verwendung der Real Time PCR Technik erzielt, und die Daten mit Hilfe von Microsoft Excel® ausgewertet. Die Ergebnisse werden durchwegs als „MNE“ (mean normalized expression value) und in Abhängigkeit vom mittleren MNE-Wert zweier Kalibratoren angegeben.

In den untersuchten Kolorektalkarzinomproben der Patienten kann Hochregulierung von sechs Transportern, 1B3, 2A1, 3A1, 4C1, 4A1 und 5A1 nachgewiesen werden. Der Transporter OATP1B3 zeigt mit MNE-Werten zwischen 4.32 und 8.27 in vier

Proben signifikante Hochregulierung, wobei die bemerkenswertesten Ergebnisse von OATP4A1 (MNE 9.85) erzielt werden. Interessanter Weise ist im umliegenden gesunden Gewebe der Kolonkarzinompatienten keine Hochregulierung dieser OATPs feststellbar.

Zusätzliche Real Time PCR Screenings von Lungenkarzinom- und Ovarialkarzinom-Zelllinien zeigen, dass auch die Transportergene 3A1, 1A2, 4A1, 4C1 und 5A1 hoch reguliert werden. Ungeachtet dessen sind die Expressionsraten durchwegs eher homogen. Besonders sticht allerdings die Expression des OATP3A1 in der Zelllinie DMS 456 (Zelllinie des humanen kleinzelligen Lungenkarziom) mit 14.7-facher Hochregulierung hervor.

Zusammenfassend zeigen die erhaltenen Daten, dass die Transporter der OATP Superfamilie im Kolorektalkarzinom weit verbreitet vorkommen, und daher zukünftig als Marker im Zuge einer Krebstherapie verwendet werden könnten.

2 AIMS OF THE THESIS

Colorectal cancer is one of the most frequent malign diseases worldwide, accounting for thousands of cases every year. As dietary factors seem to play a vicious role in the formation of cancerous colorectal tissue, the highest incidence can be observed in the civilized Western countries.

Colorectal cancer can be of hereditary origin, or it can be caused by inflammations in the gastrointestinal tract. In most cases, however, it appears without predisposition.

The treatment of colorectal carcinoma is primarily surgical, obtaining a rate of success of approximately 75 %. In cases where surgical intervention does not lead to satisfying results, either palliative resection can be applied, or the patient needs to undergo chemotherapy. Most frequently, the fluoropyrimidines 5-fluoruracil (5-FU) and floxuridine, and thiotepa are applied (Lui et al., 2005).

The organic anion transporting polypeptides form a group of transporter proteins of which 11 different human subtypes are known until now. They account for the transport of organic anionic substances, for instance bile salts or thyroid hormones. However, the most important aspect of this transporter superfamily is the fact that they are closely associated with drug absorption and excretion. Hence, they are considered to play an important role referring to drug-drug or drug-food interactions, drug side effects and drug efficacy in general. As the transporters' expression is up-regulated in certain tumors they might be the reason for inefficient chemotherapy, leading to poor prognosis for the patients, and also increased side effects.

The expression of the 11 known human SLCO (solute carrier organic anion transporter family) genes is the objective of the present thesis. By investigating the expression rates in four different types of samples, including samples from cancer patients as well as established cancer cell lines, additional knowledge about the upregulation of the OATP family in cancerous tissues should be achieved.

The aim of the thesis will be to elucidate the expression pattern of OATPs on the mRNA level by real time PCR. The results obtained by real time PCR then are analyzed by calculation programs. In addition attention will be paid on the distribution of the OATPs within the different sections of the colon to show whether upregulation of the transporter might be coupled not only with tumor stage but as well with the

tumor’s localization within the gut. OATP transporters therefore may act as tumor markers in future anticancer treatment.

3 INTRODUCTION

3.1 The Organic Anion Transporting Polypeptides OATPs

3.1.1 General Information

The organic anion transporting polypeptides (OATPs in human, Oatps in rodents) are sodium-independent membrane transport proteins mediating the transport of various generally amphipathic organic compounds, including bile salts, organic dyes, thyroid hormones, steroid conjugates, anionic oligopeptides and numerous drugs, and other xenobiotics (Hagenbuch and Meier, 2003).

In general, OATPs' substrates are of high molecular weight (>450 KD) and show high plasma albumin binding under physiological conditions.

Some OATPs are involved in the hepatic clearance of albumin-bound compounds (Meier and Stieger, 2002). However, most OATPs are expressed in a great number of tissues, including the blood-brain barrier, choroids plexus, heart, lung, kidney, placenta, testes and intestine. The liver specific OATPs/Oatps are localized at the sinusoidal (basolateral) plasma membrane domain of the hepatocytes.

OATPs show 12 transmembrane domains (TM). All of them represent a large extracellular domain (extracellular loop 5, between TM 9 and 10) containing mostly conserved cystein residues with disulfide bonds, N-glycosylation sites in extracellular loops 2 and 5, and the consensus superfamily signature between loop 3 and TM 6 (Hagenbuch and Meier, 2003).

In addition, Hagenbuch and Meier (2004) described that it consists of 643–722 amino acids, and are glycosylated. Glycosylation has been demonstrated to influence membrane localization and transport function. Also intact disulfide bonds in extracellular loop 5 are important. Without these requirements the transporter is not expressed and shows no transport function (Hänggi et al., 2004).

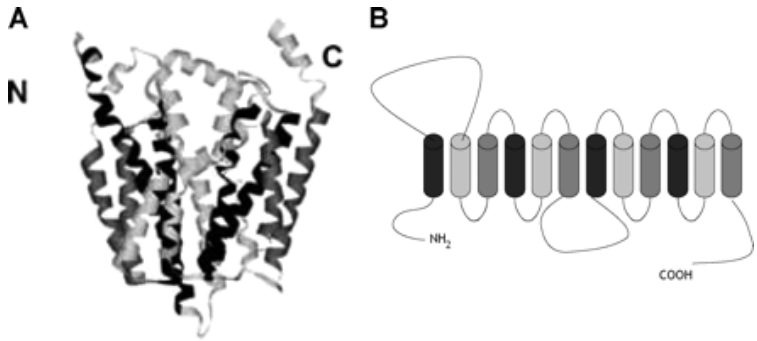

Figure 1: A three-dimensional model of hOAT1 based on the structure of GlpT. (Perry et al., 2006)

OATPs/Oatps belong to the SLC21/SLCO gene family (solute carrier class) as members of the SLCO family (solute carrier organic anion transporter family) (Hagenbuch and Meier, 2004).

The first Oatp has been identified and isolated from rat liver in 1994 by Jacquemin et al. Numerous additional OATPs/Oatps have been isolated from various species, including human, rat, mouse (Hagenbuch and Meier, 2004; Mikkaichi et al., 2004) cow (Geyer et al., 2006), horse (Brown et al., 2007), quail (Nakao et al., 2006), and other non-mammalian species (fruitflies, bees, nematodes, sea urchins, catfish, zebrafish, pufferfish, frogs, chicken, and pig).

So far, Oatps were only found in species either belonging to the clade of protostomia or the clade of deuterostomia, but not in bacteria and yeast.

Up to now, 11 human OATPs have been discovered and, following the new nomenclature systems (Hagenbuch and Meier, 2004), have been classified into 6 families, OATP1 – OATP6. All together, 36 OATPs/Oatps have been identified in humans and rodents.

3.1.2 Classification and Nomenclature

Formerly, OATPs/Oapts have been named organ-specifically rather than functionally, creating similar terms for different gene products in different species on the one hand, and vice versa. By establishing a new species-independent system based on divergent evolution and defining an OATP/Oatp-gene superfamily that can be further

divided into sub-families and individual genes and gene products, further confusions can be avoided. This evolutionary based system of nomenclature has been approved by the HUGO Gene Nomenclature Committee (HGNC).

Instead of using “SLC21/Slc21”, which does not permit classification in sub-families, the root “SCL” is kept and extended with “O”, symbolizing the first letter of the abbreviation OATP/Oatp. Thus, the “SLCO/Slco” is used to classify genes, whereas “OATP/Oatp” stands for the corresponding protein.

Genes, respectively proteins with >40% amino acid sequence identity are grouped in families and designated by Arabic numbering (e.g. SLCO1). Individual families include genes/proteins showing >60% amino acid sequence identity, and are designated by letters (e.g.SLCO1A). If the subfamily embraces further individual genes/gene products, additional Arabic numbering is added, based on the chronology of identification (e.g. SLCO1A1, SLCO1A2).

Until the new nomenclature is broadly accepted and, most important of all, used, the familiar old names should supplement the new classification by being given in parentheses. (Hagenbuch and Meier, 2004)

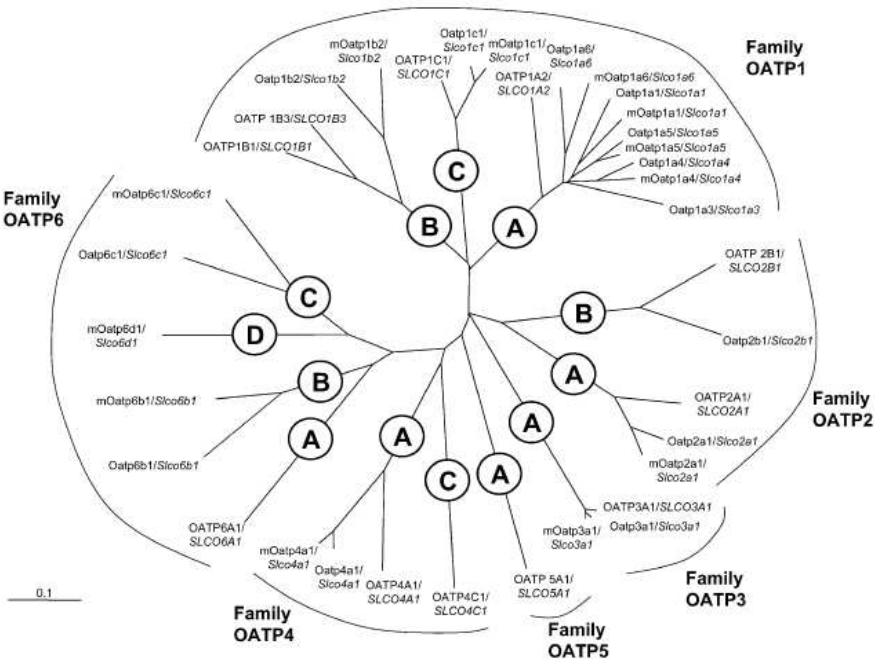

Figure 2: Phyllogenetiv classification and new nomencalture of human and rodent members of the OATP/SLCO superfamily (Hagenbuch and Meier, 2004).

3.1.3 Function, physiological importance and pharmacological aspects

As their name already indicates, OATPs/Oatps mediate sodium-independent transport (Jacquemin et al., 1994; Kullack-Ublick et al., 1995; Noé et al., 1997; Walters et al., 2000) of organic anionic compounds, but the mechanism of transport is not fully understood yet. It seems to be anionic exchange, coupling the uptake of organic compounds with the efflux of another (e.g. bicarbonate, glutathione and/or glutathione-S-conjugates) (Shi et al., 1995; Satlin et al., 1997; Li et al., 2000; Li et al. 1998).

All members of the OATP/Oatp superfamily seem to be able to mediate bidirectional transport while the direction of the substrates' transport likely depends on the substrates' local concentration gradients. In addition, the transport appears to be pH-dependent and electroneutral (Nakao et al., 2006).

Most OATPs/Oatps mediate the transport of a wide range of amphipathic compounds. This broad and partially overlapping substrate specificity has been documented for mostly all members of the OATP1A- and OATP1B-subfamilies.

Therefore, they might play an important role in overall drug absorption and disposition, together with the P-glycoproteins (MDRs/Mdrs) and the muldtidrug resistance associated proteins (MRPs, Mrps).

Data provided by Fattinger et al. and Vavricka et al. (2000) indicate that co-administration of specific OATP inhibitors may increase bioavailability of drugs otherwise being highly metabolized by hepatic first-pass effect.

As a conclusion, OATPs/Oatps may have important effects on drug development and therapy.

Table 1: Substrates of the OATP Superfamily. (Hagenbuch and Gui, 2008; Hagenbuch and Meier, 2004)

Substrates of the OATP superfamily		
OATP	**substrates**	**notes**
1A2	APD-ajmalinium, atrasentan, bamet-R2, bamet-UD2, bilirubin, BQ-123, bromosulfophtalein (BSP), chlorambuciltaurocholate, cholate (CA), ciprofloxacin, CRC220, dehydroepiandroserone-3-sulfate (DHEAS), deltorphin II, [D-penicillamine-2,5]enkephalin (DPDPE), enoxacin, oestradiol-17β-glucuronide, oestrone-3-sulfate, fexofenadine, gatifloxacin, gd-B20790, glycocholate (GCA), Levofloxacin, lomecloxacin, methotrexate, microcytin, N-methylquinidine, N-methylquinine, norfloxacin, ouabain, pitavastatin, prostaglandin E2 (PGE2), reverse triiodothyronine (rT3), rocuronium, rosuvastatin, saquinavir, taurocholate (TCA), taurochenodeoxycholate (TCDCA), tauroursodeoxycholate (TUDCA), thyroxine (T4), TR-14035, triiodothyronine (T3), unoprostone metabolite	BQ-123, cyclic pentapeptide endothelin receptor antagonist; CRC220, peptidomimetic thrombin inhibitor; Gd-B20790, gadolonium-18-((3-(2-carboxylbutyl)-2,4,6-triiodophenyl)amino)-3,6,9-tris (carboxymethyl)-11,18-dioxo-3,6-9,12-tetraazaoctadecanoic acid; TR-14035, α4β1/α4β7 integrin dual antagonist.
1B1	ACU154, arsenic (arsenite, arsenate), atrovastatin, atrasentan, bamet-R2, bamet-UD2, benzylpenicillin, bisgluvuronosyl bilirubin, bosentan, BQ-123, bromosulfophthalein (BSP), caspofungin, CDCA-NBD, cholate (CA), [D-Ala2, D-Leu5]enkephalin (DADLE), dehydroepiandroserone-3-sulfate (DHEAS), demethylphalloin, [D-penicillamine-2,5]enkephalin (DPDPE), enalapril, oestradiol-17β-glucuronide, oestrone-3-sulfate, fluvastatin, glycocholate (GCA), glycoursodeoxycholate (GUDCA), leukotriene C4 (LTC4), leukotriene E4 (LTE4), methotrexate, microcystein, monoglyucuronosyl bilirubin, olmesartan, phalloidin, pitavastatin, pravastatin, prostaglandin E2 (PGE2), rifampicin, Ro 48-5033 (bosentan metabolite), rosuvastatin, SN-38, taurocholate (TCA), tauroursodeoxycholate (TUDCA), thromboxane B2 (TXB2), thyroxine (T4), TR-14035, triiodothyronine (T3), troglitazone sulfate, valsartan	ACU154, metabolite of PKI166, an epidermal growth factor receptor kinase inhibitor; BQ-123, cyclic pentapeptide endothelin receptor antagonist; CDCA-NBD, chenodeoxycholyl-(Nε-NBD)-lysine; Ro 48-5033, bosentan metabolite; SN-38, 7-ethyl-10-hydroxycamptothecin; TR-14035, α4β1/α4β7 integrin dual antagonist

Table 1 ctd.: Substrates of the OATP Superfamily. (Hagenbuch and Gui, 2008; Hagenbuch and Meier, 2004)

