universität wien

DIPLOMARBEIT / DIPLOMA THESIS

Titel der Diplomarbeit / Title of the Diploma Thesis

„Metabolization of CE-123 in *in vitro* 2D Hepatic Cell Culture Models"

---

verfasst von / submitted by

Verena Carina Johler

angestrebter akademischer Grad / in partial fulfilment of the requirements for the degree of

Magistra der Pharmazie (Mag. pharm.)

Wien, 2020 / Vienna 2020

Studienkennzahl lt. Studienblatt / degree programme code	UA 449
Studienrichtung lt. Studienblatt / degree programme	Diplomstudium Pharmazie
Betreut von / Supervisor:	Univ. Prof. Mag. Pharm. Dr. Thierry Langer

“Remember to look up at the stars and not down at your feet” – Stephen Hawking

Acknowledgements

Univ.-Prof. Mag. Dr. Thierry Langer for making this Diploma Thesis possible, his vast expertise, kindness, and support.

ao. Univ.-Prof. Mag. Dr. Walter Jäger for answering an infinite amount of questions and for making his resources available to me.

Univ.-Prof. Dr. Lukas A. Huber, for his guidance and support through my beginnings and for enabling me to do what I love.

Mag. Stefan Poschner for taking the time and sharing his expertise very generously.

Daniel Dobusch, MSc (FH) for being a great colleague, teacher and, moreover, a friend.

Lisa Horvat for her enthusiasm for cell culture and her untiring cheerfulness.

Susanne Wiederkum, who even though under no obligation provided me with all necessities and practical knowledge.

My parents, Christina and Werner, for their continuous support, love and guidance.

My sisters Marlene and Sylvie for supporting and challenging me, and my entire family - you are all so very amazing.

My partner Claudio, for his contagious confidence, unwavering determination, humour and serenity. You never cease to amaze me.

Table of contents

1 Abstract/Zusammenfassung

1.1 Abstract

CE-123 is a newly synthesized compound that was derived from the narcolepsy medication modafinil. Modafinil is pharmacologically characterized as an atypical dopamine reuptake inhibitor that is suspected to act through the inhibition of the dopamine transporter DAT. Modafinil has numerous adverse effects, which limit its application. CE-123 was developed as a more selective alternative with the goal to reduce adverse effects. CE-123 was probed in an *in vitro* metabolism study that was conducted with two hepatic cell lines, namely HepG2 and HepaRG. The main purpose of the utilization of the HepG2 cell line was to establish an assay suited to form and subsequently detect possible metabolites of CE-123. HepG2 however are known to have a limited metabolic capacity in respect of Phase I metabolization enzymes. Therefore, HepaRG were procured to ameliorate the metabolic output, as HepaRG cells have significantly higher Phase I metabolic capacities, compared to HepG2. As modafinil is an established drug it was employed to help assess the functionality of the assay, as well as the applicability of the experiments. In order to establish a functional assay, variations of the experiment were conducted with HepG2 cells, including the omission of antibiotics, to rule out metabolic nuisances. The established protocol was then used for the experiment with HepaRG. For the prediction of metabolites and support of metabolite identification the Meteor™ nexus software was utilized. The samples were measured via LC-MS, utilizing the internal standard method for quantification. A decrease in concentration of both compounds was observed with HepG2 as well as HepaRG, it was however not possible to distinguish metabolites in the experiments conducted with HepG2 cells. The HepaRG cells however presented the capacity to form at least one metabolite corresponding to the prediction conducted via the Meteor™ nexus software.

1.2 Zusammenfassung

CE-123 ist eine neu synthetisierte Verbindung, die von dem Narkolepsie Medikament Modafinil abgeleitet wurde. Modafinil ist pharmakologisch als ein atypischer Dopamin-Wiederaufnahmehemmer charakterisiert, der vermutlich durch die Hemmung von DAT wirkt. Modafinil hat jedoch zahlreiche unerwünschte Wirkungen, die seine Anwendung einschränken. CE-123 wurde als selektiverer Alternativwirkstoff entwickelt, mit dem Ziel, die Nebenwirkungen zu reduzieren. CE-123 wurde in einer *in-vitro*-Metabolismus Studie untersucht, die mit zwei hepatischen Zelllinien, nämlich HepG2 und HepaRG, durchgeführt wurde. Der Hauptzweck der Verwendung der HepG2-Zelllinie war die Etablierung eines Assays, der geeignet ist, mögliche Metaboliten von CE-123 zu bilden und nachzuweisen. Es ist jedoch bekannt, dass HepG2 Zellen nur begrenzte Phase I Kapazitäten hat. HepaRG Zellen wurden eingesetzt, um die metabolische Kapazität des Assays zu verbessern, da HepaRG-Zellen im Vergleich zu HepG2 eine signifikant höhere metabolische Kapazität der Phase I Enzyme haben. Da Modafinil ein etabliertes Medikament ist, wurde es eingesetzt, um die Funktionalität und die Anwendbarkeit des Assays zu beurteilen. Um einen funktionellen Assay zu etablieren, wurden Variationen des Experiments mit HepG2-Zellen durchgeführt, einschließlich des Verzichts auf Antibiotika, um Störungen der metabolischen Aktivität auszuschließen. Das entwickelte Protokoll wurde dann für den Versuch mit HepaRG Zellen verwendet. Für die Vorhersage von Metaboliten und zur Unterstützung der Metabolitenidentifikation wurde die Software Meteor™ nexus eingesetzt. Die Proben wurden mittels LC-MS gemessen, wobei die interne Standardmethode zur Quantifizierung verwendet wurde. Sowohl bei HepG2 als auch bei HepaRG wurde eine Abnahme der Konzentration beider Verbindungen beobachtet, dennoch war es nicht möglich, Metaboliten in den mit HepG2-Zellen durchgeführten Experimenten zu finden. Die HepaRG-Zellen zeigten jedoch die Fähigkeit, zumindest einen Metaboliten zu bilden, der der Vorhersage entspricht, die über die Meteor™ nexus-Software durchgeführt wurde.

2 Introduction and Related Work

2.1 Narcolepsy

Narcolepsy is a chronic sleep disorder that is characterized by excessive daytime sleepiness (EDS). Patients experience episodes of irresistible sleep and acute rapid eye movement (REM) episodes, which may be caused by a lack of inhibition within REM activating structures (Thorpy, 2012). The neuronal activity related to REM phases appears to be constituted in the brain stem, particularly the *locus coeruleus* and the *pontine tegmentum* (Peever & Fuller, 2017; Walsh et al., 2011). REM phases can be identified by REM atonia (inhibited skeletal muscle activity), discontinuous muscle convulsions, faster frequency and reduced amplitude cortical electroencephalogram (EEG), changes in respiratory activity, body and brain temperature, and an increased excitation threshold (Peever & Fuller, 2017; Siegel, 2005). Additional less specific symptoms include hypnagogic and hypnopompic hallucinations (visual sensations during the transition to or from sleep), sleep paralysis and parasomnias. Common comorbidities include obesity, headache, cognitive deficits like impairment of memory and concentration, as well as depression. While the frequency of these symptoms is variable, excessive daytime sleepiness is persisting, though may decrease with increasing age. Cataplexy may cease spontaneously with or without treatment. Sleep paralysis and hypnagogic hallucinations may be intermittent. Interrupted noctule sleep however does not tend to ameliorate. In the Caucasian population the prevalence of narcolepsy amounts to approximately 25 – 40 per 100 000, which equals about 0.05% of the population, with a higher prevalence in women than men (Ahmed & Thorpy, 2010; Kanbayashi et al., 2011; Oertel, Deuschl, & Poewe, 2012; Silber, Krahn, Olson, & Pankratz, 2002).

Three forms of narcolepsy are described in the International Classification of Sleep Disorders:

- *Narcolepsy with cataplexy*
  Narcolepsy with cataplexy is thought to be caused by the loss of the neuropeptide hypocretin in the cerebrospinal fluid. That loss is potentially caused by an autoimmune defect that causes a decrease of hypocretin by more than two thirds of normal levels. Though ≤10% of patients show normal hypocretin levels, indicating another defect or pathophysiological mechanism (American Sleep Disorders Association, 2001; Billiard, 2007; Kanbayashi et al., 2003; Thorpy, 2012). Cataplexy is the second most common and most significant symptom for narcolepsy with cataplexy. It is characterized by sudden partial or complete loss of arbitrary muscle tonicity while remaining consciousness (Ahmed & Thorpy, 2010; Overeem, Mignot, van Dijk, & Lammers, 2001).

- *Narcolepsy without cataplexy*
  Narcolepsy without cataplexy is indicated with a positive multiple sleep latency test, two or more sleep-onset REM episodes, sleep paralysis and hypnagogic hallucinations, but no cataplexy. It is not established if the pathology corresponds to that of narcolepsy with cataplexy, as an exceeding number of narcoleptic patients without cataplexy show normal levels of hypocretin (American Sleep Disorders Association, 2001; Kanbayashi et al., 2003; Emmanuel Mignot et al., 2002; Silber et al., 2002; Thorpy, 2012).

- *Narcolepsy as a comorbidity*
  Is implied when an individual suffers from daytime sleepiness related to a severe medical disorder (American Sleep Disorders Association, 2001; Gilhus, Barnes, & Brainin, 2011; Kanbayashi et al., 2003; Kanbayashi et al., 2011; Thorpy, 2012).

2.1.1 Treatment of Narcolepsy

Only a few drugs are approved for the treatment of narcolepsy. Modafinil and sodium oxybate present the first line therapy options for EDS (Abad & Guilleminault, 2017). However, a range of medications with various biochemical targets have shown to have alleviating effects on the major symptoms of narcoleptic patients (Romigi, Vitrani, Lo Giudice, Centonze, & Franco, 2018):

- Alerting medication, for example modafinil/armodafinil, methylphenidate and amphetamine
- Sodium oxybate
- Antidepressants e.g. selective serotonin reuptake inhibitors (SSRI), serotonin-norepinephrine reuptake inhibitors (SNRI) and tricyclic antidepressants
- H3- receptor antagonists, for example pitolisant

The effectiveness of the majority of these compounds seem to heavily depend on increased catecholamine concentrations (e.g. Dopamine, Serotonin, Noradrenaline) in the synaptic gap. These increased catecholamine concentrations are a result of catecholamine reuptake inhibition and the regulation of *locus coeruleus* norepinephrine neurons activity. Muscle tone and regulation of the sleep-wake cycle are also dependent on *locus coeruleus* norepinephrine neurons. Dysregulations may result in a decreased excitatory feed to the *locus coeruleus* and with that narcoleptic and cataplexic symptoms. As EDS and cataplexy may be alleviated through augmented catecholamine levels and/or *locus coeruleus* activity, through enhanced norepinephrine neurons modulation of GABAergic signals from the amygdala, prospective narcolepsy medication target Hert/orexine *locus coeruleus* circuit integrity (Gilhus et al., 2011; Szabo, Thorpy, Mayer, Peever, & Kilduff, 2019).

2.1.2 The Effects of Dopamine and Dopamine Transporters as Target

Dopamine (DA) is synthesized in a few core areas of the brain stem (e.g. *basal ganglia* and *substantia nigra*) by the dopaminergic neurons, from L-DOPA. Norepinephrine and epinephrine are further neurotransmitters derivatized from dopamine, all of which are catecholamines. In peripheral tissue dopamine is synthesized predominantly by sympathetic nerve fibers and other catecholaminergic cells. The clearance from the synaptic cleft is implemented by the dopamine transporter DAT or is degraded by MAO-B (Herdegen, 2020; Huppelsberg & Walter, 2013; Rassow, Hauser, Netzker, &

Deutzmann, 2016). It is a neurotransmitter whose main tasks are the regulation of motor function within the *basal ganglia*, perception, thinking processes and behaviour within the limbic system (Huppelsberg & Walter, 2013). To be able to implement these functions, DA binds to a range of receptor subtypes, all of which are metabotrop receptors. Metabotrop receptors act through a second messenger-system. The binding of a neurotransmitter stimulates a G-Protein, which either leads directly to the stimulation of an ion channel, or indirectly through cyclic adenosine monophosphate (cAMP) or Inositol triphosphate (IP_3) (Huppelsberg & Walter, 2013). After DA is released into the synaptic cleft it is quickly re-absorbed through the DA transporter DAT, (Herdegen, 2013) as shown in *Figure 1*.

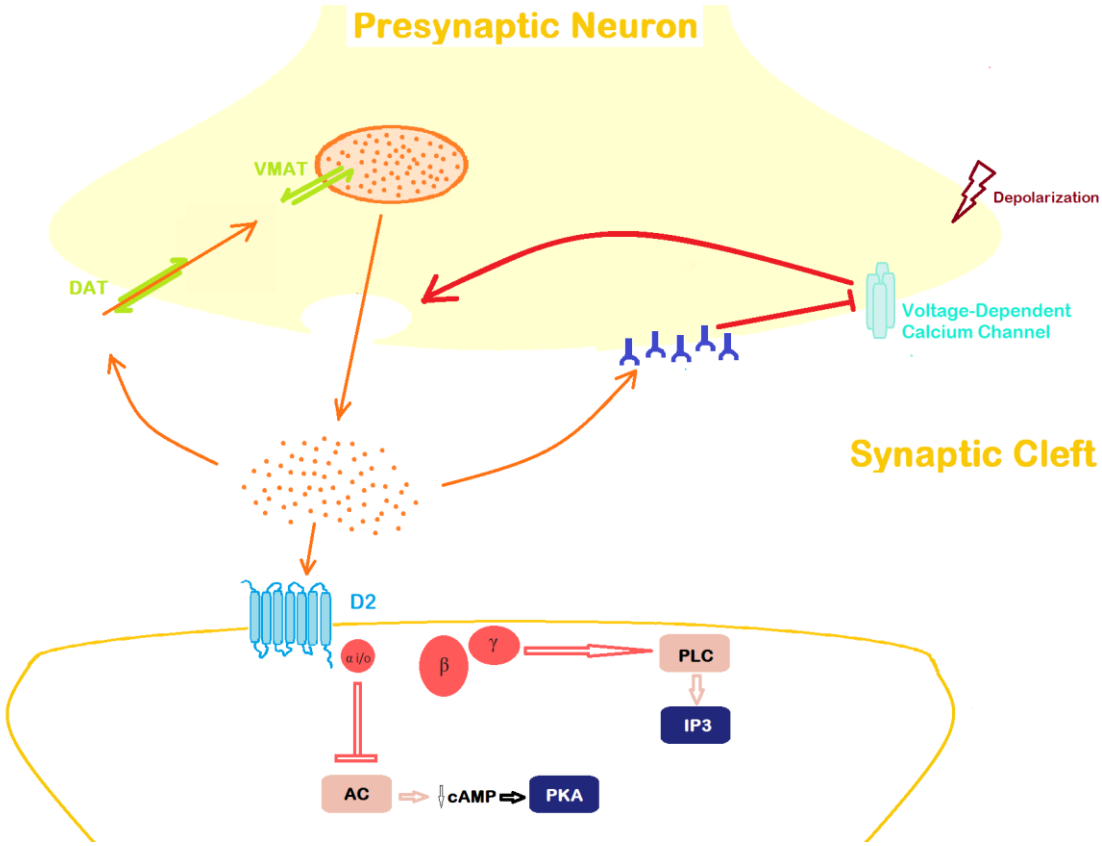

Figure 1. Schematic representation of the physiological dopamine cycle.
After the stimulation of the voltage-dependent Ca^{2+} channel, the vesicle merges with the cell membrane, where it releases dopamine (orange dots). After being released and triggering a response at the receptor on the postsynaptic neuron, the dopamine is resorbed through the dopamine transporter (DAT) and subsequently the vesicular monoamine transporter (VMAT) into a vesicle (Herdegen, 2013; Rassow et al., 2016).