Substrates of the OATP superfamily (continued)		
OATP	**substrates**	**notes**
1B3	amanitin, atrasentan, bilirubin, bosentan, BQ-123, bromosulfophthalein (BSP), CDCA-NBD, cholate (CA), cholecystokinin octapeptide (CCK-8), dehydroepiandroserone-3-sulfate (DHEAS), deltorphin II, demethylphalloin, digoxin, docetaxel, [D-penicillamine-2,5]enkephalin (DPDPE), enalapril, oestradiol-17β-glucuronide, oestrone-3-sulfate, fexofenadine, fluvastatin, fluo-3, glutathione (GSH), glycocholate (GCA), glycoursodeoxycholate (GUDCA), leuktrien C4 (LTC4), methotrexate, microcystin, monoglyucuronosyl bilirubin, olmesartan, ouabain, paclitaxel, phalloidin, pitavastatin, rifampicin, Ro 48-5033 (bosentan metabolite), rosuvastatin, taurocholate (TCA), taurochenodeoxycholate (TCDCA), taurodeoxycholate (TDCA), tauroursodeoxycholate (TUDCA), telmisartan, thyroxine (T4), TR-14035, triiodothyronine (T3), valsartan	BQ-123, cyclic pentapeptide endothelin receptor antagonist; CDCA-NBD, chenodeoxycholyl-(Nε-NBD)-lysine; Ro 48-5033, bosentan metabolite; Fluo-3, glycine, N-[2-[[[[2-[bis(carboxymethyl)amino]-5-(2,7-dichloro-6-hydroxy-3-oxo-3H-xanthen-9-yl)phenoxy]methyl]methyl]oxy]-4-methylphenyl]-N-(carboxymethyl)-pentammonium salt; Ro 48-5033, bosentan metabolite; TR-14035, α4β1/α4β7 integrin dual antagonist
1C1	bromosulfophthalein (BSP), oestradiol-17β-glucuronide, oestrone-3-sulfate, reverse triiodothyronine (rT3), thyroxine (T4), triiodothyronine (T3)	
2A1	prostaglandins, thromboxane B2	
2B1	atrovastatin, benzylpenicillin, bosentan, bromosulfophthalein (BSP), CP-671,305, dehydroepiandroserone-3-sulfate (DHEAS), oestrone-3-sulfate, fexofenadine, fluvastatin, glibenclamide, M17055, pravastatin, pitavastatin, pregnenolone sulfate, prostaglandine E2 (PGE2), rosuvastatin, taurocholate (TCA), unoprostone metabolite	CP-671,305, (+)-2-[4-({[2-(benzo[1,3]dioxol-5-yloxy)-pyridine-3-carbonyl]-amino}-methyl)-3-fluorophenoxy]-propionic acid; M17055, 7-chloro-2,3-dihydro-1-(2-methylbenzoyl)-4(1H)-quinolinone 4-oxime-O-sulfonic acid
3A1	benzylpenicillin, BQ-123, deltorphin, oestrone-3-sulfate, prostaglandin E1 (PGE1), prostaglandin E2 (PGE2), prostaglandin F2α (PGF2α), thyroxine (T4), vasopressin --- 3A1_v1 arachidonic acid, BQ-123, prostaglandin E1 (PGE1), prostaglandin E2 (PGE2), thgyroxine (T4), vasopressin --- 3A1_v2	BQ-123, cyclic pentapeptide endothelin receptor antagonist
4A1	benzylpenicillin, oestradiol-17β-glucuronide, oestrone-3-sulfate, prostaglandin E2 (PGE2), reverse triiodothyronine (rT3), taurocholate (TCA), thyroxine (T4), triiodothyronine (T3), unoprostone metabolite	BQ-123, cyclic pentapeptide endothelin receptor antagonist
4C1	cAMP, digoxin, methotrexate, ouabain, sitagliptin, thyroxine (T4), triiodothyronine (T3)	
5A1	substrates unknown	
6A1	substrates unknown	

3.1.4 OATP sub-families and their members – functional properties

OATP1A2, formerly named OATP-A, contains several rat and mouse members (Hagenbuch and Meier, 2004), and also one bovine homologue (Geyer et al., 2006). It is expressed at highest levels in brain (Kullack-Ublick, 1995; Gao et al. 2000), followed by liver (present in cholangiocytes, but not in hepatocytes (Lee et al., 2005)), the brush border of the distal nephron (Lee et al., 2005) and the apical membrane of enterocytes (Glaeser et al., 2007). It mediates the transport of a wide range of endo- and exogenous mainly amphipathic compounds, including bile salts, hormones and their conjugates, cyclic and linear peptides, toxins, organic cations and numerous drugs. Briefly, these are common OATP/Oatp substrates.

OATP1B1 (OATP-C) was cloned from human liver (Tamai et al., 2000; Hsiang et al., 1999; Abe et al. 1999; König et al., 2000a) with orthologues in rat and mouse (Hagenbuch and Meier, 2004). It is considered to be a liver specific transporter (Abe et al 1999; Hsiang et al., 1999; König et al., 2000b), expressed in the basolateral membrane of the hepatocytes (Shi et al., 1995; Satlin et al., 1997; Li et al., 2000; Li et al., 1998; König et al., 2000b), as usual for liver specific OATPs/Oatps. The transport functions have been characterized in *Xenopus laevis* oocytes (Satlin et al., 1997; Kullack-Ublick et al., 2001) and in stably transfected HEK-293 cells (Shi et al., 1995; Satlin et al., 1997; Li et al., 2000). Its substrates include the common OATP/Oatp substrates, but are extended by eicosanoids, drugs such as benzyl penicillin, methotrexate, pravastatin and rifampicin, as well as the natural toxins microcystin and phalloidin (Hagenbuch and Meier, 2003). In addition, several polymorphisms in the SLCO1B1 gene have been described (Tirona et al., 2001; Nozawa et al., 2002) identifying a number of alleles with dramatically reduced V_{max}/K_m values indicating significantly decreased transport function. The basolateral expression of OATP1B1 depends on the liver-enriched transcription factor HNF-1α (Jung et al., 2001) explaining the 50% decrease of transcriptional expression of the transporter due to primary sclerosing cholangitis (Oswald et al., 2001).

OATP1B3 (OATP8, LST-2) was cloned from human liver (Abe et al. 2001, König et al. 2000a) and holds 80 % amino acid identity to OATP1B1. It has also been detected to be a liver specific OATP (Tamai et al., 2000; Hsiang et al., 1999) which is highly expressed around the central vein (Abe et al. 2001; König et al. 2000a). In

neoplastic tissue, the transporter was found in different tumour cell lines (gastric, colon, pancreas, gallbladder, lung and brain (Tamai et al., 2000)). It transports the same substrates as OATP1B1, exclusive of the intestinal peptide cholecystokinin 8 (CCK-8) (Isamair et al., 2001) deltorphin and the cardiac glycosides digoxin and oubain (Fattinger et al., 2000). The substrate range has been investigated, as for OATP1B1, in *Xenopus laevis* oocytes and in stably transfected HEK-293 cells. Whether the increased expression around the central vein of the liver is related to a specific physiological function has not been investigated yet. The transporter is hepatically expressed depending on the levels of HNF-1α (Lee et al., 2005) and the bile acid nuclear receptor FXR/BAR (Jung et al., 2002).

OATP1C1 (OATP-F) has one human and one rodent member (Hagenbuch and Meier, 2004). This transporter is expressed at the highest levels in brain and testes (Pizzagalli et al., 2002). In the latter, it is found in Leydig cells and the blood-brain barrier (Tamai et al., 2000). Despite the common great variety of substrates, the OATP1C1 transports only a narrow range of compounds, including thyroid hormones, sulfobromophathalein (BSP), oestradiol-17β-glucuronide, and oestrone-3-sulfate. Its physiological role seems to be the delivery of thyroid hormones to their target tissues (Hagenbuch et al., 2007).

OATP2A1 (the prostaglandin transporter PGT) contains single orthologues in rat and mouse (Hagenbuch and Meier, 2004; Schuster 2002). It has been cloned from adult human kidney and is expressed in numerous other tissues (brain, colon, heart, liver, lung, ovary, pancreas, placenta, prostate, skeletal muscle, spleen, small intestine). It mediates the transport of none of the typical OATP-substrates, but prostaglandins. The transporters' physiological role is supposed to be the transport of prostaglandins and other eicosanoids. In addition, it possibly is involved in regulation of pericellular prostaglandin levels (Abe et al., 1999).

OATP2B1 (OATP-B) shows orthologues in rat and mouse (Hagenbuch and Meier, 2004), and is ubiquitously expressed. It is found in human kidney (Abe et al., 2001), lung, heart (Grube et al., 2006) liver (Pizzagalli et al., 2001), placenta (St-Pierre et al., 2002) brain, spleen, testes, ovary, colon (Pizzagalli et al., 2001), and the ciliary body (Gao et al. 2005)).

At physiological pH, it transports only a narrow range of substrates, including BSP, oestrone-3-sulfate, and dihydroepiandrosteron (DHEAS).

At acidic pH, however, also taurocholate, bilirubin conjugates, fexofenadine, statins, glibenclamide, and the loop diuretic M17055 are substrates (Hagenbuch and Gui, 2008).

OATP3A1 (OATP-D) contains orthologues in rat and mouse (Hagenbuch and Meier 2004), showing 97% amino acid sequence identity. Therefore, OATP3A1 is the most conserved protein within the OATP superfamily. Highest expression levels were reported from testes, brain, heart, lung, spleen, peripheral blood leukocytes, and thyroid gland (Adachi et al. 2003; Huber et al. 2007). It transports prostaglandins, thyroid hormones, the cyclic peptide BQ-123, and vasopressin. OATP3A1 might play an important role in transport of neuron-active peptides or thyroid hormones. However, additional studies are required to proof this hypothesis.

OATP4A1 (OATP-E), with members in rat and mouse, is expressed in various tissues, reaching highest levels in heart, placenta, lung, liver, skeletal muscle, kidney, and pancreas. Additionally it has also been detected in brain. It mediates the transport of a narrow range of substrates including oestrone-3-sulfate, oestradiol-17β-glucuronide, benzyl penicillin, PGE2, thyroid hormones (Fujiwara et al. 2001; Tamai et al. 2000), and might be important for the transport of thyroid hormones to the foetus, at least in the placenta. (Hagenbuch and Meier, 2004)

OATP4C1 (OATP-H), contains orthologues in rat and mouse with 80% amino acid sequence identity in rat, and is kidney specific. It transports a narrow range of compounds, including digoxin, oubain, thyroxine, methotrexate, cAMP, and the dipeptidyl peptidase-4 inhibitor sitagliptin. As it also transports thyroid hormones, it might be important for their delivery to the kidney (Hagenbuch and Meier, 2004).

OATP5A1 (OATP-J) represents the only member of the OATP5A subfamily. It consists of 848 amino acids. There is no further information available yet concerning its transport properties (Hagenbuch and Meier, 2004).

OATP6A1 (OATP-I) shows highest expression levels in normal testes, and additional weak signals in spleen, brain, foetal brain, and placenta. It has been found in several lung tumours, lung cancer cell lines, and bladder and oesophageal tumours. Orthologues have been discovered in rat (Oatp6b1 and Oatp6c1). They transport taurocholate, dehydroepiandrosteron sulphate (DHEAS), T3 and T4. However, additional research is required to proof relatedness of the rat homologue to the human OATP6A1 (Hagenbuch and Meier, 2004).

Table 2: Distribution of OATPs in Cancer Tissue and Cancer Cell Lines, respectively.

Distribution of OATPs in cancer tissue/cancer cell lines				
OATP	**cancer tissue**	**reference**	**cancer cell line**	**reference**
1A2	breast carcinoma	Al Sarakbi et al. 2006	T-47D, MCF-7, ZR-75-1, MDA-MB-231, MDA-MB-468	Miki et al. 2006
		Miki et al. 2006	MCF-10A, MCF-7, MDA-MB-231, ZR-75-1	Wlcek et al. 2008
			MCF-7	Nozawa et al. 2005b
	endothelial cells of blood-tumor barrier	Bronger et al. 2005		
1B1	hepatocellular carcinoma	Cui et al. 2003	MDA-MB-231, ZR-75-1	Wlcek et al. 2008
1B3	gastric, pancreatic, colon neoplasm	Abe T et al. 2001	MDA-MB-231	Wlcek et al. 2008
	gastrointestinal cancers	Abe et al. 2001; Cui et al. 2003	several cancer cell lines	Abe et al. 2001; Cui et al. 2003
	breast carcinoma	Muto et al. 2007		
	lung cancer	Monks et al. 2007		
2B1	epithelia of invasive ductal carcinoma of mammary tissue	Pizzagalli et al. 2003		
	endothelial cells of blood-brain barrier and blood-tumor barrier	Bronger et al. 2005		
	hepatocellular carcinoma	Cui et al. 2003		
	breast carcinoma	Al Sarakbi et al. 2006		
3A1			T-47D, MCF-7	Al Sarakbi et al. 2006
			MCF-10A, MCF-7, MDA-MB-231, ZR-75-1	Wlcek et al. 2008
			T-47D	Nozawa et al. 2004c
			MCF-7	Nozawa et al. 2005b; Pizzagalli et al. 2003
4A1	colon cancer	Ancona et al. 2006	MCF-10A, MCF-7, MDA-MB-231, ZR-75-1	Wlcek et al. 2008
			T-47D	Nozawa et al. 2004c
			MCF-7	Nozawa et al. 2005b; Pizzagalli et al. 2003
4C1	breast cancer	Wlcek et al. 2008	MCF-7, MDA-MB-231, ZR-75-1	Wlcek et al. 2008
5A1	breast cancer	Wlcek et al. 2008	MCF-10A, MCF-7, ZR-75-1	Wlcek et al. 2008
6A1	lung tumor, lung cancer, bladder and oesophageal tumor	Lee et al. 2004		

3.1.5 Aspects for OATPs in Drug Response

OATPs mediate the transport of a great variety of mostly amphipathic organic compounds, including physiological substances as bile salts, hormones and their conjugates, but also xenobiotics (i.e. drugs).

As uptake of drugs into cells is crucial for their therapeutic efficacy, altered function, OATPs may account for drug-drug interactions (inhibition/induction) or drug-food-interaction. This needs to be closely investigated (Hagenbuch and Gui, 2008; Shitara et al. 2005; Smith et al. 2005b; Poirier et al. 2007).

Altered function of the transporters may occur via genetic polymorphisms, embracing single nucleotide polymorphisms (SNPs) or haplotypes of the polypeptides. The resulting functional change in clearance organs as liver or kidney affects not only the drug's concentration in blood and therefore its bioavailability, but also its toxicity. Decreased capacity of the transporters leads to increased plasma and tissue concentrations. The same effect can be observed due to increased uptake activity and/or decreased efflux (see figure 3). As an example, up to now more than 40 mutations have been identified in SLCO1B1. On of these mutations results in decreased uptake of the HMG-CoA reductase inhibitor pravastatine into hepatocytes, leading to reduced inhibition of cholesterol synthesis and therefore degraded cholesterol-lowering effects. (Kazuya et al., 2008)

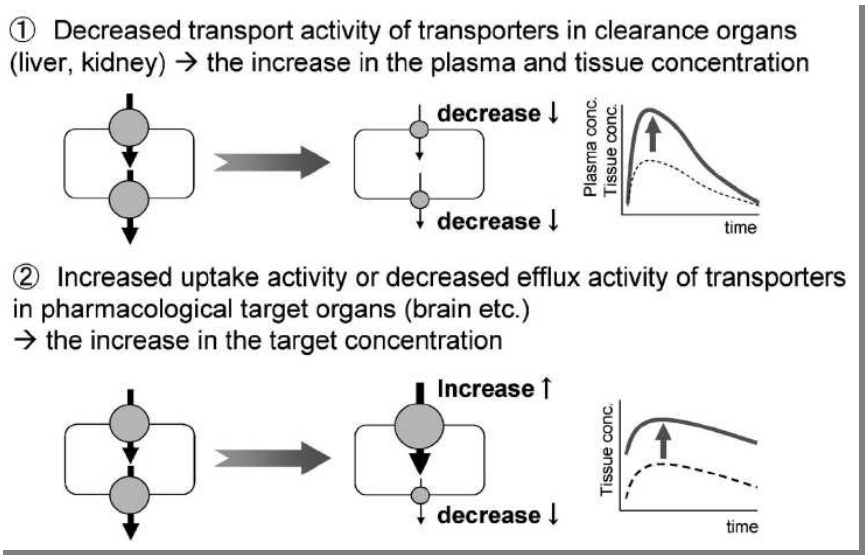

Figure 3: The effect of a functional change in transporting activity on pharmacological and toxicological actions of drugs. (Kazuya et al., 2008).