The human DAT consists of 620 amino acids (Giros et al., 1992) and presents 12 transmembrane domains, a distinctive characteristic of sodium-dependent neurotransmitter carriers (Giros, el Mestikawy, Bertrand, & Caron, 1991). The DAT, shown in *Figure 2,* features six extracellular and five intracellular loops, as well as a cytoplasmatic amino-terminus and a carboxyl-terminus, which regulate the substrate intake (Fog et al., 2006). The second extracellular loop features three N-linked glycosylation sites, which are essential for the expression and function of the DAT (Li et al., 2004).

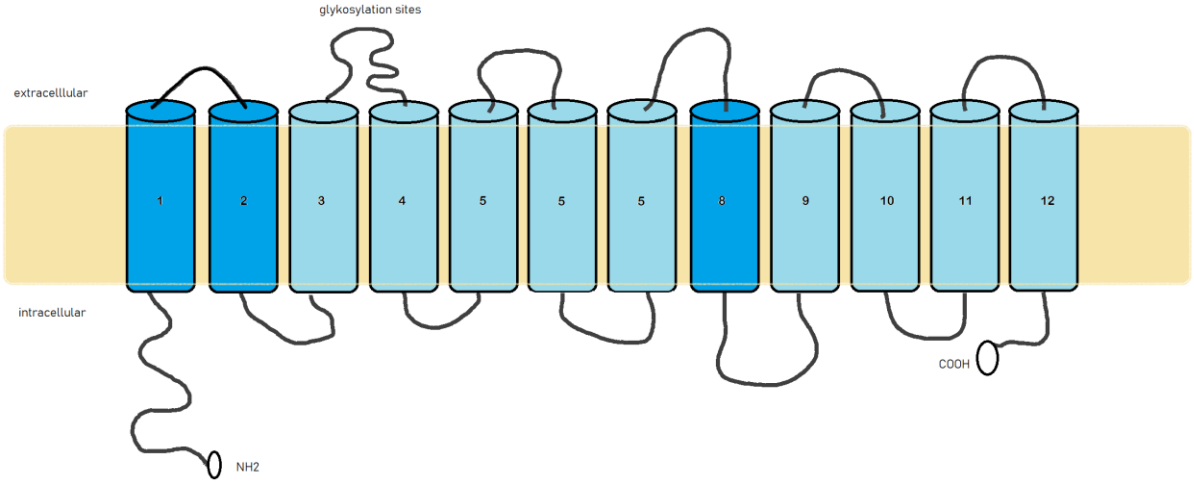

Figure 2. Schematic illustration of the DAT protein. The light blue cylinders represent the hydrophobic trans-membrane spanning domains that are linked by amino acid chains. The darker blue cylinders represent the conserved trans-membrane spanning domains that occur in DAT, the norepinephrine transporter NET, as well as the serotonin transporter SERT (1, 2, 8) (Madras, Miller, & Fischman, 2005).

2.1.3 Mechanism of Action of Dopamine Reuptake Inhibitors

The inhibition of the DAT, and with that the suppression of the Dopamine reuptake into the presynaptic neuron, illustrated in *Figure 3,* results in a surplus of Dopamine within the synaptic cleft, enhancing its effect on the postsynaptic level.

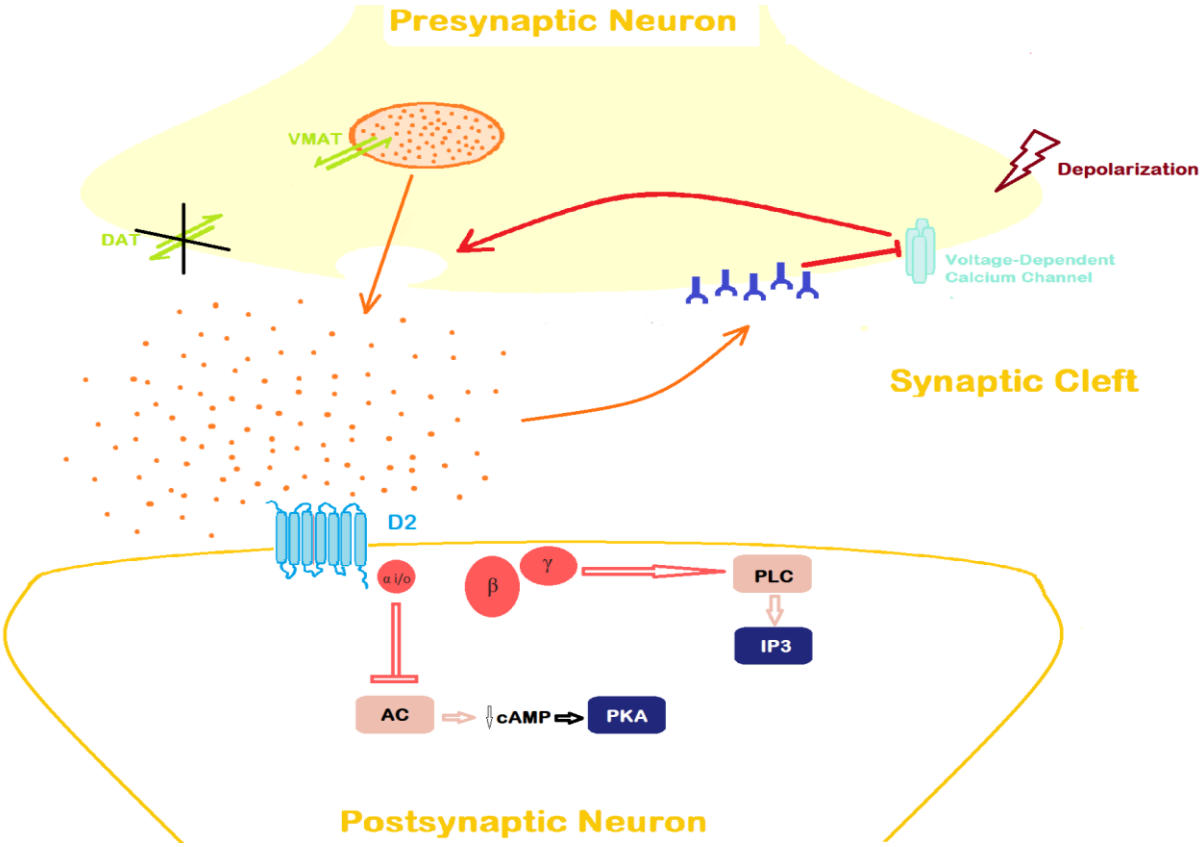

Figure 3. Schematic representation of the effect of DAT inhibition and the stimulation of dopamine receptor on the postsynaptic neuron. As dopamine cannot be reabsorbed through the DAT its concentration increases within the synaptic cleft and therefore leads to enhanced stimulation of the dopamine receptor on the postsynaptic neuron (Herdegen, 2013; Rassow et al., 2016).

Dopamine is stable within a synaptic vesicle, if however, Dopamine is not stored in synaptic vesicles, but released and not removed from the cleft, it may cause damage to the dopaminergic neuron. Dopamine is oxidized to the very reactive, radical-like dopamine quinone (Graham, 1978; Herdegen, 2013; Tse, McCreery, & Adams, 1976). Therefore the formation of dopamine quinone poses the threat of oxidative stress and with that cytotoxic effects, selective to dopaminergic neurons (Asanuma et al., 2004; Choi et al., 2003; LaVoie et al., 2005).

2.2 Modafinil

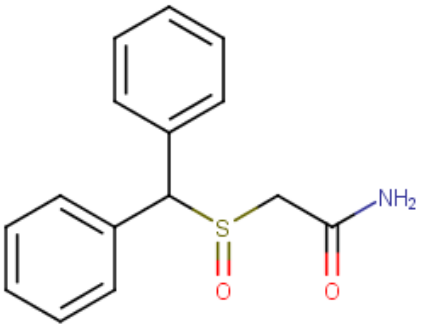

Figure 4. Chemical structure of Modafinil

The chemical structure of modafinil (*Figure 4*) is unaffiliated with dopamine and other synthetic stimulants of the central nervous system. Modafinil is suspected to act through the inhibition of DAT (SLC6A3), rather than stimulating a dopamine receptor itself (Zolkowska et al., 2009). Thereby, it is characterized as an atypical dopamine reuptake blocker. The blockage of DAT causes an increased extracellular concentration of dopamine in the synaptic gap, which leads to enhanced stimulation of dopamine receptors. The intensified stimulation subsequently leads to increased alertness through enhanced activity of wake-promoting neurons. T_{max} is reached two hours after administration. The metabolization is described in *point 2.2.1*. The elimination half-life amounts to 9 – 14 h. After two to four days the steady state is attained (Gilhus et al., 2011; Wisor et al., 2001). Modafinil might also increase the extracellular histamine levels (Ishizuka, Sakamoto, Sakurai, & Yamatodani, 2003) and also the extracellular levels of 5 - hydroxytryptamine within the brain (Luca Ferraro et al., 2002; Saint Hilaire, Orosco, Rouch, Blanc, & Nicolaidis, 2001; Tanganelli, Fuxe, Ferraro, Janson, & Bianchi, 1992). Modafinil primarily effects the parts of the brain that control wakefulness, but it does not appear to act as an alpha-agonist (E. Mignot, Nishino, Guilleminault, & Dement, 1994), to affect the glutamate reuptake, the gamma-aminobutyric acid (GABA), the glutamate synthesis (L. Ferraro, 1999), or to enhance alertness through orexin/hypocretin (Willie et al., 2005). Armodafinil, the (R)-(−)-enantiomer of the racemic modafinil, shows a prolonged plasma concentration but a similar half-life compared to modafinil (Gilhus et al., 2011).

Former indications for modafinil included obstructive sleep apnea, shift work disorder and idiopathic hypersomnia. The European Medicine Agency (EMA) revoked these indications in 2010 due to links to adverse effects, including life threatening conditions like Stevens-Johnson-Syndrome, cardiac arrhythmia, hypertension and severe psychiatric adverse reactions. Due to an increased risk of Stevens-Johnson-

Syndrome in children the EMA recommendation advises against its use in children (EMA, 2010).

2.2.1 The Metabolization of Modafinil

Modafinil is to a large part metabolised through the liver (~90%), the metabolites formed are subsequently excreted renally and only less than 10% of the administered modafinil is excreted unchanged. The main routes of metabolization are through hydrolytic deamination, aromatic ring hydroxylation, s-oxidation, as well as glucuronide conjugation. Resulting primarily in the formation of modafinil acid, but also at least six other metabolites that are detectable in urine samples. Modafinil acid along with modafinil sulfone can be found in significant concentrations in plasma samples. Preclinical tests show that modafinil acid, modafinil sulfone as well as e.g. 4-hydroxy modafinil are inactive or at least did not seem to have the effects of modafinil (FDA, 2010; Robertson & Hellriegel, 2003). Modafinil is in part metabolized by hepatic cytochrome P450 (CYP), especially by the 3A isoform CYP3A4. Modafinil has an inductive effect on CYP3A4, as well as on CYP1A2 and CYP2B6. It has inhibiting effects on CYP2C9 as well as CYP2C19. These described effects may influence the metabolization/clearance of other drugs, which can lead to drug-drug interactions, e.g. with diazepam or propranolol, which are eliminated through CYP2C19 (FDA, 2010; Gilhus et al., 2011; Robertson & Hellriegel, 2003).

2.3 CE-123

CE-123 or 5-((benzhydrylthio)methyl)thiazole, shown in *Figure 5*, is a modafinil derivate. The carboxyl-amid was replaced by a thiazole, which may be more resistant to biotransformation than the modafinils carboxyl-amide (Kristofova et al., 2018). The thiazole may also pose as a steric obstacle for any reaction at the sulfoxide partial structure.

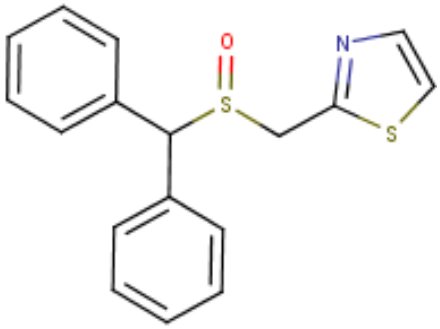

Figure 5. Chemical structure of CE-123

CE-123 was designed to fit the structure of the DAT-transporter. It shows a significant selectivity for DAT and has negligible effects on the SERT, as well as the NET in *in vitro* studies. The conducted studies imply no adverse side effects while providing performance enhancement. It is characterized as an atypical DA-reuptake blocker (Kristofova et al., 2018).

Due to their potential to ameliorate cognitive abilities drugs that block the dopamine transporter are popular. However, commercially available drugs, like Modafinil are rather unspecific for DAT, as they also inhibit serotonin and noradrenaline transporters. These drugs also have stimulating effects on dopamine release, which is leading to various adverse effects, for example oxidative stress on dopaminergic neurons (Miyazaki & Asanuma, 2009; Nikiforuk et al., 2017).

2.4 Metabolization Studies and their Relevance in Pharmacology

The aspect of metabolism has shown to be a crucial factor in clinical toxicology. Thereby not only the lack of toxicity of a compound has to be proven, but also the potential toxicity of metabolites, to ensure the safety and success of new drugs. A drug is subject to Phase I and Phase II biotransformation. The aim of phase I metabolization or functionalization reactions is to enhance a xenobiotics hydrophilicity. Phase I includes oxidation, hydrolyzation and dealkylation (Peters & Meyer, 2011). Due to the reactions involved in Phase I, its metabolites have a high potential to be pharmacologically active, which makes safety evaluations necessary (U.S. Department of Health and Human Services, FDA, CDER, 2016). In Phase II, which consists of conjugation reactions, a compound or its phase I metabolites are modified through glucuronidation with glucuronic acid, sulphation with sulphonic acid, or methylation. Metabolic enzymes are either situated in the smooth endoplasmatic reticulum, for example cytochrome P450 monooxygenases, flavin-monooxygenases (FMOs), and uridine-5-diphospho(UDP)-glucuronosyltransferases (UGTs), which are all partially membrane bound, in the mitochondria like Monoamine oxidases (MAOs) (also membrane-bound), or within the cytosol e.g. sulfotransferases (SULTs) or the catechol-*O*-methyltransferase (COMT) (Peters & Meyer, 2011).

The effect of active metabolites is hard to estimate, as they might bind to the targeted receptors, but might as well stimulate other targets. From the analytical perspective, chemically reactive metabolites or their intermediates may be hard to detect and or quantify due to their limited half-lives and their occurrence in low concentrations (U.S. Department of Health and Human Services, FDA, CDER, 2016).

2.4.1 *In vitro* Studies

Species specific enzymatic patterns and consequently differences in metabolization patterns between species suggest that animal trials are not ideal for either toxicity studies, or metabolism studies. The differences in enzyme patterns limit the transferability of the results and even though it is unlikely for an active metabolite to only occur in humans, it is likely that the proportion of formed metabolites vary from species to species. These quantitative variations arise from species specific genetic distinctions and pose as a risk factor in the evaluation of drug toxicity (U.S. Department of Health and Human Services, FDA, CDER, 2016).

Primary human hepatocytes are recommended as an attractive alternative to animal testing, providing the natural enzyme clusters and drug transporters, with the limitation of limited lifespan and challenges in handling and maintenance. Immortalized cell lines provide an alternate easy-to-handle, easy-to-maintain option. As these cell lines originate from mutated tissue, they show dedifferentiated behaviour and their characteristics are not corresponding to their parent tissue. Examples for dedifferentiated behaviour are over- or under expression of genes, excessive growth, significant resistance and abnormal energy metabolism. Immortalized cell lines are, for example often highly resistant to metabolic toxins and reactive species affecting the mitochondria, due to an underemployment of the mitochondria, which leads to unreliable results regarding these targets. These characteristics are the reason for their robustness, but also the reason for their limitation in toxicity models. Nevertheless immortalized cell lines represent a practical tool for toxicity and pharmacodynamic evaluation, as they maintain some characteristics of the original tissue, with relevance to *in vivo* systems (Coleman, 2010; Szabo et al., 2019).

2.4.2 Cell Lines

As primary human hepatocytes are difficult to acquire and to handle, hepatoma cell lines, especially HepG2 are frequently used as an alternative in *in vitro* testing. Immortalized cell lines have the virtue of availability, ease of use, unlimited and quick mitosis and a stable phenotype. Unfortunately, their limitation is a significantly decreased expression of metabolic enzymes, compared to primary hepatocytes (María Teresa Donato, 2014).

Kanamori et al. (2015) and Wohlfarth et al. (2013) concluded that HepaRG cells may be a suitable substitute to imitate human metabolism with the restrictions of an in vitro system (Richter, Flockerzi, Maurer, & Meyer, 2017).

2.4.2.1 HepG2

HepG2 cells were retrieved from a liver biopsy of a Caucasian male suffering from a differentiated hepatoblastoma, in the early 1980s (Knowles, Howe, & Aden, 1980). It is a nontumorigenic cell line with an epithelial-like morphology and high proliferation rates that show various differentiated hepatic functions (Fearn & Hirst, 2006), for example insulin signalling, metabolism of cholesterol, triglycerides, lipoproteins and the synthesis of bile acid, glycogen and plasma proteins (Dongiovanni et al., 2008; Forte, McCall, Knowles, & Shore, 1989; Javitt, 1990; Knowles et al., 1980; Meier, Klein, Kramer, Drenckhan, & Schütt, 2007).

It is the most frequently used cell line in drug hepatotoxicity and metabolism studies (Donato, Tolosa, & Gómez-Lechón, 2015). Unfortunately, its CYP expression is variable and rather low for CYPs like 1A1 and 1A2, some CYPs expression is even switched off completely. This also applies for the expression of other phase I metabolizing enzymes, like aldehyde dehydrogenases, flavin-containing monooxygenases, alcohol dehydrogenases (Coleman, 2010; Szabo et al., 2019). While phase II enzyme expression levels are similar to those of HepaRG, apart from some UGTs (Gerets et al., 2012; Hart et al., 2010; Kittler, Fessard, Maul, & Hurtaud-Pessel, 2014; Richter et al., 2017).

2.4.2.2 HepaRG

The HepaRG cells were retrieved from a female patient suffering from the Hepatitis C Virus (HCV) and as a result, of cholangiocarcinoma. The genome of HepaRG cells stays unchanged. The HepaRG cell line relies on promoting and utilising the available properties of the genome. HepaRG carries properties of early hepatic progenitor cells, as well as mature hepatocytes, enabling novel hepatic cell models. HepaRG were not genetically modified, which leaves them with various hepatic features and makes them applicable to many means, for example for the investigation of lipid and sugar metabolism (BIOPREDIC INTERNATIONAL, 2011; Gripon et al., 2002). Their gene expression of metabolizing enzymes is equivalent to primary human hepatocytes (Gerets et al., 2012; Wohlfarth et al., 2015), with the exclusion of CYP2D6 (Hart et al., 2010), which metabolizes various drugs of abuse (DOAs) (Peters & Meyer, 2011; Richter et al., 2017).

2.4.3 Metabolization Studies

The main routes for biotransformation are Uridine 5'-diphospho-glucuronosyltransferase (UDP)-glucuronosyltransferases, glutathione S-transferases and the Cytochrome P450 oxidases. Functional groups make molecules vulnerable for (bio)transformation, large, planar, lipophilic, molecules with few functional groups are rather stable and are unlikely to be metabolized. If there are no functional groups, but aliphatic and aromatic structures, the preferred point of attack is any aliphatic group or non-aromatic ring. But even the stable aromatic structure can be altered by the highly potent CYP1A1. It is highly effective in oxidizing aromatic structures, making them more hydrophilic, but also more reactive. Benzene as the simplest aromatic structure can be bio-transformed to a more reactive and more hydrophilic structure, namely a phenol. The phenol is, contrary to the benzene, vulnerable to glucuronidation and sulphation during phase II (conjugative) metabolization and enables the clearance of xenobiotics with aromatic partial structures (Coleman, 2010).

Cytochrome P450 oxidases are omnipresent within the body, as they can be found in any tissue, but the bio-transformational capacity is foremost located in liver and gut. They fulfil numerous duties like xenobiotic clearance, steroid biosynthesis and metabolic conversion. The CYPs located in the liver or gut are mainly responsible for the processing and clearance of diverse xenobiotics. CYPs in other organs only

account for a small part of the overall clearance of a drug. They are however also responsible for the formation of toxic intermediates of xenobiotics. So far about 60 variations of CYPs have been discovered, of these 60 about half fulfil a highly specific bio-modulatory task that differ from high-volume chemical oxidation. A good example is the brain, where CYPs are found in rather low concentrations (often ≤ 2% compared to the hepatic CYP levels), depending on the area of the brain, as CYPs are not evenly distributed. Their role in the central nervous system (CNS) does not include large-scale metabolization like their hepatic counterparts, but to catalyse certain neuronal functions by regulating endogenous units such as neuro-steroids.

The hepatocytes however have to fulfil two tasks simultaneously:

1. Metabolization of the substances absorbed in the gut
2. Processing substances already in the peripheral blood circulation.

Due to the blood circulation routes a xenobiotic will pass through the liver for bio-transformation without exception regardless of the form of admission (Coleman, 2010).

2.5 Liquid Chromatography-Mass Spectrometry (LC-MS)

Liquid Chromatography-Mass Spectrometry is a liaison between liquid chromatography and mass spectrometry, linked through an interface and processed via a data system. The schematic structure of an LC-MS is outlined in *Figure 6.*

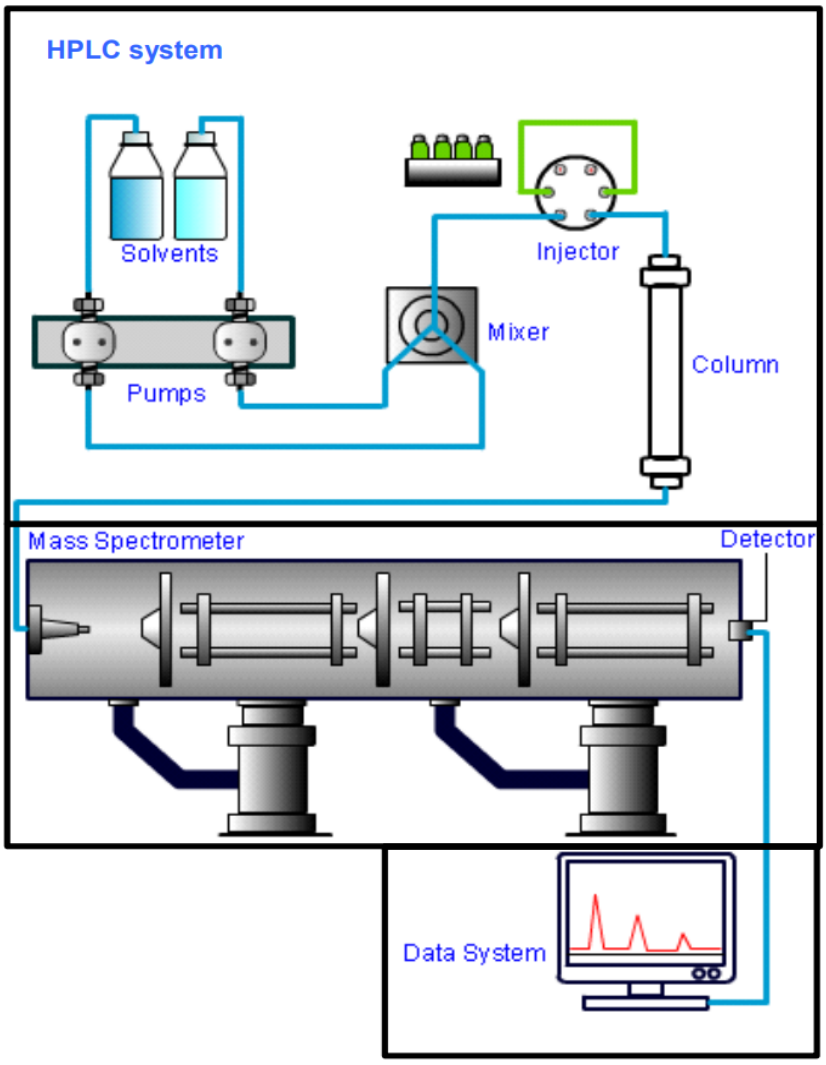

Figure 6. Schematic description of an LC-MS. It consists of four main parts: the UHPLC (solvent vessels, one or several pumps, a mixer, an injector, a column and one or several detectors), the Interface, the MS (a heated vacuum chamber, an ionization chamber, a quadrupole and a Time of Flight (TOF) detector) and a Data System. This image was acquired from (CHROMacademy by Crawford Scientific, n.d.).

2.5.1 Ultra-High-Performance Liquid Chromatography

Ultra-High-Performance Liquid Chromatography (UHPLC) was developed on the bases of column liquid chromatography. It separates the compounds of a heterogeneous mixture due to their physicochemical properties. The basic principal relies on the adsorption capacity of the stationary phase and the mobile phase, which result in different retention times. UHPLC is an advanced version based on that principle. The use of high pressure allows a more densely packed stationary phase with smaller particles, which leads to a higher amount of theoretical plates and with that superior resolving power. Another way to enhance or adapt the separation of a heterogenous mixture is by adapting the mobile phase (liquid), by utilizing either an isocratic elution, where the mobile phase is an invariable composition of solvents, or by employing a gradient elution, where the mobile phase composition is gradually changed (Lundanes, Reubsaet, & Greibrokk, 2014).

2.5.2 Mass Spectrometry

Mass spectrometry is an analytical method that is often used as a complementation of nuclear magnetic resonance (NMR) for the determination of the chemical structure of unknown organic compounds. It is however not sufficient to solely identify an unknown compound. Combined with chromatography (e.g. LC-MS) it is the ideal tool to separate and quantify single compounds from complex matrices. An ion source converts the sample's molecules into negatively and positively charged, as well as neutral fragments. These fragments are later separated according to their mass-to-charge ratio (m/z), before being captured by a detector. The positive ions are usually of the most relevance, even though the fragmentation patterns of negative ions resemble the fragment patterns of positive ions, most of which correlate with their parent compound. The formation of the surplus of fragments can be predicted or traced back by established fragmentation patterns (Manfred H. Gey, 2008; Thompson, 2017). After the ionization the fragments are funnelled through to the mass analyser. Common mass analyser types are time-of-flight, quadrupole, ion trap-, and fourier transform analysers (FT) (Lundanes et al., 2014). However the predominant majority of the injected sample is not fragmented and measured, but is removed from the ionization chamber with high vacuum. Due to the nature of the method, samples are subjected to destructive fragmentation, in contrast to other analytical-instrumental methods. The

downside is that fragmentation renders the sample useless for other analytical methods. The upside is that only a very small amount of a nanogram is needed to obtain a mass spectrum. Limits of detection can be as low as a nano- (ng) or femtogram (fg), which makes the method eligible for structural information of small samples (Manfred H. Gey, 2008; Thompson, 2017; Woods & Darie, 2014).