3.1.6 Drug-drug interactions

As OATPs/Oatps play an important role in the hepatic first pass clearance of numerous drugs, co-administration of specific OATP modulators may rise or lower oral bioavailability of highly metabolized compounds.

Organic anion transporting polypeptides show broad substrate specificity with a certain degree of overlapping. By examining the K_i values of inhibitors of both OATPs and OAT (organic anion transporter) it could be demonstrated that cephalosporins, probenecid, cyclosporine A and rifampicin have the potential to lead to clinically significant drug-drug interactions (Shitara et al., 2005).

Poirier et al. (2007) stated that inhibition of OATPs may result in altered pharmacokinetics, possibly interfering with drug safety and efficacy. However, the influence of the CYP enzyme family has to be considered as another factor of important influence on drug-drug interactions in the course of drug metabolism.

Inhibition of some OATP/Oatp gene expression by rifampicin occurs via PXR-receptors (i.e. pregnan X receptors). Therefore rifamycin may cause higher plasma levels, which on the one hand is desirable – concerning drugs being highly metabolized via OATP/Oatp mediated transport –, or on the other hand might lead to (more) adverse drug effects, decreased efficacy and/or therapeutic failure due to lowered uptake of the compound into its target tissue. Otherwise, induction may even increase the already strong first pass effect of OATPs/Oatps and lead to insufficient plasma levels. (Hagenbuch and Meier, 2004; Fattinger et al. 2000)

3.1.7 Drug-food Interactions

Dresser et al. (2002) reported for the first time that fruit juices are able to inhibit OATP1A2. Subsequently, studies have been performed on the effect of citrus juice and its components as well as of the influence of diverse flavonoids and herbal extracts on OATP1A2, OATP1B1, OATP1B3 and OATP2B1 (Hagenbuch and Gui, 2008; Ismair et al. 2003; Satoh et al. 2005; Wang et al. 2005b; Fuchikami et al. 2006; Lu et al. 2006a; Bailey et al. 2007; Glaeser et al. 2007).

Most flavonoids indeed inhibited the OATPs stated above. For instance, naringin inhibited the transport of fexofenadine via OATP2A1 (Bailey et al. 2007). Other

compounds, such as hesperidin from grapefruit juice (Bailey et al. 2007), biochanin A, genistein, and epigallocatechin-3-gallate (Wang et al. 2005), glycyrrhizin (Ismair et al. 2003), green tea and ginkgo extract (Fuchikami et al. 2006) showed an additional inhibitory potential on OATP1B1, OATP1B3 and OATP2B1 mediated transport.
Hence, these results clearly suggest that transport via OATPs can be affected by the intake of fruit juices and this may lead to unexpected side effects and changes in bioavailability (Hagenbuch and Gui, 2008).

3.1.8 OATPs and Cancer

The organic anion transporting polypeptides family is broadly expressed in human tissue, in most members ubiquitously. They play a fundamental role in uptake of various substrates into cells, as well as their excretion and, therefore, in the course of body detoxification.
Many studies have been performed yet to investigate the OATPs' distribution not only in normal tissue, but also in cancer tissue and cancer cell lines.
The majority of OATPs was found to be expressed in at least one malign tissue or cancer cell line. Their frequent expression of OATPs might play a key role in tumour genesis, possibly by rendering cells sensitive to hormonal growth stimuli.
Further information about the individual OATP expressed in a certain tumor might bring useful information and improve the specific targeting of cytotoxic drugs (Hagenbuch and Gui, 2008).

4 METHODS AND MATERIALS

4.1 Colorectal Tumor Tissue Samples

Samples of stages of colorectal cancer are obtained from patients suffering from different stages of colorectal carcinoma, and are kindly provided by Prof. Dr. Enikö Kállay (Department of Pathophysiology, AKH Wien). Research investigation is approved by the hospital's Ethics Committee.

4.2 Cell Culture – Maintenance of Cells

Materials

- Roswell Park Memorail Institute (RPMI) 1640 Medium (PAN™ Biotech GmbH, Aidenbach, GER)
- Fetal bovine serum (FBS) (PAN™ Biotech GmbH)
- Penicillin (10.000 U/ml) – Streptomycin (10.000 U/ml) Solution (P/S) (Gibco/Invitrogen, Carlsbad, CA)
- Trypsin/EDTA Solution (10x) (Gibco, Carlsbad, CA)
- 10x PBS (phosphate buffered saline) pH 7.4

Potassium chloride (Merck, Darmstadt, GER)	2g
Sodium chloride (Merck)	80g
Potassium dihydrogen phosphate (Merck)	2g
Disodium hydrogen phosphate (Merck)	14.4g
ddH2O	ad 1 l

- Dimethyl sulfoxide (DMSO) (Sigma, St. Louis, MO)
- Cell culture flasks 25 cm^2 and 75 cm^2 with vented cap (Iwaki scitech div., Asahi Technoglass, Ltd., Tokyo, JP)
- Sterile pipettes 10 ml (Bibby Sterilin Ltd., Stone, Staffordshire, UK)
- Sterile centrifuge tubes 15 ml and 50 ml (TPP Techno Plastic Products AG, Trasadingen, CH)
- Sterile Pasteur pipettes (VWR International, Darmstadt, GER)
- Cryovials /Bibby Sterilin Ltd., Stone, Staffordshire, UK)

- Incubator Cytoperm Heraeus (Kendro Laboratories GmbH, Vienna, AT)
- Centrifuge Hettich Rotixa/RP (Hettich AG, Bäch, CH)
- Laminar air flow (Heraeus, Hanau, GER)
- Mikroscope Olympus CK2
- Cell strainers, 40 µm (Falcon™, Becton Dickinson Biosciences, San Jose, CA)
- Eppendorf pipettes (Eppendorf AG, Hamburg, GER)

4.2.1 General

All cells are from the C205 cell line, i.e. human colorectal adenocarcinoma cells derived from a 70 years old person. Cell culture work is performed exclusively in the cell culture room, where access is only allowed to people wearing a lab coat. Every action performed on the cells needs to be done in a sterile cabinet, the laminar air flow (Heraeus, Hanau, GER).
The cells were cultivated in either 25 cm² or 75 cm² flasks. They were incubated at standardized conditions (37℃, 5 % CO_2, 95 % relative humidity; incubator: Heraeus cytoperm 2). To avoid contamination of the cells with microorganisms, sterile conditions are applied and hands are disinfected with ethanol (70 %) before work.

4.2.2 Thawing of Cells

For long term storage cells are put into liquid nitrogen (– 196℃). When needed, cells should be thawed at 37℃ at approximately the same velocity as they have been frozen. The thawed cell suspension is centrifuged at 1000 rpm for 3 minutes at room temperature and the supernatant is discarded. The cell pellet is resolved in 4 ml medium (RPMI-1640, containing 10 % FBS and 1 % penicillin/streptomycin (P/S)) by pipetting the suspension up and down several times. It is then transferred into a sterile culture flask (either 25 cm² or 75 cm²) and incubated at 37℃.

4.2.3 Maintaining Cells in Culture – Washing and Feeding

To culture cells, RPMI 1640 (PAN™ Biotech GmbH) supplemented with 10 % FBS (fetal bovine serum) and 1 % P/S is used. The medium is exchanged approximately every third day. For this purpose, the cells need to be washed with PBS first. Both, PBS and the RPMI medium, shall be brought to room temperature before being put onto the cells to avoid stress for the cells. Cells are washed routinely once with the same amount of PBS as medium used. PBS is aspirated and fresh medium was added to the cells. When adding either PBS or medium, it is important to avoid putting the reagents directly onto the cells to avoid detaching them from the flask.
Cells shall not be kept outside the incubator for a long time. To guarantee sufficient CO_2 exchange in the incubator, the flasks are either closed with gas permeable screwtops, or non-permeable screwtops are left partially open.
When the cells reach confluence (80–90%), they have to be split in order to avoid overgrowing and decease of cell growth.

4.2.4 Splitting of Cells

Before splitting cells, confluence must be checked by light microscopy. Cells are washed twice with PBS to remove dead cells and cell parts. To detach the cells from the flask, 1 ml (25 cm²) trypsin is added and the flask is incubated at 37℃ for approximately 3 minutes. Afterwards the flask is rocked gently several times and detaching of the cells is watched under light microscope.
After adding new medium (4 ml for a 25 cm² flask, 12 ml for a 75 cm² flask), two drops of cell suspension are put into a flask of small size. For a flask of medium size approximately 6 drops have to be added. The flasks then are incubated at 37℃ again.

4.2.5 Freezing of Cells

For long term deposit cells are frozen when confluence is up to 80%.

The medium is aspirated and the cells are washed with PBS twice, which is aspirated again. Trypsin is added (1 ml for a small flask), followed by 3 minutes of incubation at 37℃. The flask is agitated and detaching of cells is checked under light microscope. Thereafter, 4 ml medium are added to the trypsinized cells (serum inactivates trypsin), the cells are re-suspended by pipetting them up and down several times, and then transferred into a 15 ml centrifugal tube. The suspension is centrifuged at 200 x g for 5 minutes at 4℃. The supernatant is discarded and the tube containing the pellet is placed on ice.
To freeze cells, a special freezing medium was prepared (table 4).

Table 3: Preparation of Freezing Medium for C205 cells

Freezing Medium C205	
800 µl	medium (RPMI-1640)
100 µl	FBS
100 µl	DMSO

The freezing medium needs to be pre-cooled to 4℃. Cells are suspended in the medium by pipetting them up and down several times. Then cells are quickly transferred into cryotubes (1ml/tube) and kept on ice. It is essential to work quickly, because DMSO (i.e. dimethyl sulofoxide, used as a cryoprotectant) in the freezing medium seriously harms the cells. The cryotubes are transferred on –80℃, and for longtime storage the next day into liquid nitrogen (–196℃).

4.3 Mycoplasma Testing

Materials

Agarose Gel Electrophoresis

- Agarose UltraPure™ (Invitrogen, Carlsbad, CA)
- 10x Tris-acetate EDTA (TAE) buffer pH 7.2

TRIS (Merck, Darmstadt, GER)	96.8g
Sodium acetate (Merck)	54.5g

EDTA (Merck)	7.6g
ddH2O	ad 2 l

- Ethidium bromide (5 mg/ml) (Sigma-Aldrich Chemie GmbH, Steinheim, GER)
- Gene Ruler™ 100bp DANN Ladder (MBI Fermentas, Vilnius, LT)
- 6x Loading dye solution (MBI Fermentas)
- Transluminator and camera Herolab E.A.S.Y. 429K (Herolab, Wiesloch, GER)
- Gel electrophoresis unit HE 133 (Hoefer, San Francisco, USA)
- Power supply Power Pac 3000 (Biorad Laboratories, Hercules, CA)
- Microwave oven MIK 4600 (Elin, AT)

Before cells are frozen for longtime storage they needed to be tested for mycoplasma contamination.

Mycoplasmas do not have a cell wall (Ryan et al., 2004) and therefore remain unaffected by many commonly used antibiotics, e.g. penicillin and other beta-lactam antibiotics which target cell wall synthesis.

Contamination occurs frequently in cell culture due to contaminated media or individuals that work with the cells. Mycoplasmas induce cell alterations, including chromosome aberrations and changes in metabolism and cell growth. Severe contamination can destroy a whole cell line or, if remaining undetected, adulterate research results.

Mycoplasm are the smallest bacteria (i.e. < 1 µm), and therefore are difficult to detect by microscopy. Methods used to test cells for contamination are with mycoplasma PCR detecting their DNA.

In this thesis, the mycoplasma testing is performed using the Myco Sensor® PCR Assay Kit. To save resources several cell lines in culture are tested for mycoplasma contamination.

The supernatant taken from the cell culture is boiled at 95℃ for 5 minutes in a heating block (Eppendorf Thermomixer comfort). The reaction is done accourding to the Myco Sensor® PCR Assay Kit. To save reagents samples of 50 µl, requiring only 5 µl of StrataClean, are used. The reagent is added to the sample, vortexed and centrifuged. 30 µl of the supernatant are transferred into an Eppendorf tube and either stored for further use at –20℃, or directly processed.

According to the instructions given in the manual of Myco Sensor® Assay Kit, the master mix was prepared as follows:

Table 4: Preparation of Master Mix for Mycoplasma PCR and halved amounts.

Master Mix for a single PCR reaction			**MM halved amounts**	
PCR water	fill to 50	µl	fill to 25	µl
$MgCl_2$ (2.0 nM)	depends on conc.	µl	depends on conc.*	µl
10x Taq buffer	5.0	µl	2.5	µl
dNTP mix	1.0	µl	0.5	µl
Mycoplasma primer mix	2.0	µl	1	µl
internal control template	4.0	µl	2	µl
Taq Polymerase DNA (5 U/µl)	0.5	µl	0.25	µl
sample (or positive control)	5.0	µl	2.5	µl
total volume of reaction	50.0	µl	25.0	µl

The $MgCl_2$ solution available in the laboratory featured a concentration of 1 nM/ µl. To accomplish, for the required concentration of 2 nM 2 µl of Master Mix are added*.

According to the instruction manual, 0.5 µl of Taq DNA polymerase with a concentration of 5 U/µl are used for one reaction, resulting in 2.5 U/0.5 µl. As half the amount of reaction volume is used, and 0.63 µl of Taq polymerase is added for one PCR reaction (see table 5).

Table 5: Preparation of Master Mix for Mycoplasma PCR II – single reaction and amounts for 8 reactions

Master Mix for a single PCR reaction			**MM for 8 reactions**	
PCR water	13.9	µl	111.2	µl
$MgCl_2$ (2.0 nM)	2.0	µl	16.0	µl
10x Taq buffer	2.5	µl	20.0	µl
dNTP mix	0.5	µl	4.0	µl
Mycoplasma primer mix	1.0	µl	8.0	µl
internal control template	2.0	µl	16.0	µl
Taq Polymerase DNA (5 U/µl)	0.63	µl	5.04	µl
sample (or positive control)	2.5	µl	/	µl
total volume of reaction	25.0	µl	200.0	µl

The reagents are pipetted into reaction tubes (eightstrip tubes). Tubes are sealed thoroughly and placed into the PCR cycler (Biometra Personal Cycler).

According to the manual, parameters for PCR are set as shown in table 8. For detection of the PCR products, an agarose gel is used.

Table 6: PCR cycling parameters

PCR Cycling Parameters (1 cycle)	
temperature	**time**
94℃	30 sec
55℃	1 min
72℃	1 min

Table 7: Preparation of Agarose Gel

Agarose Gel (~1.5 %)	
agarose	0.450 g
buffer	360 ml
ethidium bromide	1.5 µl

The results of the mycoplasma testing are all negative. Hence, none of our cell lines is contaminated.

4.4 Spheroid Inducing Cell Culture

To induce the formation of spheroids, the C205 cells need to be transferred from normal RPMI 1640 medium to serum free medium containing special growth factors, but lacking antibiotics and FBS. For this purpose, the growth factors EGF (epidermal growth factor) and FGF-2 (fibroblast growth factor) are applied. The C205 culture then is called “SPH” (for spheroid).
Growth factors are stored as lyophilisates, from which stock solutions are prepared. Stock solutions are stored at –20℃ in the cell culture room.
To prepare EGF working solution, the stock solution (c = 2 µg/µl) is diluted 1:200. To 2 µl stock solution 398 µl sterile double distilled water are added. This results in 400 µl working solution with a concentration of 10 ng/µl.
FGF-2 stock solution (25 ng/µl) is diluted 1:2.5. To 20 µl stock solution 5 µl of sterile double distilled water are added. This results in 25 µl working solution featuring a concentration of 20 ng/µl.

As C205 cells stop growth at all when being put on SPH medium, no splitting step is done the medium is aspirated before cells are washed twice with PBS. Then, serum free RPMI is added (13 ml/flask) and the growth factors (13 µl EGF and 13 µl FGF-2) are dissolved into it. The appropriate amount of growth factor working solution has to be refreshed every third day, but the medium can be kept.