2.5.2.1 Electron Spray Ionization

A significant development for LC-MS was the invention of electron spray ionization (ESI). It allows the analysis of intact molecules and represents a soft ionization method. ESI allows a direct desorption and ionization from the liquid phase as it is based on the formation of charged droplets. With the evaporation of the solvent, the surfaces charge increases, so as soon as the repulsion of surface charges surpass the surface tension, the droplets disintegrate. This process iterates until gas phase ions are formed (D. Y. Lee, Bowen, & Northen, 2010). This technique is significantly less invasive than the bombardment with electrons and with that enables new bioanalytical methods.

2.5.2.2 Quadrupole Mass Analyser

As the name suggests the quadrupole mass analyser is built from four parallelly arranged poles, each of which generates an oscillating electric field by employing a direct current (DC) and radio frequency (RF). Due to the entry of ions in the z-direction, they oscillate in the x- and y-directions. The quadrupole detects only the ions that have a consistent trajectory and consequential are not colliding into one of the four pols. The deployment of distinctive DC and RF values ensure that only ions with a specific m/z value reach the detector (Lundanes et al., 2014).

2.5.2.3 Time-of-flight (TOF) Analyser

As implied by the name the TOF-analyser measures the time it takes an ion from the entrance in a field-free drift tube to the detector. It is made of a pulsed source and two plates with a large potential difference. When the ions attain similar kinetic energy, the source accelerates the ions and moves them towards the drift tube. Heavy ions have a longer TOF compared to the TOF of light ions due to the reversed proportionality of velocity and m/z value. As a result a high resolution separation can be achieved (Lundanes et al., 2014).

2.5.3 LC-MS Interface

The LC-MS interface represents the intersection between LC and MS. The main task of the LC-MS interface is the transfer of analytes from the liquid phase of the LC to the vaporized gas phase of the MS and subsequently the ionization (M. S. Lee, 2002).

2.5.4 LC-MS as Analytical Tool and its Integration into Drug Development

LC-MS-based techniques are employable to a broad variety of substances. LC-MS allocates analytical figures of pharmaceutical interest like selectivity and sensitivity and provides other positive attributes like cost and time effectiveness. With that the LC-MS as an analytical tool meets the focus of quantitative, concise and prompt characterization of new compounds. These features allowed a shift from pure samples to complex sample matrices like natural products including animal and human products. These are the reasons why LC-MS is now the method of choice for numerous pharmaceutical analyses (M. S. Lee, 2002).

2.6 Analysis

2.6.1 Metabolite Analysis

Peters and Meyer (2011) pointed out that metabolite standards are crucial to be able to identify and quantify a metabolite, as well as to confirm the suggested mass spectrometric results. These standards are however presumably not commercially available and might be difficult to synthesise. Another rather costly possibility would be a custom-made metabolite standard.

Due to these reasons alternative strategies were crafted, to be able to bypass this procedure:

Staack et al. (2004) and Theobald and Maurer (2007) assumed a linear relationship between metabolite response and its concentration in the sample. The responses were utilized as a representative value for metabolite concentrations. To underline their reasoning, they used compounds with analogue structures compared to the metabolites. Linearity experiments were conducted and enabled a 'relative' quantification (Peters & Meyer, 2011).

Springer, Staack, Paul, Kraemer, and Maurer (2003, 2005), assumed that a structurally alike internal standard provides approximately the concentration-response relationship of the targeted metabolite. Relying on this postulation the absolute concentration of the metabolite was determined (Peters & Meyer, 2011).

From an analytical perspective neither method is optimal, but it is plausible to presume that the analytical data is accurate as all isoenzymes were evaluated with the same quantification tool (Robertson & Hellriegel, 2003).

2.6.2 Quantification

The specification of the concentration of analytes is called quantification. For quantification purposes, the detector response to the sample, which is either defined by the height of the peak or the area of the peak, has to surpass a threshold of a 10:1 signal-to-noise ratio, whereas the limit of detection is defined by a signal-to-noise ratio of at least 3:1 (Lundanes et al., 2014).

For quantification purposes four different methods can be employed:

1. *Normalized peak areas method*

   Is the simplest, but also the least accurate method. The total of all peak areas is summated and the concentration of every compound is calculated in area %, under the presumption of equal detector response. This method only provides a relative concentration of compounds within a sample (Lundanes et al., 2014).

2. *External standard method*

   The foundation of the external standard method is the creation of a calibration curve by measuring solutions with known concentrations of the analyte. The detector responses (peak area/peak height) y are applied as a function of the analyte concentration x. Resulting in the calibration equation y=ax+b (Lundanes et al., 2014).

3. *Internal standard method*

   In the internal standard method samples and calibration standards are supplemented with a defined concentration of an internal standard. The internal standard is a diligently selected compound that has to fulfil multiple requirements. It has to provide equivalent physicochemical behaviour to that of the analyte and consequently a retention time analogical to the analytes retention time, a similar peak height, as well as availability in high purity and robustness. The calibration curve describes the ratio of analyte to internal standard signal intensity, as a function of the ratio of the concentration of the analyte and the concentration of the internal standard (Lundanes et al., 2014). The internal standard also represents a correction factor that may be utilized to equalize sample manipulations or technical problems during sample

preparation, injection or ionization. This method is indicated for complex samples (Lavagnini, 2010).

4. *Standard addition method*

   This method can be utilized for the stipulation of a known analyte but an unknown concentration in a complex matrix (Lavagnini, 2010). It is used seldomly, as it is elaborate and time consuming to conduct. The procedure includes multiple analyses per sample, as each sample is divided into aliquots, and each is supplemented with a different quantity of standard analyte. The results are dependent on the standard analyte concentration, which are then utilized to calculate the analytes concentration within the sample through extrapolation of the values of the calibration curve (Lundanes et al., 2014).

3 Project

The goal of this study was to determine whether metabolization of CE-123 can be achieved in a hepato-cell-culture-model and if so, identify, characterize and quantify possible metabolites of the modafinil derivate CE-123. The FDA provides the following groundwork on how to design nonclinical studies, and points out important factors to consider while doing so, (U.S. Department of Health and Human Services, FDA, CDER, 2016):

1. *Analogy of the metabolite to the parent molecule*
   As there is no known metabolite, it was necessary to consult a source of information. For this project, this source was the Meteor™ nexus software that provided suggestions to what the possible metabolites could be.

2. *Chemical or pharmacological characterization*
   CE-123 is a 5-((Benzhydrylsulfinyl)methyl)thiazole, which is chemically similar to Modafinil a 2-[(diphenylmethyl)sulfinyl]acetamide.
   CE-123 is characterized as an atypical Dopamine reuptake inhibitor.

3. *Solubility*
   CE-123 is not soluble in water, soluble in DMSO (when heated), soluble in ACN.

4. *Phase I versus Phase II metabolites*
   There was no special focus, as the goal was to identify any metabolite.

5. *Relative amounts detected in humans versus the amounts detected in animals*
   There is no data available, as there were no clinical studies conducted so far. In animal trials however, 10 mg/kg CE-123 racemate bodyweight were administered intravenous. The maximal plasma concentration 5 minutes post dose amounted to about 9.3 µg/mL, according to internal unpublished research.

HepG2 cells were utilized to establish the basic experimental conduct. As described the HepG2 cells have a rather low cytochrome P450 activity. Especially CYP 1A1 and 1A2 are being expressed on a significantly lower rate than in intact human hepatocytes. This might be of interest, as CYP1A1 is responsible for the transformation of benzene to phenol. Also the levels of other phase I enzymes like flavin-containing monooxygenases, alcohol dehydrogenases and aldehyde dehydrogenases are relatively low (Coleman, 2010; Richter et al., 2017; Westwick, 1976).

Nevertheless, HepG2 is an easy to handle cell line, so the HepG2 cells were used to establish and optimize the assay. The initial experiment was adapted for example by dosage adjustment and elimination of interfering factors within the experiment and in the sample preparation process. After establishing the outlines of the assay, HepaRG cell line were used as an alternative, with enhanced metabolic performance in comparison to HepG2 and comparable metabolic capacities to primary human hepatocytes. Due to limited resources only one experiment was conducted with HepaRG cells.

4 Materials and Methods

4.1 Materials

4.1.1 Cell Lines

HepG2 Cells were provided by the Faculty of Life Sciences of the University of Vienna. Differentiated HepaRG cells were purchased from Biopredic International.

4.1.2 Chemical Compounds

4.1.2.1 R-Modafinil

Dr. Predrak Kalaba, MSc and Vladimir Dragačević, MSc synthesized R-Modafinil, for our purposes (Jung et al., 2012). R-Modafinil is a (2-[(diphenylmethyl)sulfinyl]acetamide), with a chiral centre located at sulfoxide function. The sum formula is $C_{15}H_{15}NO_2S$, the molecular mass is 273.35 g/mol. R-Modafinil is practically insoluble in water, it is however soluble in ACN and DMSO. R-Modafinil was used as reference substance as well as an internal standard in LC-MS analysis.

4.1.2.2 CE-123

CE-123 was synthesised by Dr. Predrak Kalaba, MSc and Vladimir Dragačević, MSc. The sum formula of 5-((Benzhydrylsulfinyl)methyl)thiazole or CE-123 is $C_{17}H_{15}NOS_2$ and the molar mass amounts to 313.44 g/mol. It is a derivate of modafinil, but is due to its structure much more selective to its target DAT (Kalaba et al., 2017). CE-123 and Modafinil share the diphenylmethane partial structure. The difference in their structures is limited to the thiazole (CE-123) or amide (Modafinil) part of the compound.

4.1.2.3 Diazepam

The utilized Diazepam was obtained from Vitrum AB, Stockholm, Batch No 1563, and was used as an internal standard for the experiments conducted with Modafinil. The sum formula is $C_{16}H_{13}ClN_2O$ and the molar mass amounts to 284.74 g/mol.

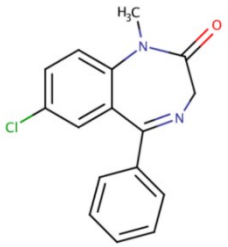

Figure 7. Structure of Diazepam

4.1.2.4 Other Chemical Compounds

A complete account of utilized chemicals is listed in *point 7.2 Liquids and Chemicals*.

4.1.3 LC-MS

4.1.3.1 Liquid Chromatograph

UltiMate 3000 RSLC-series system (Dionex); Thermo Fisher Scientific, Inc., Gemering, Germany.

Kinetex Phenyl-Hexyl 2.6 µm 100 Å LC column (50x2.1mm I.D), Phenomenex, Inc., Torrance, CA, United States.

4.1.3.2 High Resolution Mass Spectrometer

maXis HD ESI-qq-TOF mass spectrometer, Bruker Corporation, Bremen, Germany.

4.1.4 Centrifuge

Eppendorf Centrifuge 5804 R, Eppendorf AG, Hamburg, Germany

4.1.5 Metabolite Prediction

Metabolite prediction software Meteor™ nexus by Lhasa Limited, Leeds, United Kingdom.

4.2 Methods

4.2.1 Cell Cultivation

4.2.1.1 HepG2

The adherent HepG2 cells were cultured in either 25 cm^2 or 75 cm^2 flasks. For cultivation of HepG2 cells Dulbeccos's Modified Eagle's Medium (DMEM) high glucose was used as a basis and supplemented with 10% fetal bovine serum (FBS), 2% Glutamate and 1 % Penicillin and Streptomycin to prevent bacterial infections. As the HepG2 cell line is rather robust, the cell splitting cycles were adapted to the presented conditions. Splitting was conducted approximately once every six days. The HepG2 cellular growth and cluster development is shown in *Figure 8 - Figure 10.* The media was changed every two to three days. HepG2 cells were washed with phosphate buffer saline (PBS) twice to remove dead cells, cell debris and cell products like collagen and other excreted cellular proteins. After purging, new media was administered to insure ideal conditions for cell growth. When confluent (at approximately 80%), usually after five to six days, cells were splitted in a 1:5 ratio. After purging the cell monolayer twice with PBS, trypsin was added to detach the cells from the surface and allow splitting. For the trypsinisation 0.25% trypsin in 1 mM ethylene-diamine-tetra-acetic-acid (EDTA) was used. The trypsinisation process was stopped after about four minutes, providing efficient and swift detachment of cells from the culture bottle. Experiments were conducted between passages three to ten, in 6-well plates in a corresponding volume of 2 mL of completed DMEM. After 24 h of incubation a monolayer was obtained and the completed DMEM was removed and replaced with completed DMEM supplemented with CE-123 or modafinil. The experiments were conducted in triplets and a blank, with variable incubation time spans and variable concentrations (see *point 5.1*. *Experiments*).

All these steps were conducted in the sterile environment of the Lamina Airflow. Cells were incubated at 37 °C and 5% CO_2 in a humidified atmosphere.