4.5 Protein Purification

Materials

- Protease inhibitor cocktail P8340 (Sigma, St. Louis, MO)
- PBS (Merck, Darmstadt, GER)
- 1.5 ml reaction tubes (Biozym Biotech Trading GmbH, Vienna, AT)
- Plastic vials Polyvials® (Zinsser Analytic GmbH, Frankfurt, GER)
- Eppendorf centrifuge 5415D (Eppendorf AG, Hamburg, GER)
- Lysis buffer (see below)

To measure the expression of CD133, cells are cultured on serum free medium enriched with growth factors (EGF working solution, 10 ng/µl; FGF working solution, 20 ng/µl) on day 1 and 3. Two 75 cm² flasks are cultured.
Protein extraction is done on day 4 and 5. On day 4, total protein is extracted. On day 5, both, cytosolic and membrane protein is extracted.
All steps of protein purification are performed outside the cell culture room in the laboratory, however, working on ice.

4.5.1 Protein Purification 1 – Total Protein

Cells are washed twice with 10 ml of pre-cooled PBS (4℃). It is important to aspirate PBS quantitatively. For protein isolation, 1 ml of PBS is added and the cells are detached from the flask using a cell scraper. Thereafter, the suspension is transferred into a sterile 1.5 ml tube and centrifuged at 200 x g for 5 minutes at 4℃. The supernatant is discarded, and the pellet is resolved in 500–600 µl of lysis buffer (table 10).

Table 8: Preparation of Lysis Buffer

Lysis Buffer	
PBS	5000 µl
Triton X-100	0.1%
protease inhibitor mix	0.1%

To extract membrane proteins, the suspension is agitated on ice for 30 minutes and then centrifuged at 14.000 x g for 10 minutes at 4°C. The supernatant is transferred into a sterile 1.5 ml tube and kept frozen at –80℃ . The pellet is dissolved in 500 µl of lysis buffer and stored at –80℃ for possible furth er use, although.

4.5.2 Protein Purification 2 – Cytosolic and Membrane Protein

Cells are washed twice with pre-cooled PBS (4℃), a nd then lysis buffer (pH = 7.4) is added to the cells.

First, stock solutions of Na_2HPO_4 and NaH_2PO_4 are prepared. 10 mM is the target concentration, wherefore both stock solutions are diluted to this concentration. The pH is adjusted to pH 7.4. All solutions are stored at +4℃. The lower pH at +4℃ has to be adjusted again. Therefore 0.3 M saccharose is added to 50 ml of Na_2HPO_4/ NaH_2PO_4 buffer. Just before applying the lysis buffer to the cells, 2 µl/ml protease inhibitor mix is added.

Table 9: Calculations for Lysis Buffer Preparation

Calculations for Lysis Buffer Preparation		
Na_2HPO_4 (*$2H_2O$)		
MW	177.99 g/l	1 M
ws	0.88995 g/500 ml	10 mM
NaH_2PO_4 (* H_2O)		
MW	137.99 g/l	1 M
ws	0.68995 g/500 ml	10 mM

saccharose		
MW	342.4 g/l	1 M
ws	5.1345 g/50 ml	0.3 M

MW	*molecular weight*
ws	*weighted sample*

Cells are detached from the flask using a cell scraper. The suspension is transferred into a sterile tube and put on ice. Homogenisation is done by repeated freezing and thawing, using liquid nitrogen and a heating block (Eppendorf Thermomixer comfort). The homogenate is centrifuged at 3.000 x g for 10 minutes at 4℃. The supernatant then is transferred into a Beckman Microfuge tube and centrifuged at 48.000 x g (equals 28.000 rpm for Beckman Optima TLX centrifuge) for 60 minutes at 4℃. The supernatant, containing the cytosolic proteins, is transferred into a fresh tube again. The pellet containing the membrane proteins is resuspended in 50 µl of lysis buffer and stored at –80℃ for further use.

4.6 Bradford Protein Assay

In the Bradford test the specific colorant Coomassie Brilliant Blue G bind to proteins via hydrophobic and electrostatic interactions.

The detection limit of the Bradford Protein Assay is 1 µg protein/ml under optimized conditions (Spector, 1978; Read and Northcote, 1981). The sample should be free of detergents, e.g. SDS, triton or nonidet P-40. Detergents in a concentration higher than 0.1 % inhibit the specific binding of the staining reagent to the proteins, and therefore need to be removed before analyzing the sample.

The staining reagent, Coomassie Brillant Blue G, appears mostly in protonated, cationic form in acidic solutions, and shows its absorption maximum at 470 nm (red). It balances with its anionic form. In contrast, by binding to the protein the anionic form of the staining reagents is stabilized, showing a new absorption maximum at 595 nm (blue) (Compton and Jones, 1985).

As the extinction coefficient of the colorant-protein-complex exceeds the coefficient of the unbound colorant, the increase of absorption at 595 nm can be measured easily and with high sensitivity against the unbound staining reagent by photometry. Variations in the rate of yield concerning the formation of the colorant-protein-complex are due to variability of the proteins. (Eckert and Kartenbeck, 1997)

Samples and protein standard (i.e. IgG) are thawed on ice. For the standard curve,

5 µl, 10 µl, 15 µl and 20 µl of protein standard are pipetted into Eppendorff tubes and mixed with 1 ml of Bradford reagent, adding one tube containing only the reagent as starting point of the standard curve (blank). Every probe is analyzed twice.

If the values fit into the standard curve, the volume of sample can be used for double specification. If the value exceeds the upper limit of the standard curve, fewer sample needs to be used, or the sample has to be diluted.

Before measurement, the device settings need to be checked. The sipper has to be unlocked and the drip has to be opened. Sipping time is set to two seconds. At the end of the procedure, the sipper is washed once with distilled water, and once with pure Bradford reagent; afterwards the absorption is set to zero manually, and the measurement can be started.

Table 10: Procedure for the Bradford Assay on Protein from Tissue Samples.

Bradford Protein Assay - Results

sample ID	spec. 1	spec. 2		note		calculation - average concentration of protein (µg/µl)
1st measurement, using 5 µl of sample						
1	38,18	37,25	⇨	use 1 µl		
2	23,37	24,97	⇨	use 2.5 µl		
3	18,40	19,60	⇨	use 2.5 µl		
4	27,71	28,27	⇨	use 2.0 µl		
2nd measurement, using altered volumes of sample						
1	31,48	20,74	⇨	dilute 1:2		
2	19,11	22,76	⇨	use 1 µl		
3	14,88	12,13	⇨	o.k.	⇨	[(14.88 + 12.13)/2]2.5 = **4.852 µg/µl**
4	15,84	15,76	⇨	o.k.	⇨	[(15.84 + 15.76)/2]/2 = **7.88 µg/µl**
3rd measurement, using altered volumes of samples 1 and 2						
1	21,29	23,07	⇨	dilute 1:10		
2	8,51	9,86	⇨	o.k.	⇨	(8.51 + 9.86)/2 = **9.185 µg/µl**
4th measurement, sample 1 and tissue samples						
1	14,19	11,22	⇨	o.k.	⇨	[(14.19 + 11.22)/2]*10 = **141.9 µg/µl**
a (1 µl)	14,8	16,91	⇨	o.k.	⇨	(14.80 + 16.91)/2 = **15.885 µg/µl**
a (5 µl)	38,81		⇨	x		
b (1 µl)	14,65	16,92	⇨	o.k.	⇨	(14.65 + 16.92)/2 = **15.785 µg/µl**
b (5 µl)	36,39		⇨	x		

legend

sample ID	sample	
1	11.5.09 supernatant C205	
2	11.5.09 dissolved pellet C205	
3	12.5.09 cytosolic proteins C205	
4	12.5.09 membrane proteins C205	
5	669 T	} tissue samples (colorectal carc.)
6	669 SH	}

Due to complications referring to the storage of IgG antibody, no Western Blot has been performed. The results obtained by real time PCR will be confirmed by Western Blot in further investigations. However, these are not part of this diploma thesis.

4.7 Polymerase chain reaction (PCR)

Materials

- RNase free water
- Sense and antisense primer, 100 pmol/µl (MWG Biotech, Ebersberg, GER)
- Taq Man gene expression assays (AB Applied Biosystems, Foster City, USA)
- TaqMan® gene expression master mix (Applied Biosystems)
- ABI PRISM™ optical 96-well plates and caps (Applied Biosystems)
- ABI PRISM™ 7700 sequence detector (Applied Biosystems)
- Safe seal tips (Biozym Biotech Trading GmbH, Vienna, AT)
- Sigma laboratory centrifuge 4K15C (Linder Labortechnik, Vienna, AT)

4.7.1 General Information

Polymerase chain reaction (PCR) was invented by Kary Mullis in 1983 (Bartlett and Stirling, 2003), for which the Nobel Prize was awarded to Mullis. It is a technique central to molecular biology research currently used in a variety of applications ranging from cloning, gene expression analysis, genotyping, sequencing, resequencing, and mutagenesis. In addition, it is applied in diagnostics for infectious diseases and in forensic investigations.

The instrument to perform real-time PCR and all chemistries used are products of Applied Biosystems Inc.

Real-time PCR (RT PCR) gives the researcher the ability to monitor the progress of PCR as it occurs (i.e., in real time). Data is collected throughout the process, revolutionizing the PCR-based quantitation of DNA and RNA. Real-time PCR characterizes reactions by the point when amplification of a target is first detected rather than the amount of target accumulation throughout the progress. Hence, the higher the starting concentration of copy numbers of the cDNA, the sooner a

significant increase in fluorescence level can be observed. This is contrary to the so called “endpoint assay” or “plate read assay”, where the amount of accumulated PCR products at the end of all PCR cycles is measured. (Applied Biosystems Inc.)
An outstanding advantage of real-time PCR is its sensitiveness. Whereas agarose gels’ resolution is rather poor, about 10 fold, real-time PCR can detect two-fold changes.
Further advantages in comparison with traditional PCR technique are

- collection of data in the exponential amplification phase,
- direct proportionality of fluorescent signal to number of generated amplicons (i.e. cDNA copies),
- cleaved probe providing a permanent record amplicication of an amplicon,
- increase dynamic range of detection, and
- no post-PCR processing necessary.

(Applied Biosystems Inc.)

4.7.2 Phases of PCR

PCR can be broken up into 3 basic phases (figure 4): First, the exponential phase including the exact doubling of product and accumulation at each cycle (assuming a 100% efficiency of reaction). The exponential phase is very specific and precise. Second is the linear phase, which shows quite high variability. From one cycle to the next components are consumed, thus the reaction is slowing down and the products begin to degrade. The last phase is the plateau, where the reaction ends and products begin to degrade. (Applied Biosystems Inc.)

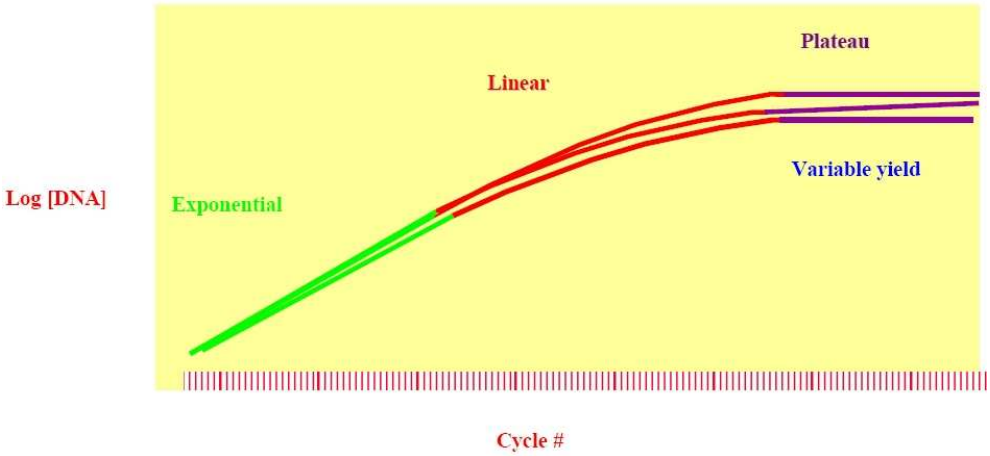

Figure 4: Phases of PCR amplification: exponential, linear, and plateau phase (Applied Biosystems Inc.)

During the exponential phase the product is doubled at every cycle. Throughout the process, components are consumed, namely at different rates for each replicate. The reaction slows down and the PCR product is no longer doubled at each cycle. This is when linear phase starts. Here the samples begin to diverge into their quantities. Each sample reaches the plateau at a different point due to different reaction kinetics.

Whereas traditional PCR takes its measurements at the plateau phase, for real-time PCR it is a lot more precise to measure during the exponential phase (Applied Biosystems Inc.).

4.7.3 Data Analysis

To simplify the analysis of the data, the linear graph is changed to log scale, where the exponential phase of the reaction can be detected more easily. To analyze the samples, the baseline needs to be set either automatically or manually, as in my case. The baseline allows excluding the noise signals occurring at the beginning of amplification. If the baseline is set correctly, background signals can not distort the results. The second step is to set the threshold, which can also be performed either automatically or manually. The threshold marks the number of cycles that are necessary to amplify the cDNA to the point where it is detectable by fluorescence. It is set in the middle of the linear domain of the log-scale, where the lines should be

parallel and each pair of lines (every sample is double-determined) should swap (Applied Biosystems Inc.).

(a) linear scale **(b)** log scale

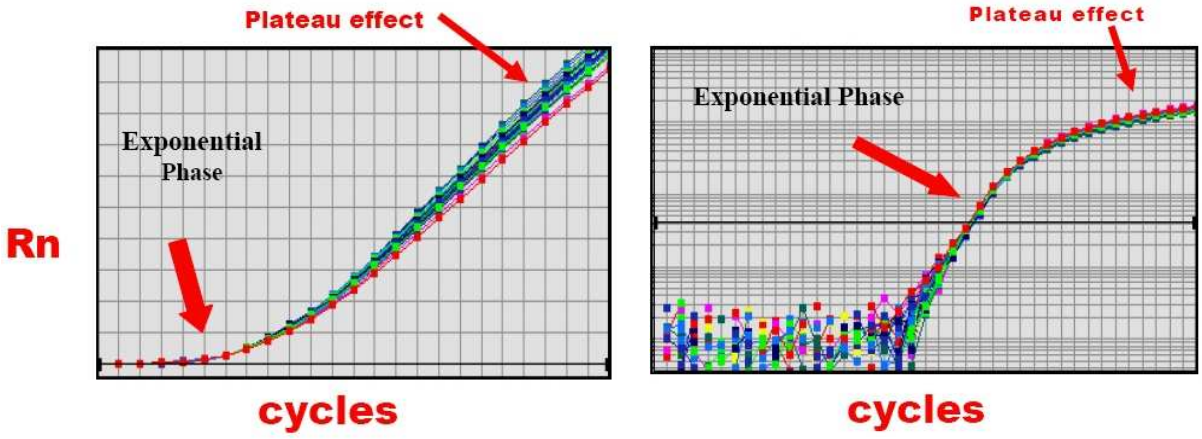

Figure 5a and 5b: Simplification of analysis by changing from linear to log scale. (modified after Applied Biosystems Inc.)

4.7.4 Calculation

To calculate the results of the quantitation assays, either absolute or relative quantitation can be used (Applied Biosystems User Bulletin #2: Relative Quantitation of Gene Expression (PN 4303859)).

Absolute quantitation is used to quantitate unknown samples by using a standard curve. In contrast, relative quantitation assays are used to analyze changes in gene expression in a given sample relative to another reference sample. Calculation can be performed by the standard curve method or by comparative CT method, which has been chosen in this case. By applying the CT method the need for a standard curve is eliminated. Hence, time and amount of work can be reduced.

The difference between the standard curve method and the comparative CT method is the use of arithmetic formulas to achieve the results (Applied Biosystems Inc.).

The amount of the target is normalized to an endogenous reference and set relative to a calibrator, and thus described as

$$2^{-\Delta\Delta C_T}$$

The equation describing the exponential amplification of PCR is:

$$X_n = X_o \times (1 + E_X)^n$$

X_n	number of target molecules at cycle n
X_o	initial number of target molecules
E_x	efficiency of target amplification
n	number of cycles

The threshold cycle is calculated as

$$X_T = X_o \times (1 + E_X)^{C_{T,X}} = K_X$$

X_T	threshold number of target molecules
$C_{T,X}$	threshold cycle for target amplification
K_X	constant

Calculation of the endogenous reference reaction by

$$R_T = R_o \times (1 + E_R)^{C_{T,R}} = K_R$$

R_T	threshold number of reference molecules
R_o	initial number of reference molecules
E_R	efficiency of reference amplification
$C_{T,R}$	threshold cycle for reference amplification
K_R	constant

To correlate the threshold number of target molecules (i.e., X_T) with the threshold number of reference molecules (i.e., R_T), these values have to be divided:

$$\frac{X_T}{R_T} = \frac{X_o \times (1 + E_X)^{C_{T,X}}}{R_o \times (1 + E_R)^{C_{T,R}}} = \frac{K_X}{K_R} = K$$

Their exact values depend on various factors, namely

- reporter dye used in the sample,
- sequence context effects on the fluorescence properties in the sample,
- efficiency of sample cleavage,
- purity of the sample, and
- setting of the fluorescence threshold.

Hence, K does not need to equal one.
To simplify the calculation, the efficiency of target and reference are assumed to be equal ($E_X = E_R = E$), leading to

$$\frac{X_o}{R_o} \times (1 + E)^{C_{T,X} - C_{T,R}} = K$$

or

$$X_N \times (1 + E)^{\Delta C_T} = K$$

X_N	X_o/R_o, the normalized amount of target
(delta)C_T	$C_{T,X} - C_{T,R}$, the difference in threshold cycles for target and reference

Via rearranging and division of X_N for any sample q by X_N for the calibrator (cb) the final equation is made out to

$$\frac{X_{N,\,q}}{X_{N,\,cb}} = \frac{K \times (1+E)^{-\Delta C_{T,\,q}}}{K \times (1+E)^{-\Delta C_{T,\,cb}}} = (1+E)^{-\Delta\Delta C_T}$$

(delta delta) C_T (delta) $C_{T,q}$ – (delta) $C_{T,cb}$

If the amplicon's size is < 150 bp, the efficiency is close to one. Hence, the amount of target is given by the simple equation $2^{-\text{deltadeltaC}_T}$ (Applied Biosystems User Bulletin #2: Relative Quantitation of Gene Expression (PN 4303859)).

4.7.5 Detection

To detect the PCR products, two chemical approaches can be used. TaqMan® method (i.e. fluorogenic 5' nuclease chemistry) enables the detection of by intercalating into the DNA double strands. Former intercalator deys detected both specific and nonspecific products. By improving TaqMan® chemistry fluorogenic-labeled probes were introduced, using the 5' nuclease activity of Taq DNA polymerase, detecting only specific amplicons and hence eliminating the need of post-PCR processing (Applied Biosystems Inc.).

First, an oligonucleotide is constructed. It contains a reporter fluorescent dye on the 5' end and a quencher on its 3' end. As long as the probe is intact, the proximity of quencher and reporter disables fluorescence by fluorescence resonance energy transfer (FRET) through space. When the probe encounters the target sequence it anneals to it downstream from on of the primer sites. As the primer is extended, the probe is cleaved by the 5' nuclease activity of Taq DNA polymerase. The cleavage of the probe separates the reporter from the quencher, resulting in increasing fluorescence signals. In addition, the probe is removed from the target strand. Hence, the primer can continue to extend the template strand to its end. With every cycle more reporter dye molecules are cleaved from their respective probes, resulting in increasing fluorescence signals.

The second method is SYBR Green I Dye Chemistry®. This dye immediately binds to all double-stranded DNA in the sample. The further the amplification progress advances and the more amplicons are produced, the more dye can bind. This results

in increasing fluorescence, direct proportional to the amount of amplicons. As the SYBR Green I Dye® binds to all double-stranded DNA in the sample it might give false signals due to impurities of the samples (Applied Biosystems Inc.).

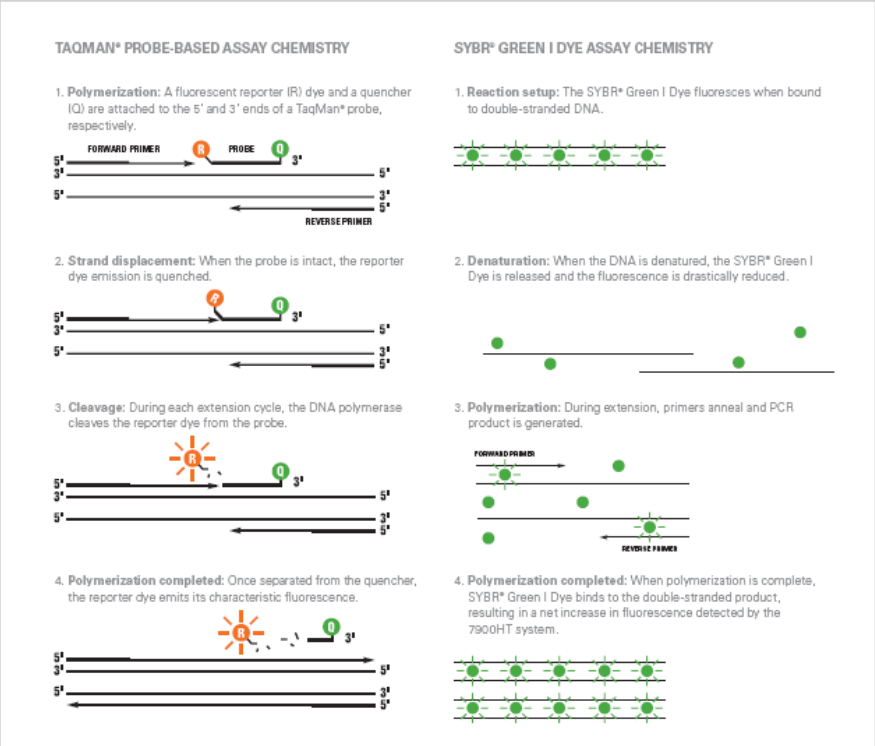

Figure 6: How TaqMan® and SYBR® Green I Dye Assay work. (Applied Biosystems Inc.)

TaqMan® Chemistry is used for all eleven OATPs and for 3 out of 4 house keeping genes. The forth house keeping gene is analyzed using SYBR Green I Dye Chemistry® (Applied Biosystems Inc.).

4.7.6 Implementation

The cDNA, kindly allocated by the working group of Prof. Dr. Enikö Kállay features a concentration of 100 ng/µl. The target concentration for Real Time PCR is 5 ng/4 µl. Therefore, samples have to be diluted in two steps, i.e. once 1:1 with PCR water, reaching a concentration of 50 ng/µl, and secondly 1:40, resulting in a concentration of 1.25 ng/µl, which equals 5 ng/4 µl.

The master mix has to be prepared separately for every plate, naturally. As the assay varies depending on the target of interest, 15 different master mixes have to be prepared. It is essential to pipet the master mix just before usage, and to keep all its components on ice while working.

Table 11: Composition of Master Mix for RT-PCR

Master Mix RT-PCR				
1 reaction			**x 115**	
2x PCR Master Mix	5	µl	575	µl
20x Assay (Primer/Probe)	0.5	µl	57.5	µl
PCR water	0.5	µl	57.5	µl

The master mix is pipetted into a 1.5 ml Eppendorf tube first, followed by the appropriate amount of PCR water. Finally, the assay is taken out of the freezer and thawed, vortexed and dispersed into the solution. The whole master mix is vortexed carefully and then distributed to eight-strip tubes (8 x 84 µl), due to application of an eight-channel pipet to accelerate work. With this eight-channel pipet, 6 µl of respective master mix are pipetted into each well. The plate then is sealed with foil.
All plates are pipetted at once to minimize working effort and reduce the frequency of freezing and thawing, which can harm the cDNA. In total, 15 plates are prepared by pipetting 4 µl of sample in each well, and sealing the plate with foil. Afterwards the plates are stored at –20℃ for further usage.

For investigation of the expression rates, the accordant gene expression assays are added into the wells. The plate is sealed with foil again with especial attention paid to not touching the foil. Before the plate can be placed into the real time PCR device it has to be centrifuged at 2000 x g for 1'.

Table 12: Real Time PCR – Thermal Cycler Settings

HOLD	HOLD	**40 Cycles**	
		Step one: Denaturation	Step two: Annealing/Extension
50°2 min	95°10 min	95℃ 15 sec	60°1 min

The results are exported into a Microsoft Excel® data sheet and analyzed with the kind help of Mag. Martin Svoboda. The relative quantities are expressed as MNE, i.e. mean normalized expression values (Muller et al., 2002), and refer to the average value of the calibrators.

5 RESULTS AND DISCUSSION

The main objective of the present thesis is to investigate the expression rates of the eleven human SLCO genes on the mRNA level in samples from tumors and tumor cell lines, including human colorectal cancer samples, derived from patients hospitalized at the AKH Wien, human colon cell lines, human lung cell lines and human ovary cell lines.
Extraction of RNA and preparation of cDNA is done by Mag. Martin Svoboda and other colleagues of the Department of Pathophysiology on the AKH Wien. cDNA can directly be used to perform real time PCR. The results obtained are analyzed using Microsoft Excel®.

Some information about the cell lines I worked with are given in chapter 5.1 (see table 13).

5.1 Cell Lines – A Short Characterization

Apart from the twenty patient colorectal cancer samples, five colon carcinoma cell lines, eight lung carcinoma cell lines, and six ovarian carcinoma cell lines are used in the present investigations. All of them are established cell lines for laboratory use. Table 13 gives a brief overview of the cells' most worth knowing details.

Table 13: Short Characterization of Carcinoma Cell Lines, used in the present investigations.

NCI-H69	Human small cell lung carcinoma, derived from a 55 years old Caucasian (CLS – Cell Lines Service Inc.)
HBE	Human bronchial epithelial cells (CLS)
DMS-114	Human small cell lung carcinoma (CLS)
DMS-456	Human small cell lung carcinoma (CLS)

Table 13 ctd.: Short Characterization of Carcinoma Cell Lines, used in the present investigations.

GLC-16	Human small cell lung cancer cell line (Berendsen et al., 1988)
HEY cell lines	Human ovarian carcinoma cell lines, derived from human ovarian cancer xenograph (HX-62), with demonstrated different degrees of resistance against alkylating chemotherapy agents (Cellutions Biosystems Inc.)
HEY Alimta	Demonstrated resistance against pemetrexed
HEY Cis	Demonstrated resistance against cisplatin
HEY Doce	Demonstrated resistance against docetaxel
HEY Etop	Demonstrated resistance against etoposide
HEY Pax	Demonstrated resistance against paclitaxel
HEY WT	Wild type
Caco 2/15	Human colorectal adenocarcinoma, isolated from a primary colonic tumor, derived from a 72 years old male Caucasian (CLS)
Coga 1A	Human colon carcinoma cell line, derived from a pT3 stage moderately differentiated carcinoma (Brozek et al., 2008)
C205	Human colorectal adenocarcinoma (colon, metastatic), derived from a 70 years old male caucasian (CLS)
HT 29	Human adenocarcinoma (colorectal), derived from a 44 years old Caucasian (CLS)
L174T	Human adenocarcinoma (colon, colorectal), derived from a 58 years old female Caucasian (CLS)

5.2 Expression of OATPs in Human Colon Carcinoma Cell Lines

The results obtained by real time PCR were analyzed using Microsoft Excel. As the calibrator is arbitrarily set to the value of 1 the expression rates have to be understood as relative multiples. The abbreviation “MNE” stands for “mean normalized expression value”. The calibrator consists of a mixture of all 9 cell lines’ cDNAs to make the cells comparable among each other. MNE values are calculated according to the expression of the respective gene in the calibrator.

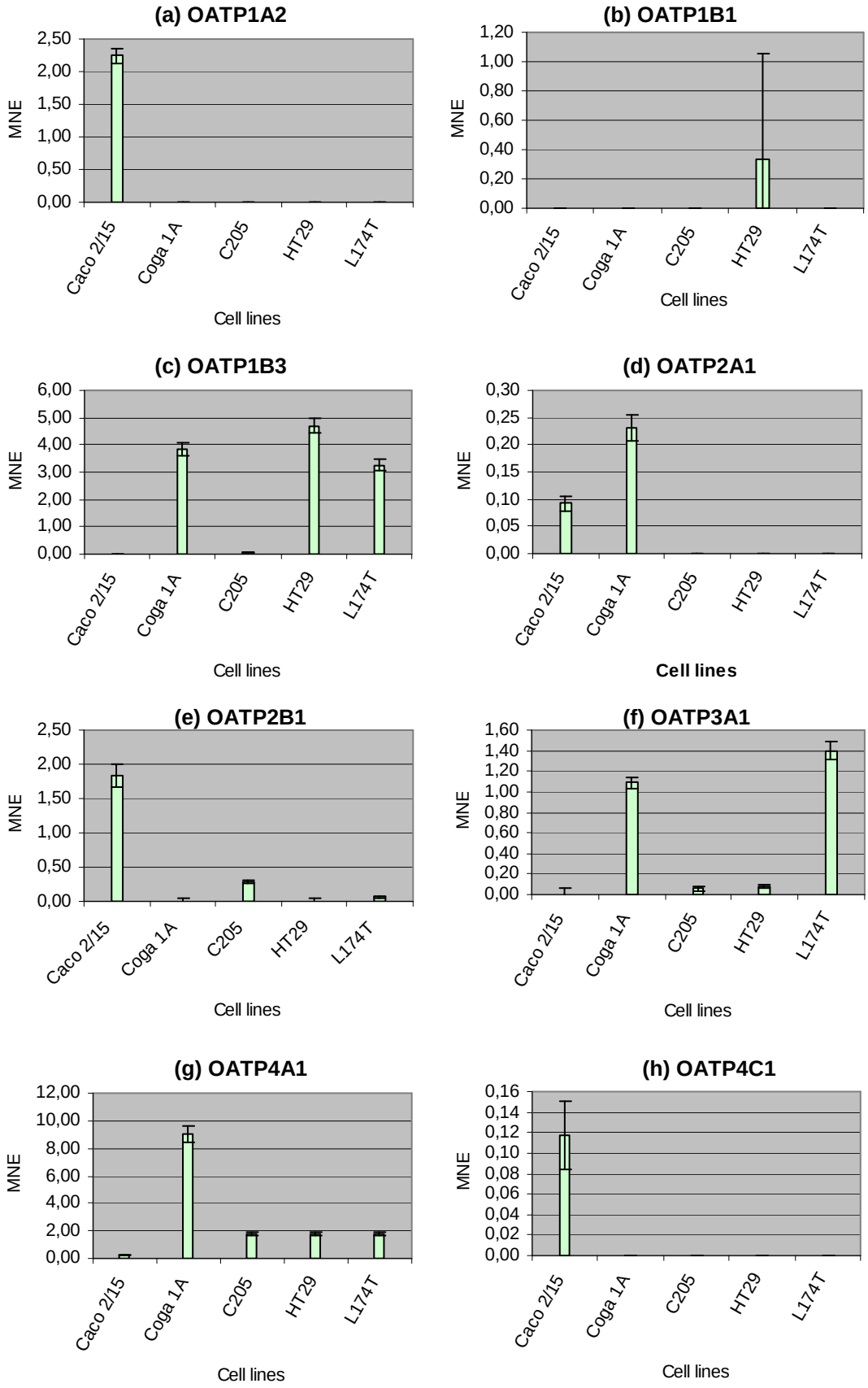

Figure 7 a–h: Expression of OATP1A2 (a) ,1B3 (b), 1B1 (c), 2A1 (d), 2B1 (e), 3A1 (f), 4A1 (g), and 4C1 (h) on mRNA level in Colon Cancer Cell Lines, shown in MNE values. Results were obtained by Real Time PCR.

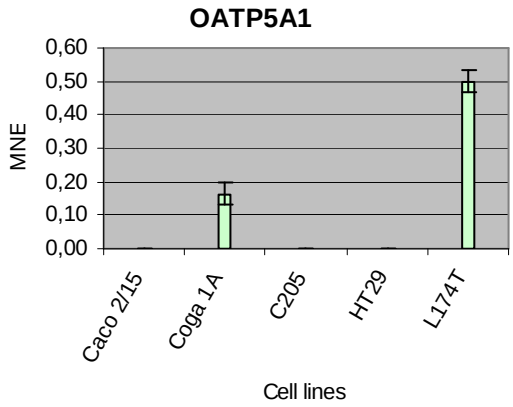

Figure 8: Expression of OATP5A1 on mRNA level in Colon Cancer Cell Lines, shown in MNE values. Results were obtained by Real Time PCR.