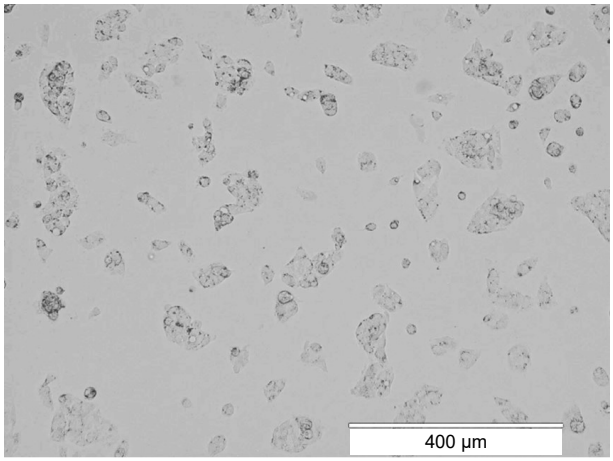

Figure 8. HepG2 24 h after splitting

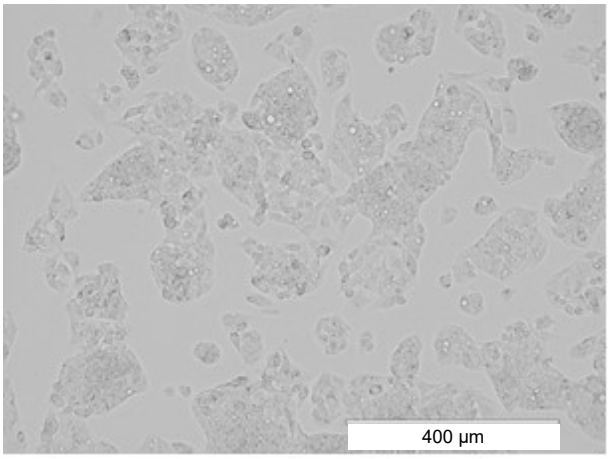

Figure 9. HepG2 60 h after splitting

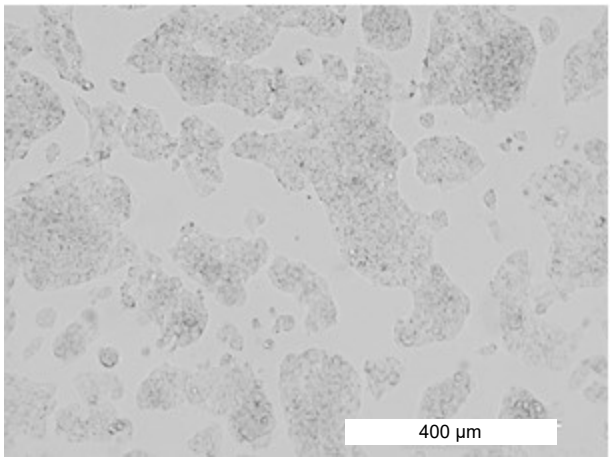

Figure 10. HepG2 72 h after splitting

4.2.1.2 HepaRG

HepaRG cells were cultivated according to the provided protocol by Biopredic International. The HepaRG cell line's creation of a tissue like structure is documented in *Figure 11-16.*

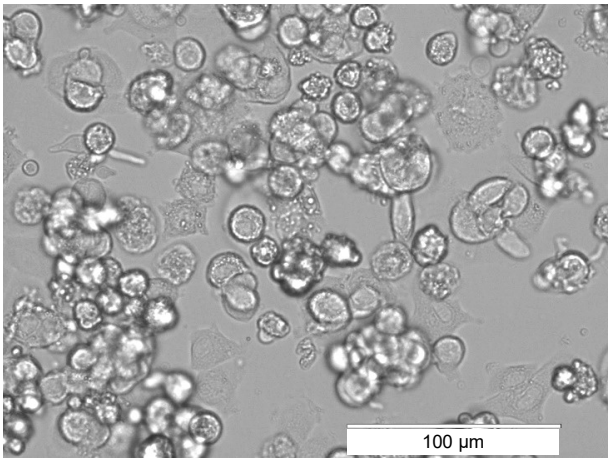

Figure 11. HepaRG 4 h after seeding

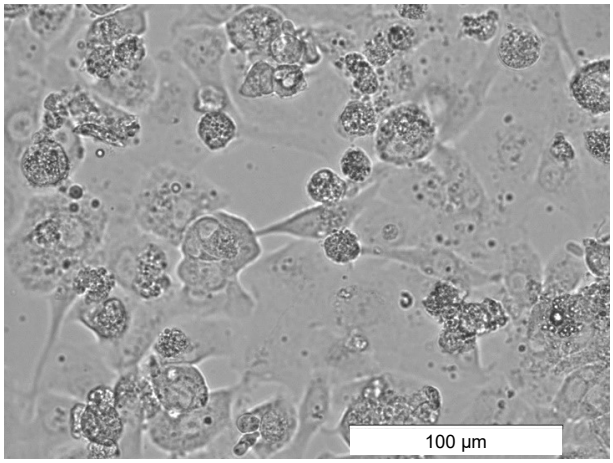

Figure 12. HepaRG 24 h after seeding

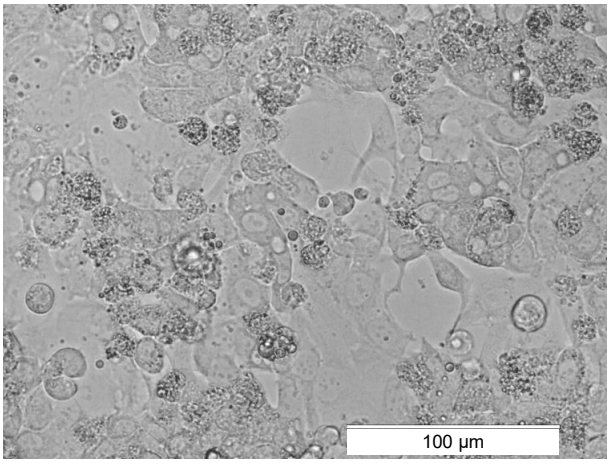

Figure 13. HepaRG 52 h after seeding

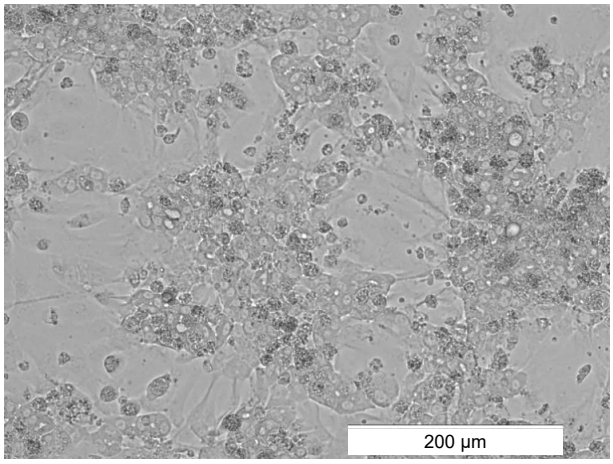

Figure 14. HepaRG 94 h after seeding

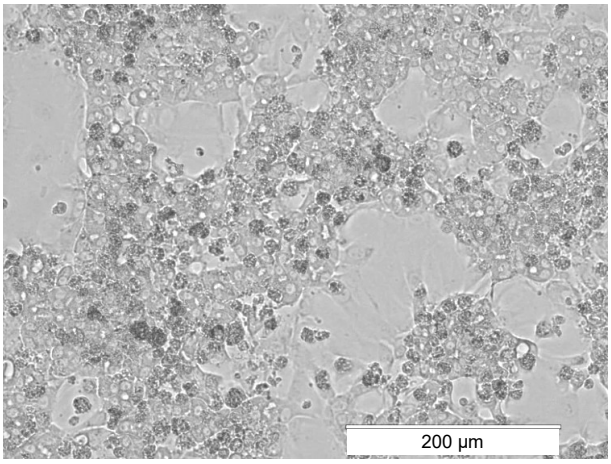

Figure 15. HepaRG 126 h after seeding

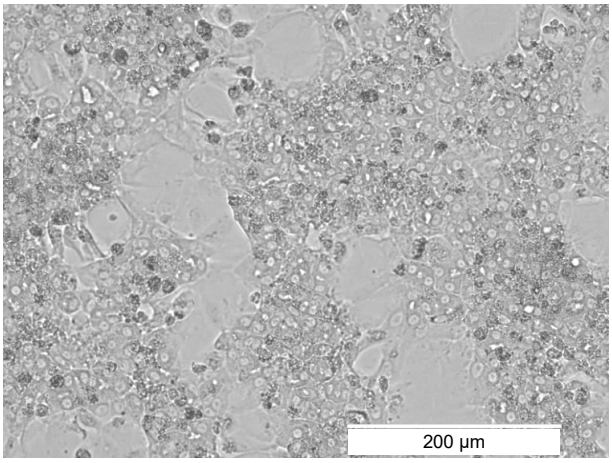

Figure 16. HepaRG 168 h after seeding

4.2.2 Dosage Finding

At the beginning various low concentrations were used. The applied dosage of CE-123 was in compliance with the maximal plasma concentration in rats that was provided by internal, unpublished research. The dosage in rats was administered intravenously. After 15 minutes the measured plasma levels were 9.3 µg/mL for CE-123. The maximal concentration used in the metabolization study was 9.5 µg/mL. The applied Modafinil concentrations were 1 µg/mL, 5 µg/mL or 10 µg/mL.

4.2.3 In Vitro Studies

For method establishment a monolayer cell culture offered the ideal access point, as it is easy to handle. Preliminary tests were conducted with HepG2 cells, with various cell counts and plates, as described in *point 5.1.1*. Experiments were conducted with HepG2 and HepaRG cells, as outlined in *point 5.1*. 1 x 10^6 HepG2 cells were seeded in a 6-well plate. 24 hours incubation provided the cells with enough time to attach to the well's surface and to proliferate. 0.8 x 10^6 HepaRG cells were seeded into 12-well plates and incubated for four hours, before substrate was added, which is in accordance with the protocol provided by Biopredic International.

CE-123 and modafinil were solubilized in ≥99.5% dimethyl sulfoxide (DMSO) and then diluted with completed DMEM media to a maximal DMSO concentration of 0.1%. The dilution was conducted in 1:10 dilution steps. The media was removed, the cells were purged twice to remove dead cells, debris and other impurifications. The CE-123 or Modafinil solution was added to the cells and was then incubated. Every experiment was conducted in triplets and included a blank.

4.2.3.1 HepG2 Experiments

Substrate preparation

Modafinil/CE-123 was weighted and dissolved in sterile filtered DMSO (≥99.5%), as DMSO is toxic for cells the solution was diluted with DMEM until the DMSO concentration amounted to 0.1%. The dilution was conducted in 1:10 steps.

Application

The media was removed, and the cells were washed with PBS twice. 2 mL of the substance solution was applied to each well. After the application the plate was returned to the incubator and the Samples were taken after the intended incubation time e.g. 24 h or 48 h.

Sample preparation

To ensure the inhibition of enzyme activity, samples were stored on ice during sample preparation. 500 µL of supernatant was transferred into a microcentrifuge tube, 500 µL iced acetonitrile (ACN) to precipitate proteins and 500 µL internal standard in ACN were added. In the beginning the samples were centrifuged at 4 °C, 10 min at 1800 rpm, then the supernatant was transferred into an microcentrifuge tube, 1:1 mixed with H_2O and sterile filtered with 0.2 µm, RC-membrane, single use syringe filters, later the sterile filtration step was cut and replaced by extensive centrifugation (5000 rpm for 30 min) to ensure that no compound would be adsorbed by the filter membrane.

4.2.3.2 HepaRG Experiment

Substrate preparation

Modafinil and CE-123 were weighted and dissolved in sterile filtered DMSO (≥99.5%), as DMSO is toxic for cells it was diluted with the provided supplemented Williams E until the DMSO concentration was 0.1%. The dilution was conducted in 1:10 steps.

Application

The cells were not completely attached to the wells after 4 hours and therefore the media could not be removed. Subsequently, 1 mL medium supplemented with an adapted concentration of CE-123 was added to the 2 mL of Williams E media to each well and gently mixed. After the application the plate was returned to the incubator. The samples were extracted after 20 h, as the metabolic activity declines after 24 h, according to the protocol provided by Biopredic International.

Sample preparation

To ensure the inhibition of enzyme activity, the samples were stored on ice during sample preparation. 500 µL of the supernatant were transferred into a microcentrifuge tube, 500 µL iced ACN to precipitate proteins and 500 µL internal standard in ACN were added. To cut out the filtration step and minimize loss of analyte the sample was centrifuged at 4° C, 30 min at 5000 rpm. A phase separation due to centrifugation was not visible (as the media is colourless). The entirety of the supernatant was extracted and mixed with H_2O (1:1). The prepared sample was pipetted into an HPLC vial and measured.

4.2.4 Metabolization Prediction by Meteor™ nexus

Meteor™ nexus was employed as a source of possible metabolites. The goal was to predict likely metabolites of CE-123 and their corresponding masses, to enable a rational approach. To estimate the adequateness of the results modafinil was analysed as a reference substance, with known metabolites.

4.2.4.1 Predicted Metabolization of R-Modafinil

The metabolization prediction of modafinil was performed, to establish if the Meteor™ nexus software prediction was in accordance with the known metabolites of modafinil and to ensure the adequate analysis and the reliability of the results. The prediction is corresponding to established experimental findings, which show modafinil acid and modafinil sulfone as main metabolites, but also includes the formation of a thioether from the sulfoxide as well as a para-hydroxylation of one of the monosubstituted benzenes, as illustrated in *Figure 17*.