Figure 7a shows the relative mRNA expression rates of the OATP1A2. Even though this transporter has been shown to be expressed in healthy tissue of liver and brain, it reaches an approximately 2.25 fold higher expression in a colon carcinoma cell line (i.e. Caco 2/15) compared to the calibrator. Nevertheless its expression in other colon carcinoma cell lines is below the detection limit.

Figure 7b displays mRNA expression of OATP1B1. This transporter is considered to be liver specific. Via PCR screening its expression in HT29 colon carcinoma cell line could be assessed on the RNA level. However, it is only 0.3 fold higher than is the calibrator.

Figure 7c depicts OATP1B3. mRNA expression of this liver specific transporter appeared in the colon carcinoma cell lines Coga 1A (3.8 fold), HT29 (4.8 fold), L174T (3.4), and with the lowest amplification levels also in C205 cells. As the transporter protein shows significant elevations in 3 out of 5 investigated tumor cell lines, it might be investigated as a tumor marker for future diagnostical aspects.

Figure 7d illustrates the expression of OATP2A1 mRNA. This transporter is the prostaglandine transporter. Its expression are low in the cell lines, namely at 0.8 in Caco 2/15 cells and at 0.225 in Coga 1A cells, and its expression levels are below the expression limit in other cancer cell lines.

Figure 7e presents OATP2B1 mRNA expression. It is one out of three transporters investigated that shows expression in every cell line, even levels are low. The highest

expression rate is reached in Caco 2/15 cells with an approximately 1.8 fold increase compared to the calibrator.
Also in C205 cells, a 0.25 fold expression is observed. Measurable expression levels could also be proven for L174T and Coga 1A cells. The OATP2B1 transporter, which is normally expressed in various healthy tissues, is clearly down regulated in tumor cell lines.

Figure 7f depicts second out of three transporters with shwon expression on the mRNA level in all five tested colon carcinoma cell lines, namely OATP3A1. It reaches highest expression rates in L174T cells (1.4 fold) and Coga 1A cells (1.1 fold), while in C205 and HT29 cells only a 0.1 fold expression is observed.

Figure 7g displays the third transporter which is expressed in every one of our investigated colon carcinoma cell lines, namely OATP4A1. It actually reaches the highest expression rates compared with the other transporters, showing a remarkable 9 fold increase in the expression rate in Coga 1A cells as compared to the calibrator. The results are interesting as OATP4A1 has not been proven to be expressed in healthy colon tissue. The significant upregulation in tumor cells seems to be especially worthy for future investigations for of colorectal cancer diagnosis.
C205, HT29 and L174T cells show approximately the same expression rate (1.9 fold increase compared to the calibrator). However, Caco 2/15 cells express the transporter at low levels (0.3 fold).

Figure 7h illustrates the expression of OATP4C1 mRNA in Caco 2/15 cells only. The transporter could not be shown to be expressed in any other of the five cell lines, and only reached low levels (1.1 fold expression).

Figure 8 displays OATP5A1 mRNA expression. It is interesting to see that this transporter is up-regulated on RNA level in L174T cells (0.5 fold) and in Coga 1A cells (0.16 fold) because very little is known about this only member of the OATP 5 family.
The expression of all known 11 human OATPs, including OATP1C1 and 6A1, was investigated. However, these two transporters did not show any expression on mRNA level at all.

In summary, the investigations showed that Caco 2/15, Coga 1A, C205, HT29 and L174T cell lines express at least four transporters each. In C205 cells OATP1B3, 2B1, 3A1 and 4A1 were found. Five transporters were found in Caco 2/15 cells, namely OATP1A2, 2A1, 2B1, 4A1 and 4C1. HT29 cells expressed OATP1B1, 1B3, 2B1, 3A1 and 4A1, L174T OATP1B3, 2B1, 3A1, 4A1 and 5A1, and 6 out of 11 transporters were found in Coga 1A cells, namely OATP1B3, 2A1, 2B1, 3A1, 4A1 and 5A1.

Especially the transporters being expressed in all of the investigated colon carcinoma cell lines, namely OATP4A1, 3A1, 2B1, could be interesting concerning new ways of diagnosing colorectal cancer in early stages and to improve the patients' prognosis. The combination of knowledge of the transporters' substrates and the fact that certain OATPs are expressed in tumor tissue might help to find better chemotherapy strategies and avoid therapy failure due to drug resistance.

5.3 Expression of OATPs in Colorectal Cancer Samples

The aim of the investigation of the OATPs' expression in tumor samples gained from patients suffering from colorectal cancer in different parts of their colon and progressed into different stages is to show whether there are differences between normal tissue and tumor tissue. To make the samples comparable two samples are taken from every patient, i.e. healthy tissue and a biopsy of the patient's tumor. It is not necessary to extract RNA and convert it into DNA via reverse transcription because I receive cDNA kindly prepared by Prof. Dr. Enikö Kállay's research group.

The method to measure the expression both in healthy and in tumor tissue from one patient each allows two ways of interpretation: on the one hand a general overview whether the transporter is up-regulated in tumor tissue at all, and on the other hand whether there is a relation between normal tissue expressing OATPs and tumor tissue where they are expressed.

The calibrator consists of a mixture of all eight samples' cDNAs to make their values comparable among each other. The MNE values are calculated relative to the calibrators' mean MNE values.

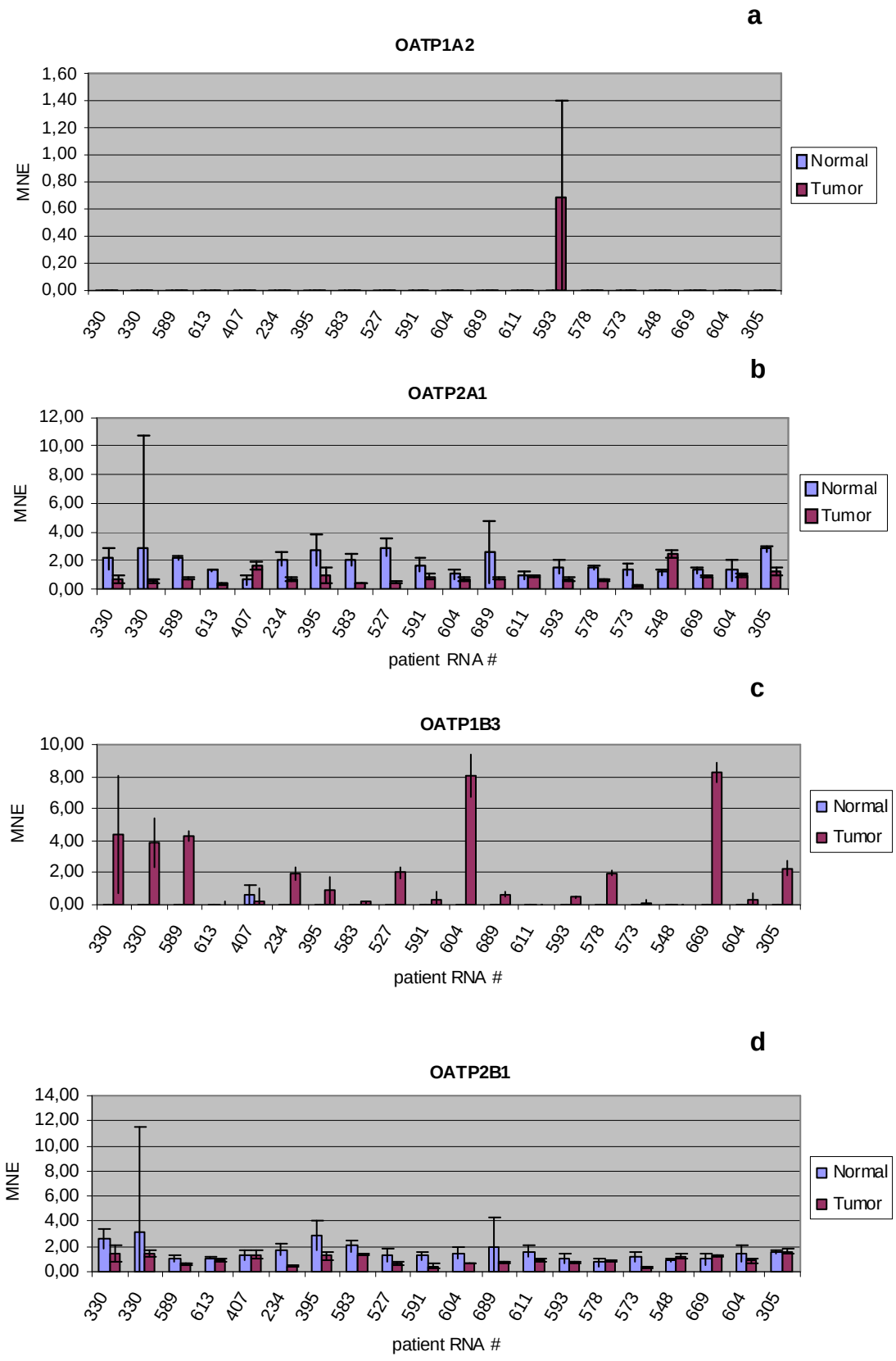

Figure 9 a–d : Expression of OATP1A2 (a), 2A1 (b), 1B3 (c), and 2B1 (d) in Colorectal Cancer Samples, shown in MNE values. Results were obtained by Real Time PCR.

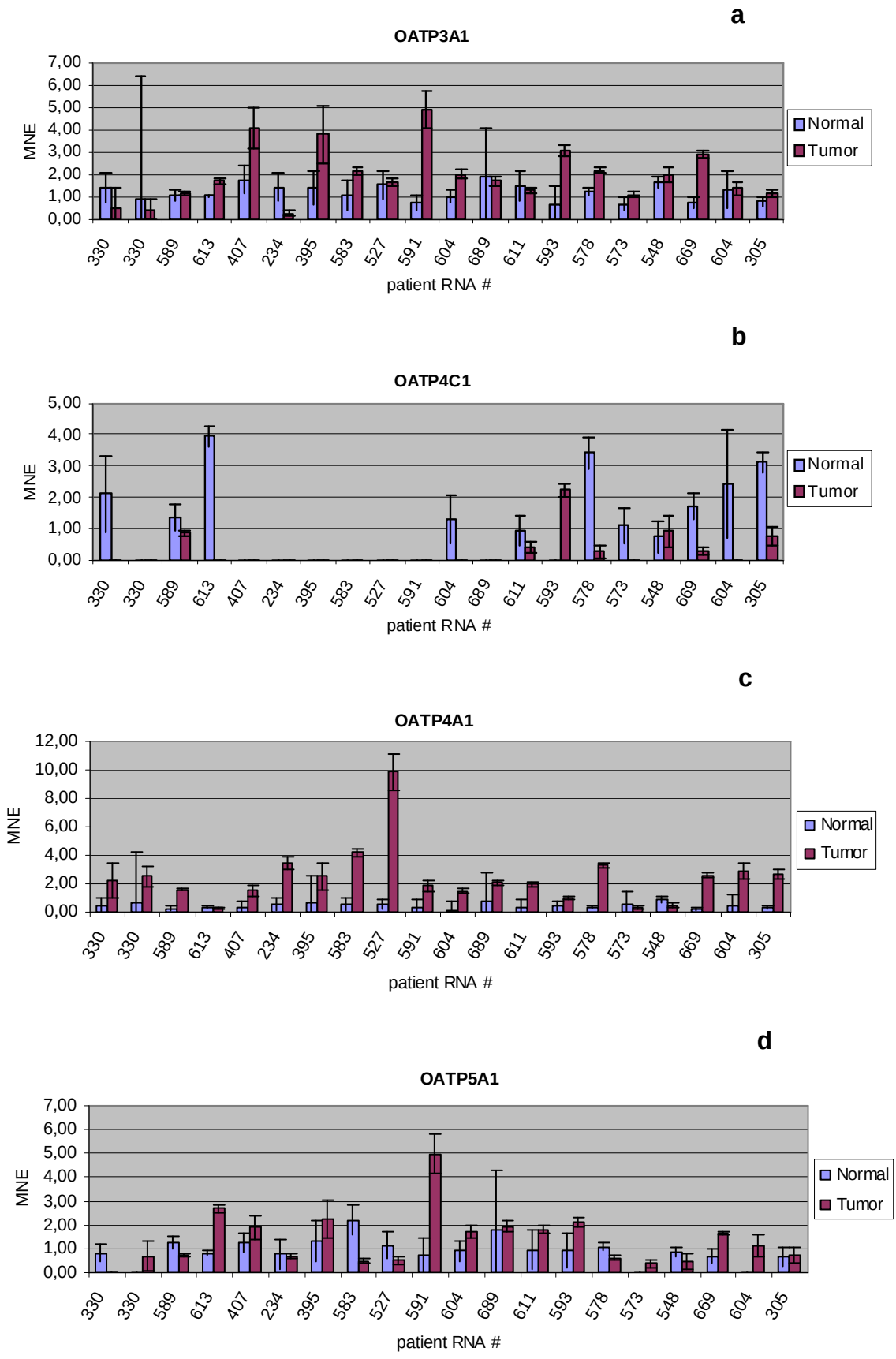

Figure 10 a–d: Expression of OATP3A1 (a), 4C1 (b), 4A1 (c) and 5A1 (d) in Colorectal Cancer Samples, shown in MNE values. Results are obtained by Real Time PCR.

Figure 9a displays the expression of human OATP1A2 in only one sample, i.e. a rectum tumor sample (stage T2), with an expression level of 0.7 fold compared to the calibrator. The healthy tissue from the same patient does not show any expression of the transporter. This suggests that the malignant transformation might have an impact on the expression of OATP transporters.

Figure 9b shows the expression of OATP2A1. In contrary to OATP1A2, this transporter is expressed in every single sample, regardless of its condition. The average expression rate in the normal tissue samples reaches a 1.80 fold enrichment compared with the calibrator, but the highest rate is shown in normal tissue of a patient suffering from cancer in the descending colon (stage T2). The tumor sample taken from this particular patient does not show significant upregulation of OATP1A2. The average level in tumor tissue amounts to 0.83 fold of that of the calibrator. Highest levels are derived from rectum cancer (stage T3) with a 2.45 fold enrichment.

Taken together, there is no relationship between the expression in normal and in tumor tissue. Some patients show high levels of OATP2A1 in their normal tissue but do not exhibit upregulation in their tumor tiusse, and vice versa. With a rate of 1.80 for tumor and normal tissue, versus 0.83 MNE, OATP2A1 is more than two fold higher expressed in normal tissue than in cancerous one.

Figure 9c illustrates the expression of the OATP1B3. It is remarkable that the OATP1B3 is the only transporter investigated which shows a significant difference in expression comparing normal tissue to the tumor samples. The transporter appears in every, but two, cancerous tissues, and reaches high levels in the tumor. The highest expression rates can be stated for one rectum carcinoma (stage T3) and one sigma carcinoma (stage T3) sample, where the MNE exceeds a value of 8.0. Lower but still significant levels are shown for three further samples (adenoma descending, stage T1, and colon ascending, stage T3), reaching MNE values of around 4.0. These are followed by four probes showing an expression rate of about 2.0 in average, found in stage T4 colon ascending, stage T1 colon descending, stage T2 rectum, and stage T4 transversum ascending. In average the expression rate amounts to 2.0, compared to an average of 0.3 MNE for normal tissue. In average, the expression rates are about 6 fold higher in cancerous than in the adjacent tissue, which makes the OATP1B3 an interesting candidate for being a tumor marker.

Figure 9d depicts the expression of OATP2B1. In contrary to OATP1B3, there is no significant difference between normal and cancerous tissue. The average expression rate in normal tissue (1.57) exceeds the tumor tissue's rate (0.96) not even twofold. In only a few samples the expression in the carcinoma tissue rises slightly above the normal tissue levels. As the transporter therefore is not significantly up-regulated in cancerous regions, OATP2B1 does not seem to become useful for the diagnosis of colorectal cancer.

Figure 10a represents the expression of the OATP3A1 gene. In 15 out of 20 cases the transporter is up-regulated in tumor tissue, in a few samples even to high extend. The highest levels can be stated for two samples of colon ascending (both of them stage T3) with 4.2 and 3.8 MNE, and a sample of colon descending (stage T3) with an MNE of 4.8. In average, the tumor tissue shows an expression rate of 1.96 compared to 1.17 in normal tissue.

Figure 10b, showing the expression of OATP4C1, depicts the only case where the transporter is mostly expressed in normal tissue, with the highest value of 3.95 MNE. However, in 8 out of 20 samples the transporter can not be detected neither in normal nor in tumor tissue.

In figure 10c high expression of the OATP4A1 is shown in almost every sample. The MNE values exceed that of normal tissue. Most remarkable the expression rate amounts to 9.85 in a sample from colon descending (stage T1). An average of 2.44 MNE can be seen in the tumor, compared with 0.46 MNE for normal tissue samples. This difference in expression seems to make the OATP4A1 a suitable target for colorectal cancer screening.

Figure 10d displays the expression of OATP5A1. This transporter shows its highest expression in a colon descending sample (stage T3), by reaching a value of 5.0 MNE. In general, the transporter is slightly higher expressed in cancerous tissue than in normal samples. However, as there does not exist much knowledge about this specific transporter, further research is required to be able to valuate its relevance in tumor therapy.

The transporters OATP1C1, 1B1 and 6A1 do not show any expression in any of the samples examined.

5.4 Expression of OATPs in Human Lung Cell Lines

Expression of SLCO genes on mRNA level is also assessed in five lung cell lines. Four out of five cell lines are human small cell lung carcinoma cells, one is derived from human bronchial epithelial cells. The expression of OATPs in lung cancer cell lines might be interesting as the lung surface is constantly exposed to a number of exogenous substances.

The calibrator consists of a mixture of all five cell lines' cDNAs to make the cells comparable among each other. MNE values are calculated according to the expression of the respective gene in the calibrator.

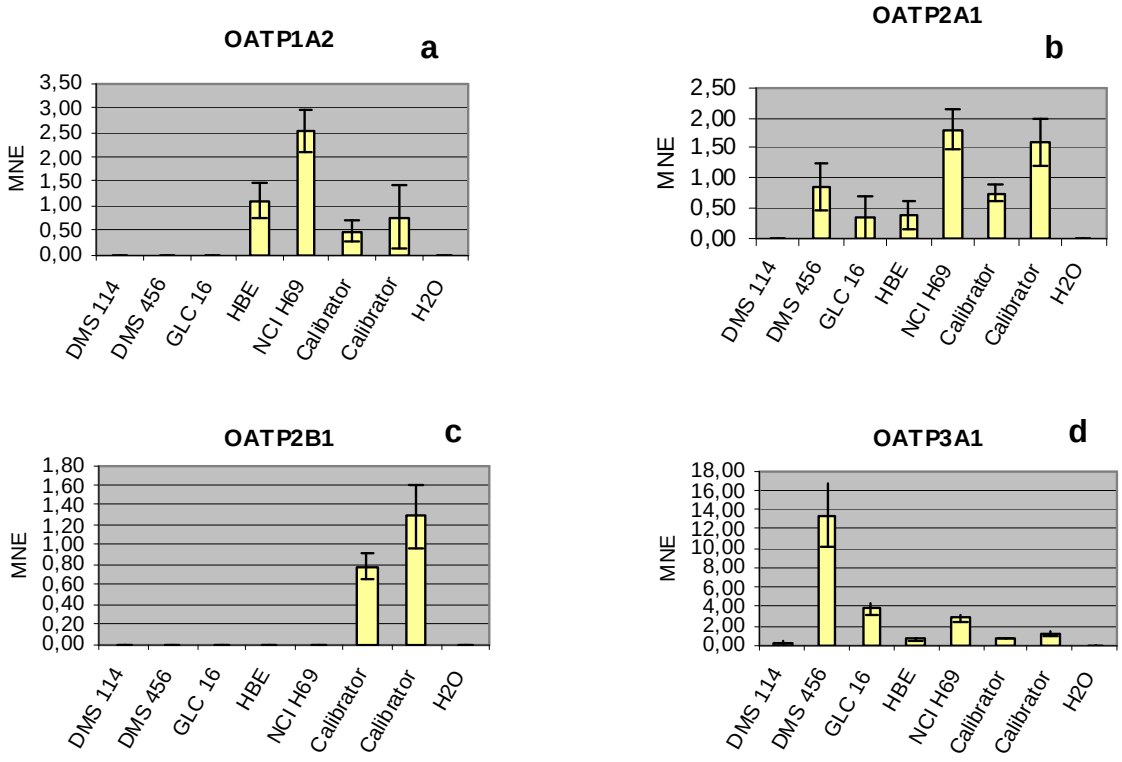

Figure 11 a–d: Expression of OATP1A2 (a), 2A1 (b), 2B1 (c), and 3A1 (d) on mRNA level in Human Lung Cancer Cell Lines, shown in MNE values. Results are obtained by Real Time PCR.

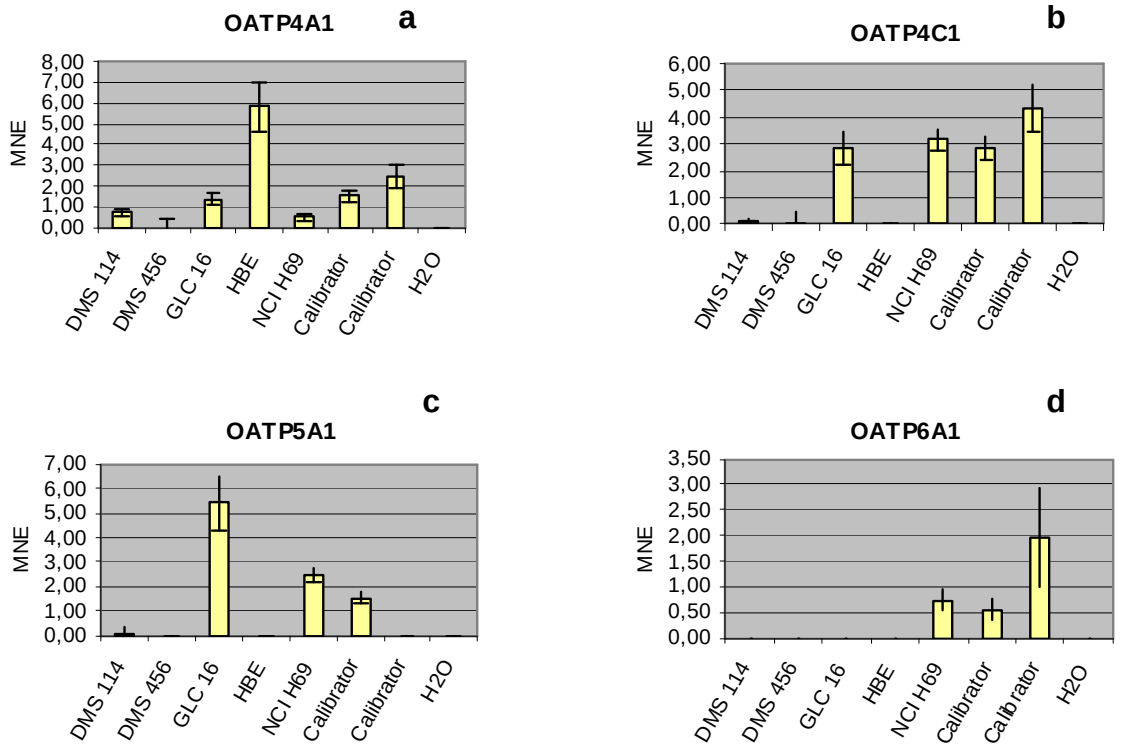

Figure 12 a–d: Expression of OATP4A1 (a), 4C1 (b), 5A1 (c) and 6A1 (d) on mRNA level in Human Lung Cancer Cell Lines, shown in MNE values. Results are obtained by Real Time PCR.

Figure 11a shows the expression of OATP1A2. The mean MNE value of the calibrator is calculated as 0.62. The highest expression is found in NCI-H69 cells with a MNE value of 2.53. In average, the expression of OATP1A2 reaches a value of 0.91 MNE. This means a slightly lower expression rate of the OATP in the cell lines, pointing out that there is no significant amount of OATP1A2 expressed in the lung cell lines on RNA level, except in NCI-H69 cells.

Figure 11b depicts the expression of OATP2A1. Again, the calibrator's mean value (1.18) doubles the average values of the cell lines, with their highest measured expression in NCI-H69 cells with a MNE value of 1.79. The average of the expression rates only reaches a value of 0.68. This suggests that there is no significant expression of OATP2A1 in lung cancer cell lines.

Figure 11c indicates that this seems to be true for OATP2B1 expression in lung cell lines as well, where no significant expression of this transporter can be found.

Figure 11d, however, demonstrates high expression of OATP3A1, which reaches a MNE value of 13.42 in DMS 456 cells. As the mean value of the calibrator equals only 0.91 MNE the expression of the transporter shows a significant upregulation. This is also reflected in the average value of the five cell lines (4.14), showing a 4.5 fold higher expression in the cell lines than in the calibrator. The GLC 16 cell line (3.76) and the NCI H69 cell line (2.80) show the second highest expression rates.
Remarkably, the transporter is also expressed in healthy lung tissue.

Figure 12a shows the expression of OATP4A1. The calibrator, reaching a mean MNE value of 1.98 is exceeded by the expression rate in HBE cells (5.80). The other cell lines show expression as well; however, it is lower than the calibrator's value and therefore can not be counted as a significant expression on RNA level.

Figure 12b depicts the results from OATP4C1. It is remarkable that the calibrator shows a very high MNE value of 3.60. Expression in the cell lines GLC 16 and NCI H69 reaches values between 2.84 and 3.18.

Figure 12c shows that the highest expression rate of OATP5A1 is reached in GLC 16 cells (5.39). The further four cell lines show either no expression or low expression levels of 0.05 MNE DMS 114 cells.
Verifying the obtained results, also by performing real time PCR on the basis of RNA gained from patient samples, could help to better understand the function of OATP5A1.

Figure 12d depicts the expression rates of OATP6A1. The average MNE value of the calibrator (i.e. 1.26) exceeds that of NCI H69 cells (MNE 0.74). No expression can be detected in the other four cell lines.

5.5 Expression of OATPs in Ovary Cell Lines

Comparative studies on the expression of OATPs on mRNA level are also done on six human ovary cell lines. Five of these six cell lines are made resistant against diverse anticancer substances, e.g. paclitaxel. The remaining cell line belongs to the HEY wild type. The calibrator consist of a mixture of all six cell lines' cDNAs and makes the results obtained comparable to each other. The MNE values are calculated according to the expression of the respective gene in the calibrator.

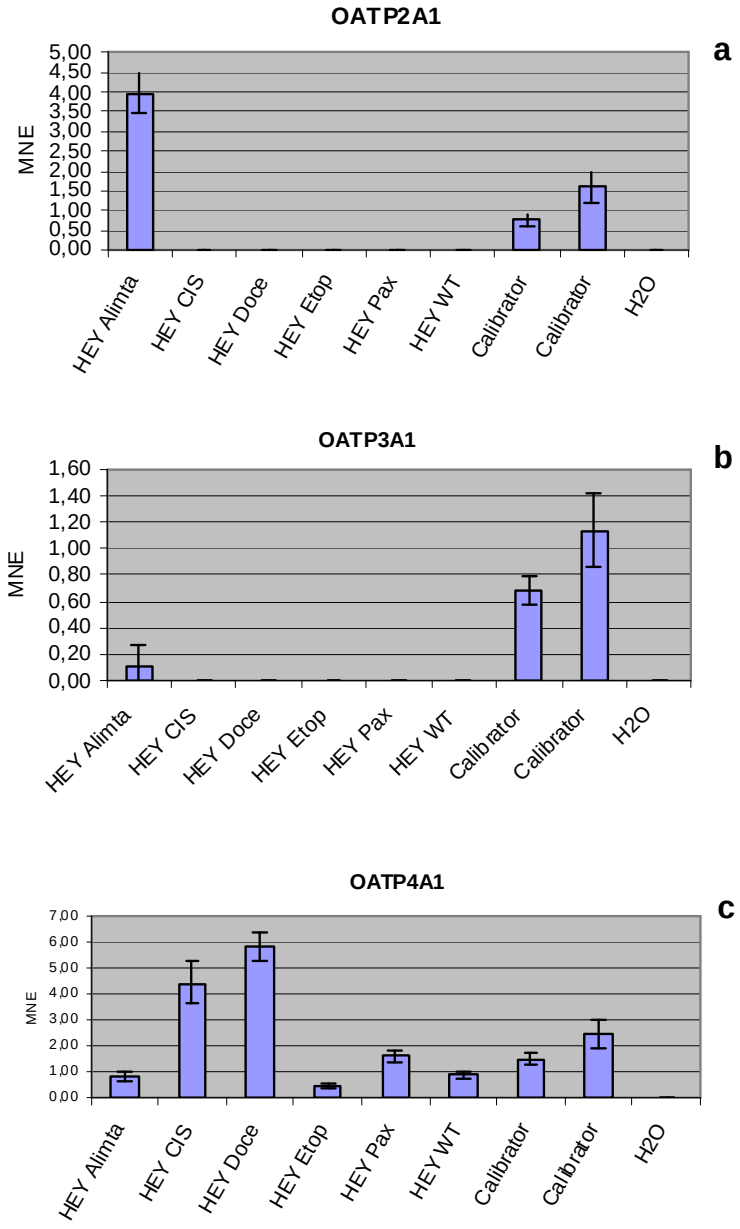

Figure 13 a–c: Expression of OATP2A1 (a), 3A1 (b), and 4A1 (c) in Human Ovary Cancer Cell Lines, shown in MNE values. Results obtained by Real Time PCR.

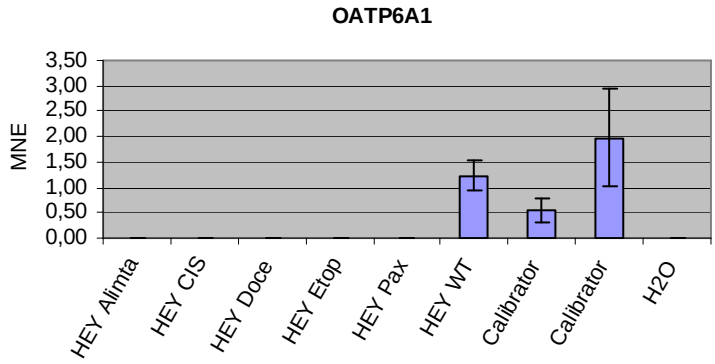

Figure 14: Expression of OATP 6A1 in Human Ovary Cancer Cell Lines, shown in MNE values. Results obtained by Real Time PCR.

Figure 13a, showing the expression of OATP2A1, points out an upregulation of the transporter. The average MNE value of the calibrator of 1.18 is approximately twofold higher than the expression in the HEY Alimta cell line (MNE 0.66). However, this cell line is the only one to express this transporter, and a twofold upregulation does not seem to be a significant marker for this cancerous tissue.

Figure 13b depicts the expression of OATP3A1. Given that the average MNE value of the calibrator lies at 0.91, the MNE value of the SLCO gene 3A1 of 0.11 is about 8 times lower and can not be count as a significant expression at all. However, it is remarkable that once again the HEY Alimta cell line is the only one to express the gene, actually.

Figure 13c describes the expression of OATP4A1. As already stated when investigating colon cancer cell lines, colorectal cancer patient samples, lung cancer cell lines, this transporter is broadly expressed in every cell line being subject of the analysis. The calibrator's MNE value lies at 1.98, which is already exceeded almost 1.5 fold by the average MNE value of all ovary cell lines, that is to say 2.98. The highest levels could be shown the HEY Doce cell line (5.84), closely followed by the HEY Cis cell line (4.42). The other cell lines only show expression rates below the value of 2.0. Hence, in these the transporter does not seem to be valuable for diagnostic purpose.