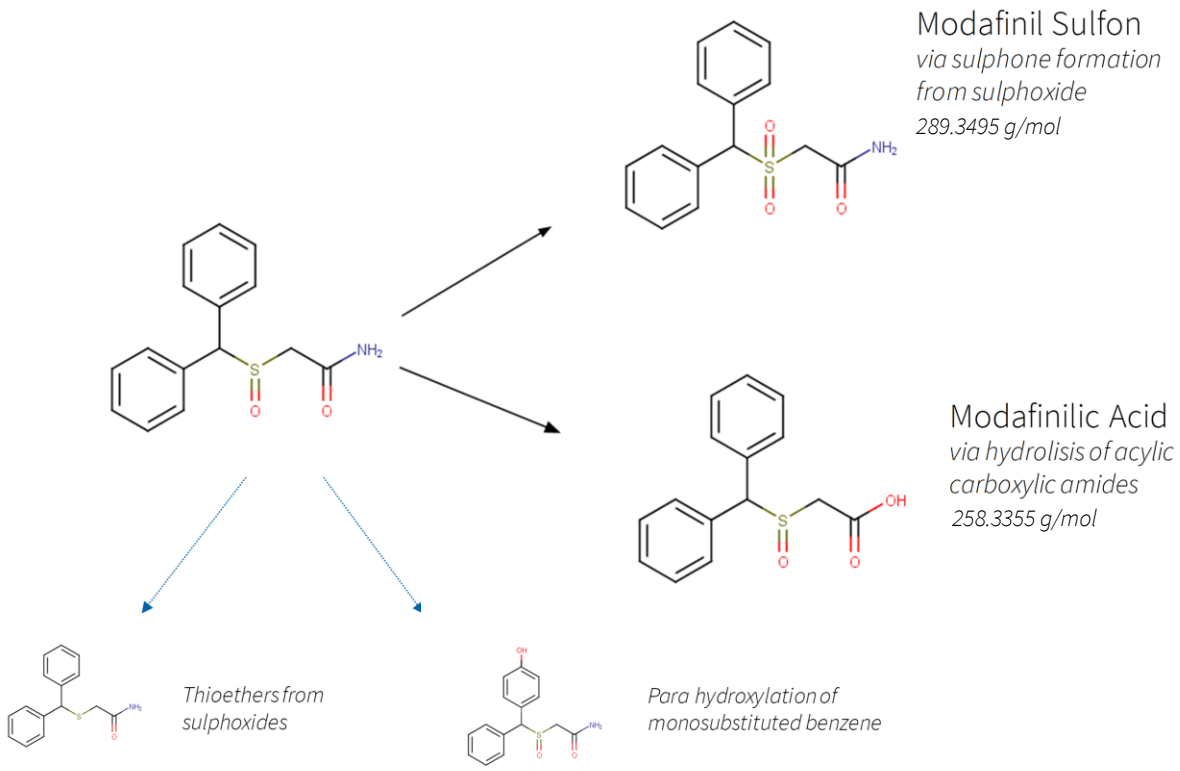

Figure 17. Selection of predicted metabolites of Modafinil by Meteor™ nexus. Modafinil sulfone and modafinil acid are the known main metabolites of modafinil. Other metabolites predicted by Meteor™ nexus are the thioether and a para-hydroxylated modafinil.

4.2.4.2 Predicted Metabolization of CE-123

In accordance to the structural similarities between modafinil and CE-123, the predicted metabolization, shown in *Figure 18,* shows distinctive similarities between the proposed metabolization of CE-123 and the metabolization of modafinil. The differences in structure and predicted metabolites are focused on the thiazole partial structure. It is not as reactive as the amid partial structure of modafinil and therefore is not predicted to being transformed. A benzene on the other hand could be enzymatically processed and transformed via para-hydroxylation to a phenol.

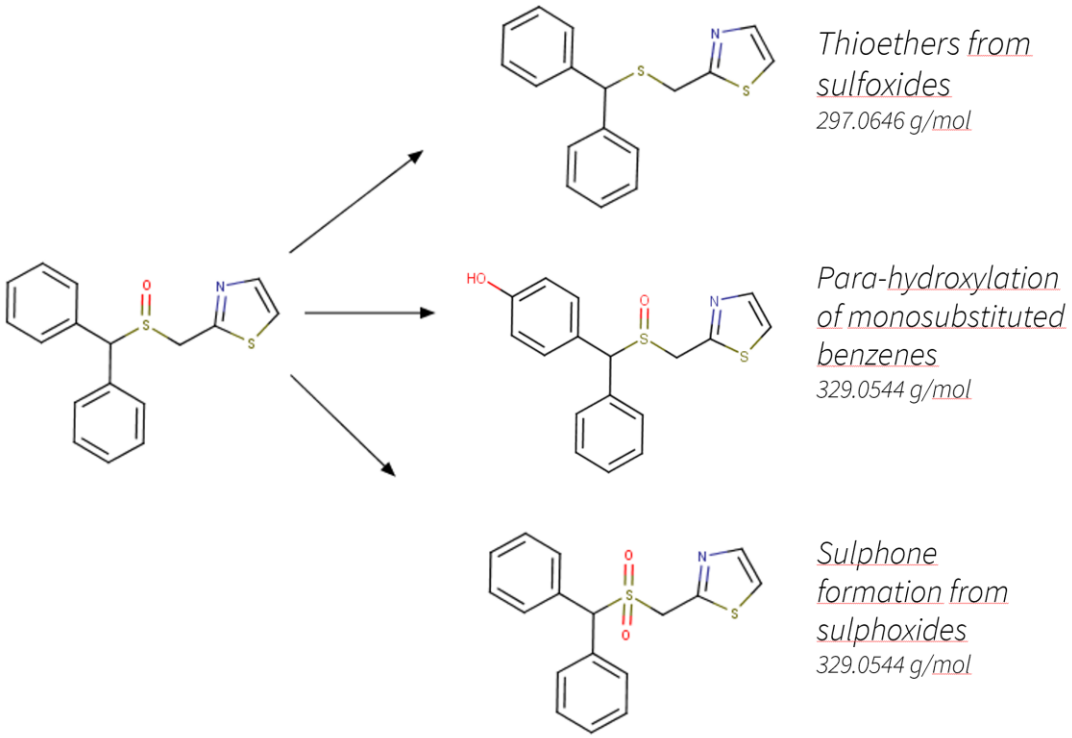

Figure 18. Selection of predicted metabolites of CE-123 by Meteor™ nexus.
The predicted metabolites of CE-123 are corresponding to the predicted metabolites of Modafinil.

4.3 Measurement and Analysis

4.3.1 LC-MS

4.3.1.1 Liquid Chromatography

The following settings listed in *Table* 1 were utilized for the UHPLC. The gradients utilized are listed in *Table 2* and *Table 3.*

Table 1. Liquid Chromatography Parameters

Column	Kinetex 2.6 µm
Column Dimension	LC Column 50 x 2.1 mm
Flowrate	600 µL/min
Injection Volume	20 µL
Column Temperature	40 °C
Pump Pressure	~ 245 bar
Mobile Phase	
Phase B	ACN (LC-MS)
Phase A	Acidic H_2O (0.1% FA)
Gradient	5% - 95% ACN
Run Time	
Preliminary Tests	15 min
Experiments	30 min
Detector	UV VIS
Wavelength	Spectrum

Table 2. UHPLC gradient preliminary tests

Mobile phase	**Time [min]**								
min	0.0	0.1	5.0	10.0	12.0	13.0	14.0	14.5	15.5
B [%]	5.0	5.0	30.0	45.0	70.0	97.0	97.0	5.0	5.0
A [%]	95.0	95.0	70.0	55.0	30.0	3.0	3.0	95.0	95.0

Table 3. UHPLC gradient experiments

Mobile phase	**Time [min]**							
min	0.0	0.0	5.0	5.1	23.0	27.0	27.1.0	30.0
B [%]	5.0	5.0	5.0	25.0	100.0	100.0	5.0	5.0
A [%]	95.0	95.0	95.0	75.0	0.0	0.0	95.0	95.0

4.3.1.2 Mass Spectrometer

The following MS parameters were utilized, for the MS measurements and are listed in *Table 4.*

Table 4. Mass Spectrometer Parameters

Capillary Voltage	4.5 kV
Nebulizer	1.0 N_2
Dry Gas Flow Rate	8.0 L/min N_2
Dry Temperature	200 ºC
Ion Transfer Parameters	
Quadrupole Ion Energy	8.0 eV
Funnel RF	400 Vpp
Multipole RF	300 Vpp
Collision Cell Parameters	
Collision energy	10.0 eV
Collision RF	1100 Vpp
Transfer time	38 µs
Pre-pulse storage	18 µs

For analysis evaluation Compass DataAnalysis 4.2 (Bruker Corporation) was used.

4.3.1.3 Analyte Specification

The listed analyte specifications in *Table 5* were applied for data processing.

Table 5. MS Analyte Specification

Analyte	Molecular weight [g/mol]	$[M+H]^+$ m/Q	$[M+Na]^+$ m/Q	Monitoring ion (m/Q)	RT [1] [min]	RT [2] [min]	Sum formula
CE-123	313.06	314.06	-	314.07	7.9	7.8	$C_{17}H_{15}NOS_2$
R-Modafinil	273.35	274.08	296.07	167.08	6.8	6.8	$C_{15}H_{15}NO_2S$
Diazepam	284.74	285.06	-	-	-	8.2	$C_{16}H_{13}ClN_2O$

[1] Preliminary tests, Experiment 1, 2

[2] Experiment 3,4,5,6

For preliminary tests and the following two experiments a gradient with 15 minutes runtime was chosen. It was later adapted to 30 minutes runtime. As a result, the retention time (RT) shifted from 4.5 min to 6.8 minutes for modafinil and from 4.5 to 6.2 minutes for CE-123. The internal standard (IS) method was applied, as it features the most desirable attributes for the intended purpose in respect to sample preparation and error avoidance. The internal standard method may countervail inadequate circumstances, like technical failure, or other process manipulations that may affect the analytical process. Modafinil was selected as IS for all experiments conducted with CE-123, and Diazepam as IS for all experiments conducted with modafinil. The selected substances met the requirements for an internal standard as they each were available in high purity, behave similarly to their analyte, but have a different retention times (Lundanes et al., 2014) . In *Figure 19* the dispersion of the modafinil peak is illustrated. Associated with the chromatogram the compound spectra of the modafinil peak is presented in *Figure 20. Figure 21* displays the less significant dispersion of the CE-123 peak, while *Figure 22* is providing the compound spectra of CE-123 that shows a significantly lower fragmentation within the CE-123 peak.

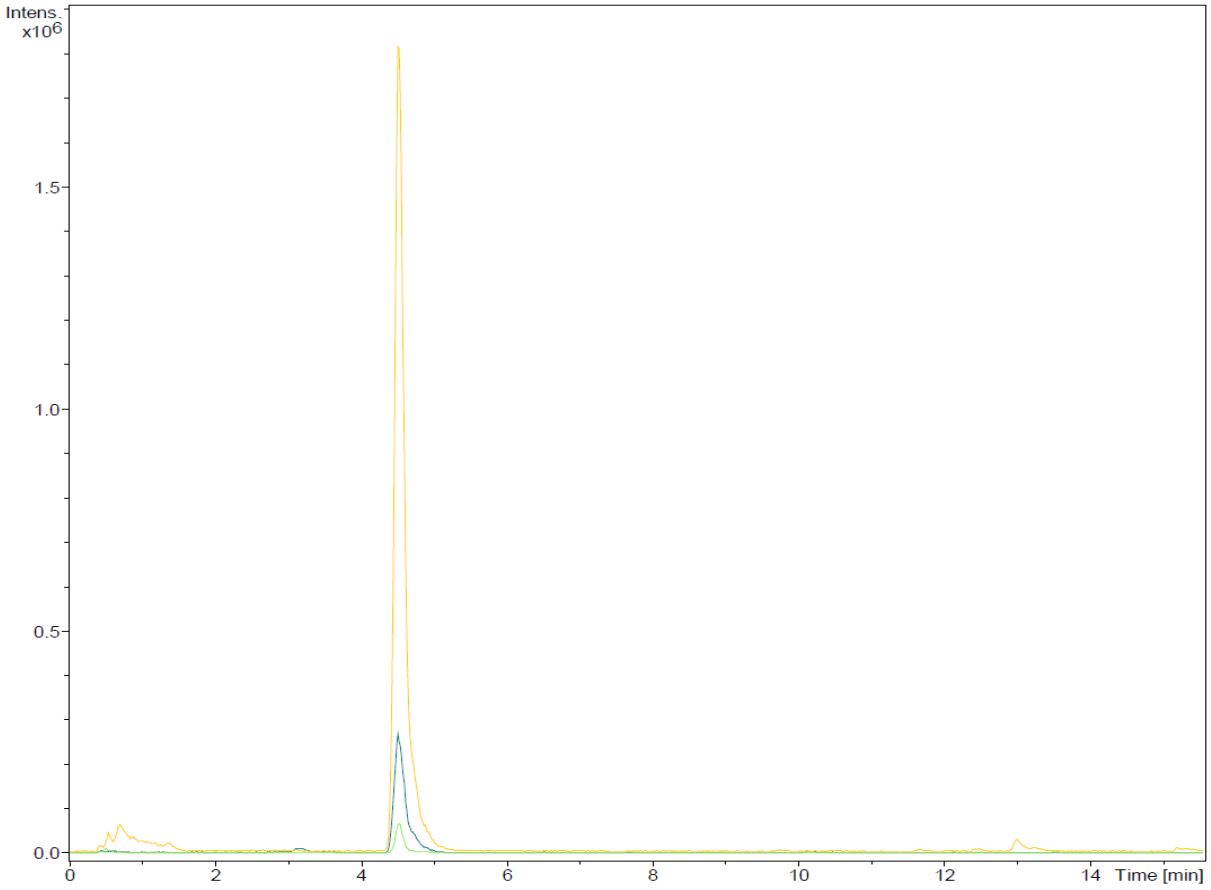

Figure 19. EIC modafinil, displaying the distribution of the modafinil signal. Yellow: m/z=167.07, represents the diphenylmethane partial structure. Turquois: m/z=296.07 represents the sodium adduct, Green: m/z=274.08 displays the relatively small amount of modafinil $[M+H]^+$. This pattern indicates insource fragmentation and reveals the structural weakness in modafinil.

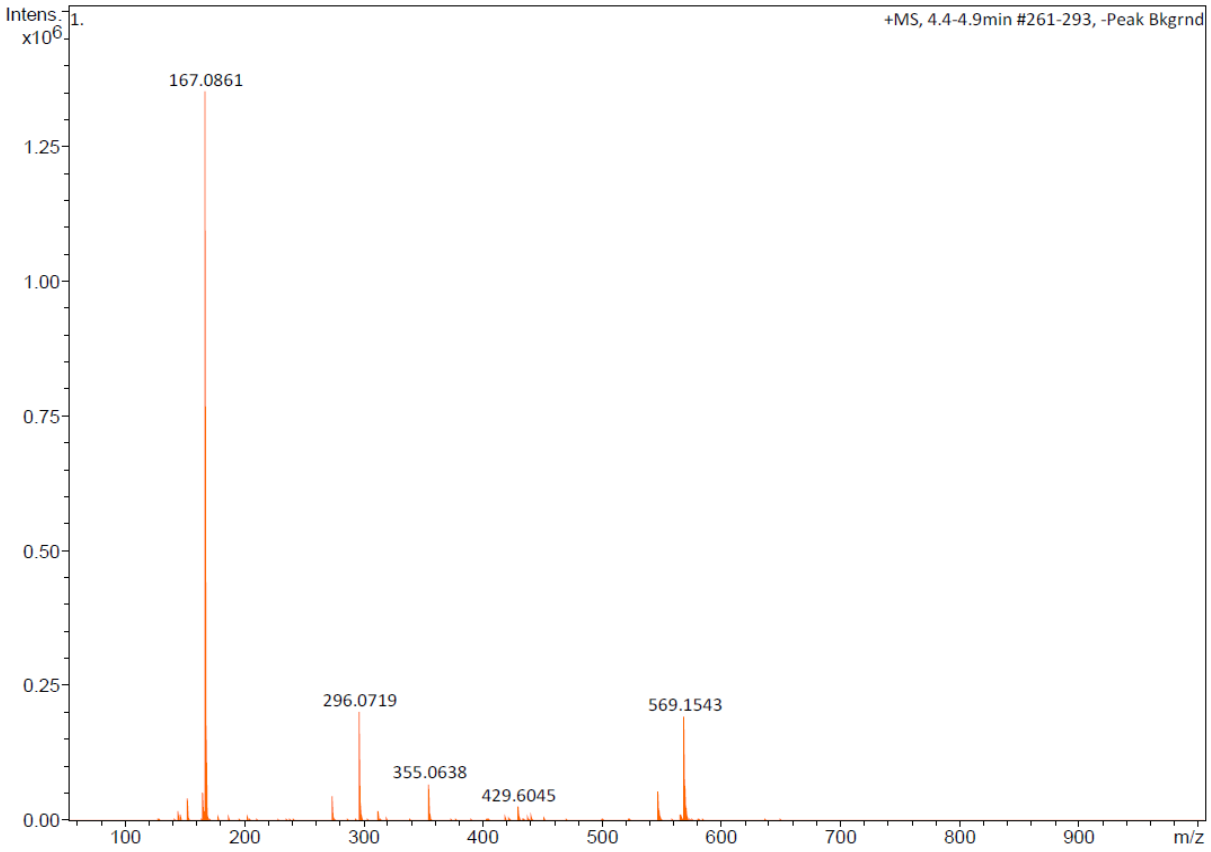

Figure 20. MS spectrum of the modafinil peak and its fragmentation. m/z=167.08 represents the diphenylmethane structure, as a result of in source fragmentation, whereas m/z=296.07 represents the sodium adduct of modafinil. The m/z=274.08 represents the modafinil $[M+H]^+$.

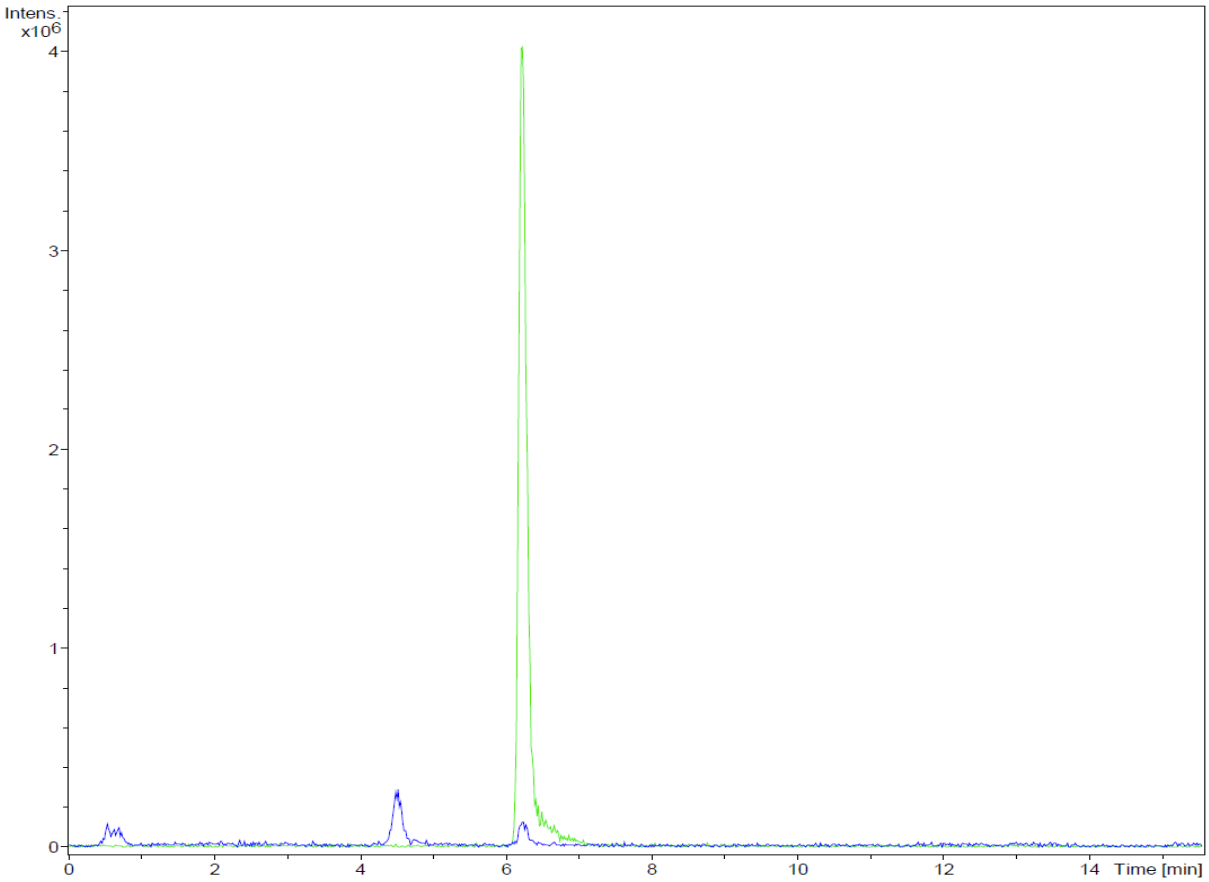

Figure 21. Extracted ion chromatogram m/z=314.06 CE-123. blue: m/z=167.07, green: m/z=314.07, significantly less in-source fragmentation than modafinil. Visible, as the blue peak at 6.2 min representing the diphenylmethane, is significantly smaller than the green peak that represents the unfragmented molecule of CE-123.

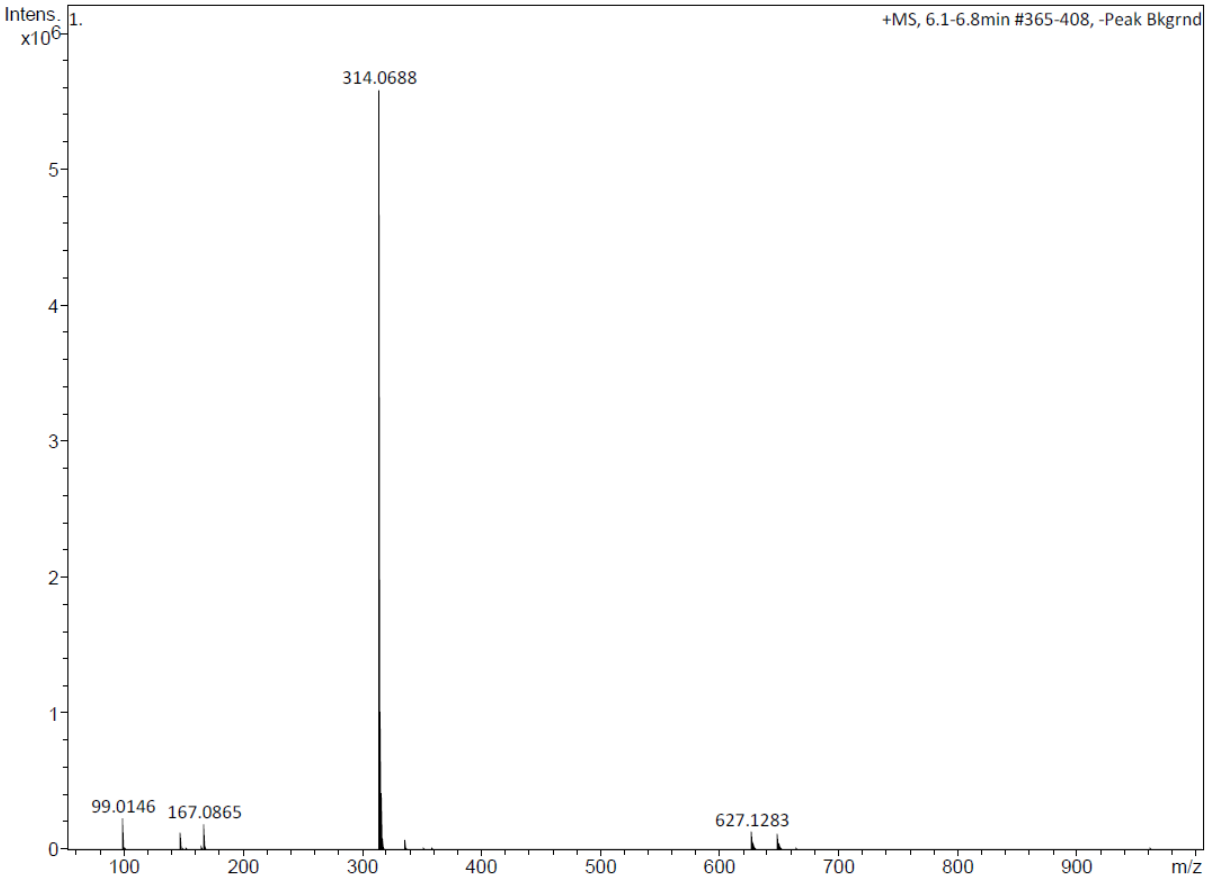

Figure 22. MS spectrum of CE-123 Peak. Only little fragmentation or in source fragmentation at m/z=167.70, but the formation of a dimer at 627.13.

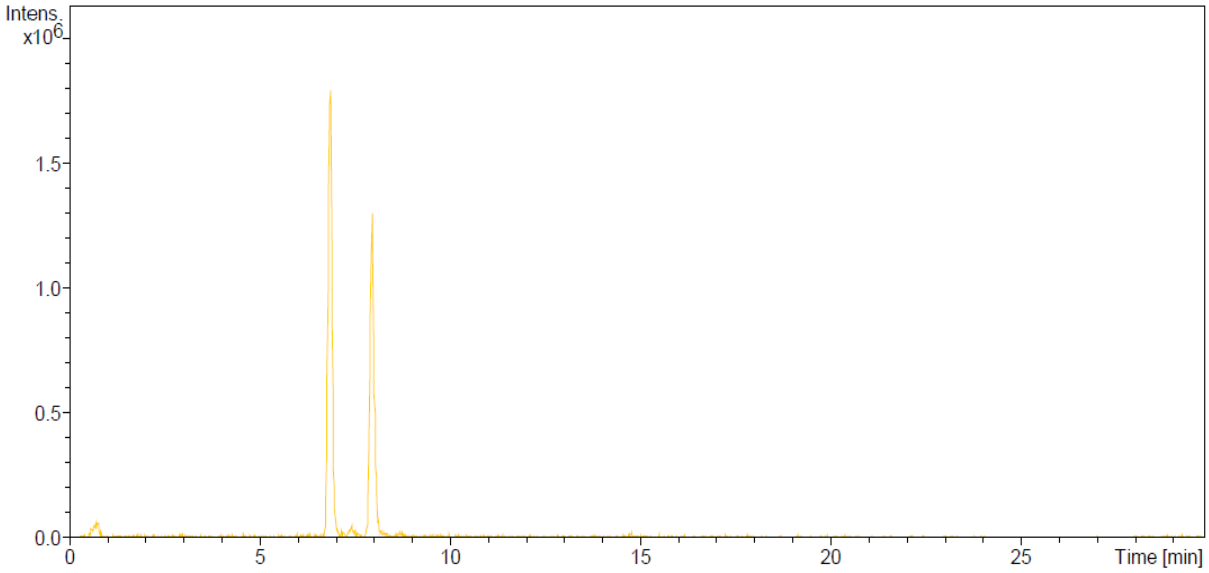

Figure 23. Chromatogram EIC m/z=167.07 Shows a comparison of m/z=167.07 peaks, representing the diphenylmethane partial structure of modafinil at 6.8 min and CE-123 at 7.8 min.

5 Results

5.1 Experiments

Experiments were conducted in triplets and included a blank. The blank sample consists of media spiked with CE-123 or modafinil, depending on the sample, but is in contrast to the samples not incubated with cells.

The sample preparation was conducted as described in *point 4.2.1.1*. (HepG2) or *point 4.2.1.2*. (HepaRG). As there was no prior experience with the substance, preliminary experiments were conducted to determine the fundamental parameters.

All relevant data is provided in *point 7.1*.

5.1.1 Preliminary Tests

Table 6. Preliminary test overview

Preliminary test	Cell line	Cell count	Substance	Concentration	Time points [h]	Well plates	Comment
1	HepG2	5 x 10^6	Modafinil CE-123	50 µM/mL	0, 1, 18, 24	24 wells, flat bottom	interrupted measurement
2	HepG2	2.5 x 10^6	Modafinil CE-123	50 µM/mL	0, 1, 20, 24	24 wells, flat bottom	Reduced cell count
3	HepG2	1 x 10^6	Modafinil CE-124	1 µg/mL	0, 24, 48	6 wells, flat bottom	Reduced cell count

An overview of the preliminary tests is outlined in *Table 6.*

The first preliminary experiment was conducted using an existing protocol. Cells were counted using a hemocytometer. 5 x 10^6 cells/well were seeded into a 24-well plate. After seeding cells were incubated for 24 h to attach to the wells. After 24 h the cells were supplemented with a 50 mM Modafinil/CE-123 solution. The incubation time points were 0 h, 1 h, 18 h, 24 h, to determine if a gradual decrease of CE-123/modafinil concentration could be observed. After extracting the supernatant, the wells were refilled with PBS, to optimize the conditions for the remaining samples. Due to a mechanical error only six samples were measured, which required an iteration of the experiment.

In the second preliminary experiment the number of cells was reduced to 2.5 x 10^6/well, as the wells did not provide enough surface for the cells to attach properly. Cells were seeded into 16 wells of a 24-well plate, the eight remaining wells were filled with PBS. After seeding, cells were incubated for 24 hours to attach to the wells. After 24 h the cells were supplemented with a 50 mM Modafinil or CE-123 solution. The incubation time points were 0 h, 1 h, 20 h, 24 h. After extracting the samples, the wells were refilled with PBS.