The last figure, figure 14, depicts the expression of the OATP6A1. Contrarily to OATP4A1, this transporter only showed expression in one single cell line, this is to say HEY WT, with a MNE value of 1.24. As the calibrator's value is 1.62, there is no upregulation in the transporter's expression rate.

The other transporters (OATP1A2, 1B1, 1B3, 1C1, 2B1, 4C1, and 5A1), do not show any expression at the PCR conditions applied.

5.6 Discussion and Conclusion

The investigations on the expression of the SLCO genes superfamily included four different sample types: human colorectal cancer samples derived from patients, human colorectal cancer cell lines, human lung carcinoma cell lines, and human ovary carcinoma cell lines.

In most of the samples, the expression rates, expressed in MNE (mean normalized expression value), remain below the value of 2.0, which is set arbitrarily to divide the expression rates in three groups and categorize the PCR results.
Group one, with MNE values ranging from 0.0 to 2.0 (first threshold), means no or very low expression rates.
Group two, with MNE values from 2.0 to 4.0 (second threshold), indicates moderate upregulation of the transporters' expression rates. These MNE values are considered to show a relevance of the transporter as tumor marker. Only a few samples, most of them within the group of colorectal cancer patient samples, but non within the group of normal tissue patient samples, exceed this second threshold.
Group three encompasses transporters with high MNE values (>4.0, third threshold), and reflects significant upregulation of mRNA expression in a sample (see table14).

Table 14: Expression of OATPs on RNA Level – Summary.

Yellow indicates first threshold (MNE <2.0), light blue indicates the second threshold (MNE 2.0–4.0), dark blue indicates the third threshold (>4.0). All thresholds have been set arbitrarily.

Expression of OATPs on RNA Level - Summary												
sample	sample ID	SLCO										
		1A2	1B1	1B3	1C1	2A1	2B1	3A1	4C1	4A1	5A1	6A1
colon carcinoma cell lines	Caco 2/15	2,24	0,00	0,00	0,00	0,09	1,83	0,00	0,12	0,28	0,00	0,00
	Coga 1A	0,00	0,00	3,84	0,00	0,23	0,01	1,09	0,00	9,02	0,16	0,00
	C205	0,00	0,00	0,06	0,00	0,00	0,29	0,06	0,00	1,83	0,00	0,00
	HT29	0,00	0,33	4,71	0,00	0,00	0,00	0,07	0,00	1,79	0,00	0,00
	L174T	0,00	0,00	3,25	0,00	0,00	0,06	1,40	0,00	1,82	0,50	0,00
colon carcinoma patient samples	330T	0,00	0,00	4,35	0,00	0,65	1,43	0,48	0,00	2,21	0,00	0,00
	330T	0,00	0,00	3,89	0,00	0,53	1,42	0,42	0,00	2,52	0,68	0,00
	589T	0,00	0,00	4,32	0,00	0,73	0,62	1,16	0,86	1,61	0,70	0,00
	613T	0,00	0,00	0,05	0,00	0,34	0,91	1,68	0,00	0,28	2,68	0,00
	407T	0,00	0,00	0,21	0,00	1,61	1,35	4,09	0,00	1,55	1,88	0,00
	234T	0,00	0,00	1,91	0,00	0,68	0,44	0,25	0,00	3,41	0,69	0,00
	395T	0,00	0,00	0,90	0,00	0,93	1,25	3,79	0,00	2,53	2,25	0,00
	583T	0,00	0,00	0,16	0,00	0,41	1,35	2,14	0,00	4,20	0,49	0,00
	527T	0,00	0,00	2,02	0,00	0,45	0,65	1,63	0,00	9,85	0,50	0,00
	591T	0,00	0,00	0,33	0,00	0,86	0,40	4,90	0,00	1,84	4,99	0,00
	604T	0,00	0,00	8,08	0,00	0,66	0,65	2,01	0,00	1,48	1,73	0,00
	689T	0,00	0,00	0,65	0,00	0,73	0,75	1,69	0,00	2,09	1,94	0,00
	611T	0,00	0,00	0,00	0,00	0,95	0,92	1,26	0,41	1,97	1,81	0,00
	593T	0,68	0,00	0,47	0,00	0,67	0,76	3,05	2,22	1,02	2,12	0,00
	578T	0,00	0,00	1,96	0,00	0,64	0,87	2,16	0,26	3,30	0,61	0,00
	573T	0,00	0,00	0,08	0,00	0,26	0,34	1,07	0,00	0,38	0,36	0,00
	548T	0,00	0,00	0,00	0,00	2,45	1,22	2,00	0,92	0,48	0,47	0,00
	669T	0,00	0,00	8,27	0,00	0,85	1,30	2,92	0,28	2,60	1,66	0,00
	604T	0,00	0,00	0,29	0,00	1,00	0,85	1,37	0,00	2,92	1,14	0,00
	305T	0,00	0,00	2,26	0,00	1,26	1,62	1,14	0,75	2,63	0,70	0,00
normal tissue patient samples	330N	0,00	0,00	0,00	0,00	2,13	2,61	1,36	2,10	0,49	0,81	0,00
	330N	0,00	0,00	0,00	0,00	2,91	3,20	0,91	0,00	0,69	0,00	0,00
	589N	0,00	0,00	0,00	0,00	2,14	1,06	1,02	1,36	0,27	1,24	0,00
	613N	0,00	0,00	0,00	0,00	1,33	1,11	1,01	3,95	0,36	0,81	0,00
	407N	0,00	0,00	0,61	0,00	0,65	1,27	1,74	0,00	0,34	1,25	0,00
	234N	0,00	0,00	0,00	0,00	2,07	1,75	1,42	0,00	0,56	0,77	0,00
	395N	0,00	0,00	0,00	0,00	2,70	2,90	1,36	0,00	0,62	1,34	0,00
	583N	0,00	0,00	0,00	0,00	2,04	2,05	1,06	0,00	0,56	2,21	0,00
	527N	0,00	0,00	0,00	0,00	2,88	1,32	1,53	0,00	0,53	1,15	0,00
	591N	0,00	0,00	0,00	0,00	1,69	1,25	0,75	0,00	0,32	0,73	0,00
	604N	0,00	0,00	0,00	0,00	1,02	1,49	0,99	1,29	0,16	0,91	0,00
	689N	0,00	0,00	0,00	0,00	2,58	1,92	1,85	0,00	0,77	1,76	0,00
	611N	0,00	0,00	0,00	0,00	0,95	1,60	1,44	0,93	0,32	0,96	0,00
	593N	0,00	0,00	0,00	0,00	1,53	1,01	0,65	0,00	0,46	0,91	0,00
	578N	0,00	0,00	0,00	0,00	1,53	0,75	1,20	3,42	0,34	1,09	0,00
	573N	0,00	0,00	0,00	0,00	1,30	1,18	0,67	1,10	0,52	0,00	0,00
	548N	0,00	0,00	0,00	0,00	1,20	0,94	1,60	0,73	0,93	0,84	0,00
	669N	0,00	0,00	0,00	0,00	1,32	0,99	0,72	1,72	0,25	0,67	0,00
	604N	0,00	0,00	0,00	0,00	1,30	1,47	1,29	2,42	0,40	0,00	0,00
	305N	0,00	0,00	0,00	0,00	2,80	1,56	0,78	3,11	0,33	0,64	0,00
lung carcinoma cell lines	DMS 114	0,00	0,00	0,00	0,00	0,00	0,00	0,15	0,16	0,72	0,05	0,00
	DMS 456	0,00	0,00	0,00	0,00	0,86	0,00	13,42	0,06	0,01	0,00	0,00
	GLC 16	0,00	0,00	0,00	0,00	0,34	0,00	3,76	2,84	1,33	5,39	0,00
	HBE	1,10	0,00	0,00	0,00	0,39	0,00	0,57	0,00	5,80	0,00	0,00
	NCI-H69	2,53	0,00	0,00	0,00	1,79	0,00	2,80	3,18	0,49	2,47	0,74
ovary carcinoma cell lines	HEY Alimta	0,00	0,00	0,00	0,00	3,96	0,00	0,11	0,00	0,81	0,00	0,00
	HEY CIS	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	4,42	0,00	0,00
	HEY Doce	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	5,84	0,00	0,00
	HEY Etop	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,48	0,00	0,00
	HEY Pax	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	1,62	0,00	0,00
	HEY WT	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,90	0,00	1,24

In colorectal carcinoma cell lines, only three transporters show upregulation, i.e. 1A2 (Caco 2/15 cell line), 1B3 (Coga 1A, HT29 and L174T) and 4A1 (Coga 1A). The most remarkable upregulation is demonstrated for the Coga 1A cell line, with an observed MNE value of 9.02.

As the main aim of the present thesis is to investigate the expression of the OATP superfamily in colorectal carcinoma samples, derived from patients suffering from different stages of the carcinoma, the findings presented in this diploma thesis could be of further value. The detection of significant upregulation could help to ameliorate diagnosis in early stage and hence the patients' prognosis. In case significant upregulation of the transporter can be demonstrated, it might be used as a marker on the one hand, and help to avoid drug-drug interactions, and drug resistance, respectively.

In total six transporters are up-regulated in cancerous tissue, i.e. OATP1B3, 2A1, 3A1, 4C1, 4A1 and 5A1. Most remarkably, OATP1B3 has high expression rates in four samples, with MNEs ranging from 4.32 up to 8.27.

The most conspicuous increase in the expression level can be demonstrated for OATP4A1, reaching a MNE value of 9.85. In addition, this transporter shows upregulation beyond the second threshold level of MNE 4.0 in thirteen out of twenty samples. These results suggest that screening patients' biopsy samples for upregulation of the transporter could become important in the diagnosis of colorectal carcinoma, particularly as upregulation is missing in the adjacent normal tissue.

Although the upregulation can be correlated neither with tumor stage, nor with the tumor's localisation within the colon, this could be due to the low number of samples.

For another transporter, namely OATP1B3 and OATP3A1, also no significant expression on the mRNA level is seen in parts of the tissue.

In any case, the results from the screening in the limited number of samples need to be verified by screening of a larger collection of samples. In addition, the transporters' expression will have to be demonstrated on protein level, too, to make the results valid for raising future perspectives. Nevertheless these initial investigations suggest that the OATP superfamily may be further examined as future tumor markers.

In comparative studies, real time PCR analysis of OATPs is also done in lung and ovary carcinoma cell lines. As these two types of cancer belong to the most frequent causes of death worldwide, new screening methods and ways to diagnose the malignancy in early stages will definitely help to ameliorate the patients' prognosis, which currently is quite poor in both cases.

Concerning lung carcinoma cell lines, OATP3A1 provided the most astonishing result. It showed an expression rate of MNE 13.42 in DMS 456 cells, what denotes a 14.7 fold upregulation compared to the calibrator. Additionally, OATP4A1, 5A1, 4C1 and 1A2 showed higher expression rates. However, to verify these first findings a screening of lung carcinoma samples Is needed in the future.

In comparison with the lung carcinoma cell lines, the results for OATP expression in ovarian cancer cell lines appear moderate. Only two transporters are up-regulated, namely OATP2A1 and 4A1. While the expression rate of OATP2A1 exceeded the first threshold simply in the HEY Alimta cell line, OATP4A1 showed MNE values beyond the second threshold (MNE 4.42 in HEY CIS, MNE 5.84 in HEY Doce).

Taken together, mRNA the expression rates for OATPs demonstrated by real time PCR do not show broad upregulation of the transporters in cancerous tissue of the colon. However, these results might build the basis of more selective diagnostic methods. Concerning colorectal cancer therapy could be ameliorated by using the OATP superfamily as a marker, helping to avoid surgical intervention due to late diagnosis on the one hand, and enhancing the efficacy of chemotherapy by avoiding inefficient drug application due to resistance on the other hand.

6 SUMMARY

Although colorectal cancer is one of the most frequent malign diseases worldwide, with the highest incidence in civilized Western countries, the prognosis of advanced metastazing colon cancer is still poor. After surgical resection of the cancerous tissue, application of anticancer chemotherapy is hampered by the development of drug resistance. Often drug resistance is due to the over or under expression of membrane transport proteins for cytostatic agents in the tumor cells. It is well established that over expression of ABC-efflux transporters causes drug resistance by mediating the rapid efflux of drugs from the cells. However, very little is known whether uptake transporters from the SLCO family might compensate increased efflux by an efficient uptake. Therefore expression of SLCO transporters is investigated in colon cancer cell lines and comparative studies are done in lung and ovary carcinoma cell lines.

From the 11 known human SLCO (solute carrier organic anion transporter family) genes only three transporters show upregulation in colorectal carcinoma cell lines i.e. 1A2 in the Caco 2/15, 1B3 in the Coga 1A, HT29 and L174T and 4A1 in the Coga 1A cell line. The most remarkable upregulation is demonstrated for the Coga 1A cell line, with an approximately 10-fold upregulation of OATP4A1. Remarkably, this transporter shows upregulation in thirteen out of twenty samples derived from human colon tumors, while its expression in the adjacent normal tissue is near the detection limit. Although, due to the low number of samples (n = 20), upregulation in colon tumors was not associated with tumor stage or localisation within the colon, these findings warrant further studies on OATP4A1 expression in colon cancer. OATP4A1 is also high in the ovarian carcinoma cell line HEY made resistant to cisplatin and doxorubicin, respectively. This further suggests that this transporter might be associated with resistance to anticancer drugs in solid tumors as well. Also in the lung carcinoma cell line HBE OATP4A1 is increased, while three other lung carcinoma cell lines over express OATP3A1 up to 14-fold (DMS456). Remarkably, also the “kidney” specific” transporter OATP4C1 is over expressed in lung cancer cell lines indicating the normal organ border of OATP expression is overcome during tumorigenesis. Another transporter, namely OATP5A1, for which organ localization and substrates are not established yet, is found to be over expressed in lung cancer

cell lines as well as in colon tumor specimens. On the other hand, OATP2A1, the high affinity transporter for prostaglandins, is greatly down-regulated in normal colon tissues as compared to colon tumors, but it is also upregulated in the HEY cell line resistant to Alimta. These transporters regulate the levels of prostaglandins, like PGE2, as inflammatory mediators in the interstitiel space. OATP2A1 down-regulation could contribute to tissue inflammation driving tumor progression.

In addition to the main objective of the present thesis on the expression of OATP transport proteins another task was to establish the spheroid model for drug screening. Spheroids were grown from established tumor cell lines and CD133 (prominin) was used to screen for potential cancer stem cells in these cell cultures. These speroid cultures resemble the physiological parameters better than tumor cells grown in a monolayer culture, and they could provide a future model for studies on tumor therapy.

In conclusion, the results of this thesis show the expression patterns of OATP transporters in human colon tumors and tumor cell lines from solid tumors, and provide the base for further functional studies on the role of OATPs in the therapy of human cancers. They may be integrated in the efforts made to reduce drug-drug, drug-food interactions and minimize drug side effects due to interactions with the transporter family.

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CURRICULUM VITAE

PERSONAL DETAILS

Name, Surname	Angela Johanna Schöffmann
Date of Birth	January 3rd, 1985
Place of Birth	Klagenfurt
Nationality	Austria

FORMATION

1991 – 1995	Primary School, St. Andrä/Villach
1995 – 2000	Bundesgymnasium/Bundesrealgymnasium BG/BRG St. Martin, Villach
2000 – 2001	Tourismusschule Bad Hofgastein, Bad Hofgastein
2001 – 2004	Bundesgymnasium/Bundesrealgymnasium BG/BRG St. Martin, Villach
June 24th, 2004	Graduation with honors
Oct. 2004 – Sept. 2009	Studies of Pharmacy, University of Vienna

PROFESSIONAL EXPERIENCES

February 2005	Pharmacy “Mag. pharm. Beate Koder GmbH”, Klagenfurt
July – August 2006	Dispensary LKH Villach
July – August 2007	Pfizer Corp. Austria, Marketing, Human Resources
Sept. 2008 – Feb. 2009	Pfizer Corp. Austria, Finance Department
March – July 2009	Diploma Thesis, Department of Clinical Pharmacy and Diagnostics, University of Vienna, in cooperation with the Institute of Pathophysiology, Medical University of Vienna, AKH Wien