In the following experiment the number of cells was further reduced to 1 x 10^6 cells/well and a 6-well plate was utilized. The concentration of modafinil amounted to 1 µg/mL, while the concentration of CE-123 was adapted to 4 µg/mL. The time points were 24 h and 48 h, to see if there was a concentration reduction/metabolization of the compounds. A slight reduction tendency was visible.

After the identifying the preliminary parameters, experiments were set up and conducted as follows:

5.1.2 Experiment 1

Table 7. Overview experimental settings Experiment 1

Cell line	Cell count	Substances	Concentration	Time points [h]	Well plates	Comment
HepG2	1 x 10^6	CE-125	4 µg/mL, 9.5 µg/mL	0, 8, 24, 48	6 wells, flat bottom	Incubated blanks

An overview of the experimental setting is provided in *Table 7*. The relevant data is provided in *point 7.1.1*.

Two concentrations, 4 µg/mL and 9.5 µg/mL CE-123 in DMEM, were used to determine potential deviations in metabolic capacities due to enzyme induction by CE-123. The concentration of the internal standard modafinil was 1 µg/mL in ACN.

The samples were incubated for 8 h, 24 h, 48 h. DMEM Media and DMEM supplemented with CE-123 (Blank) were incubated for 24 h and 48 h, to expose the blank sample to the same conditions as the samples (37°C, 5 % CO_2, humidified atmosphere). The DMEM sample was set up to identify unspecific DMEM signals and with that facilitate the identification of CE-123 specific signals and the search for possible metabolites.

The data suggests that the CE-123 concentration has a slight impact on the metabolization rate. As shown in *Figure 25* the samples with the higher concentration (9.5 µg/mL) experience a higher degree of substrate reduction, i.e. a difference of 5% in 24 h and 7% in 48 h. This allows the assumption that CE-123 may have an augmenting effect on enzyme expression. The comparison of the 24 h sample with the incubated blank shows a relative reduction of about 35 %. The 48 h sample compared with the 48 h incubated blank shows a decrease of about 28%, as shown in the chromatogram of *Figure 24* and represented in *Figure 25*. The comparison of the two blanks (24 h and 48 h incubated) shows a reduction of about 11%, as shown in *Figure 26. Figure 27* illustrates the reduction of substrate concentration of incubated blanks within the course of 24 h.

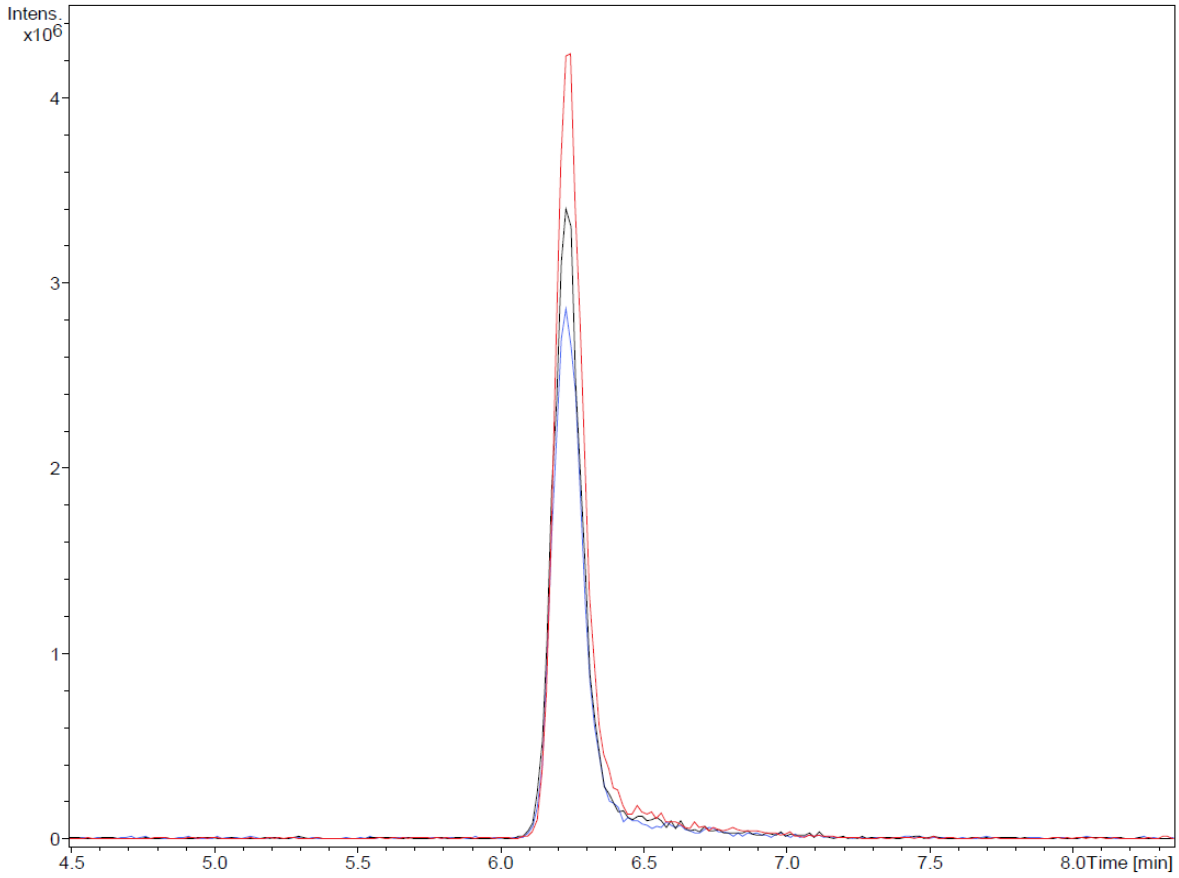

Figure 24. EIC of m/z=314.06. Reduction of CE-123 concentration over the course of 40 h. Red = 8 h, black = 24 h, blue = 48 h.

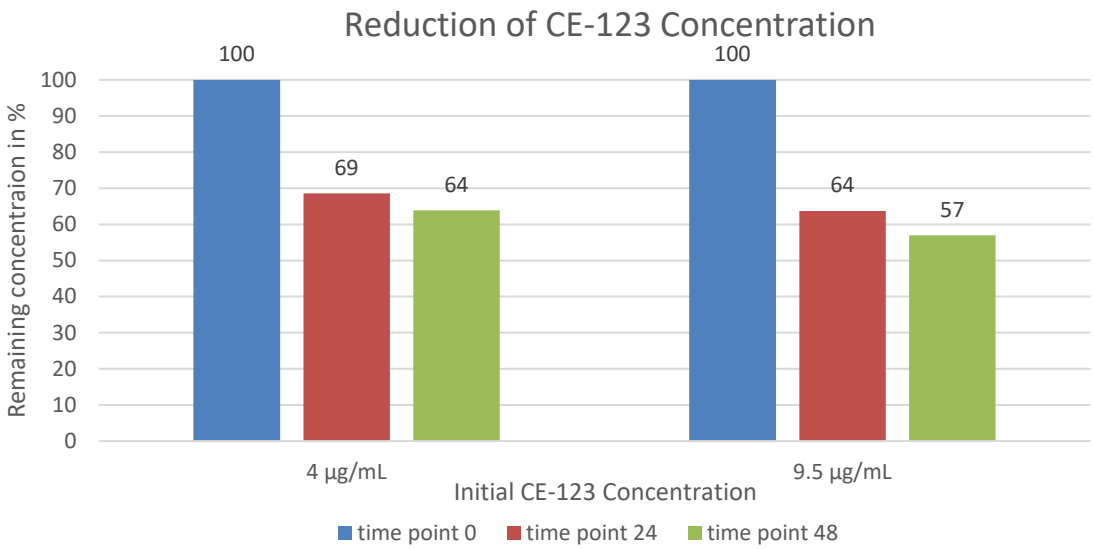

Figure 25. Illustration of the declining CE-123 concentration. After 24 h and 48 h incubation with HepG2, a graduate reduction of CE-123 concentration is visible, in comparison to the blank (time point 0). The blanks were also incubated, one blank per specification, the reduction is in relation to the corresponding blank. The displayed sample values are the mean value of three samples per specification.

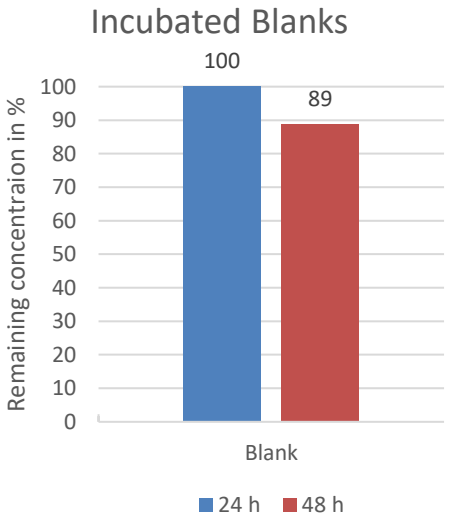

Figure 26. Illustration of the CE-123 blanks reduction in concentration. Blanks exposed to the experiments environment (incubator) for 24h, 48 h, in accordance to the samples. A distinctive reduction of about 11% in 24 h.

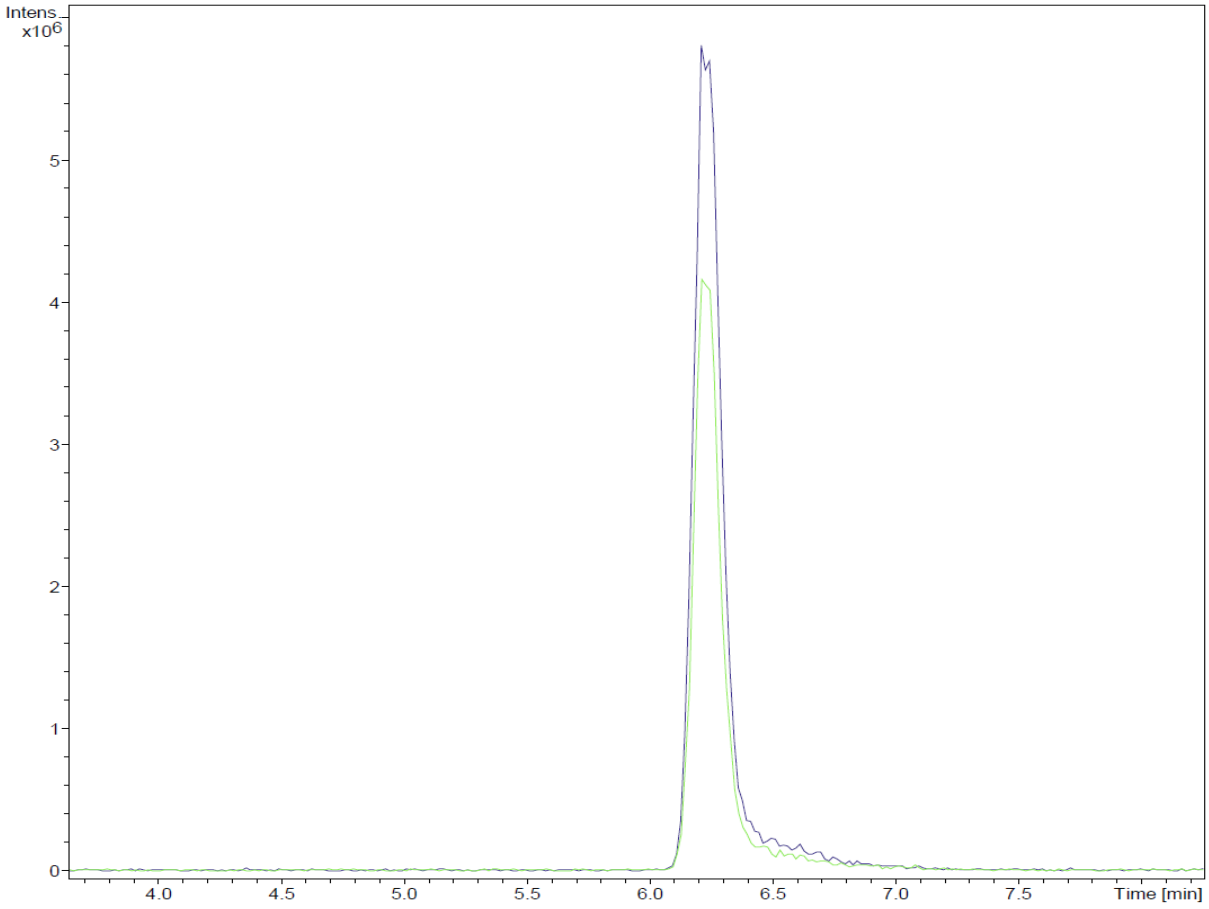

Figure 27. EIC 314.06. Comparison of an incubated blank with a sample after 48 h of incubation. The chromatogram shows a significant reduction of the CE-123 concentration in the sample (green) compared to the incubated blank (blue).

5.1.3 Experiment 2

Table 8. Overview experimental settings Experiment 2

Cell line	Cell count/well	Substance	Concentration	Time points [h]	Well plate
HepG2	1 x 10^6	Modafinil	5 µg/mL 10 µg/mL	0, 24	6 wells, flat bottom

An overview of the experimental setting is provided in *Table 8*. The relevant data is provided in *point 7.1.2.*

In previous experiments it was not possible to determine metabolites of CE-123, as the search for the unknown metabolites of CE-123 proved to be challenging. This experiment was used to scale back and search for known metabolites of the reference substance modafinil, in an effort to affirm the developed protocol. The two main metabolites of modafinil are modafinil acid and modafinil sulfone. However, no data in respect to the fragmentation of modafinil acid or modafinil sulfone was available.

A reduction of the concentration of modafinil was visible (about 12% with an initial concentration of 5 µg/mL, about 22% with an initial concentration of 10 µg/mL compared with the blank) Illustrated in *Figure 28*. Affirming the already in *point 2.2.1* described enzyme inducting effect of modafinil. But due to the lack of fragmentation patterns of the two known metabolites, neither could be determined.

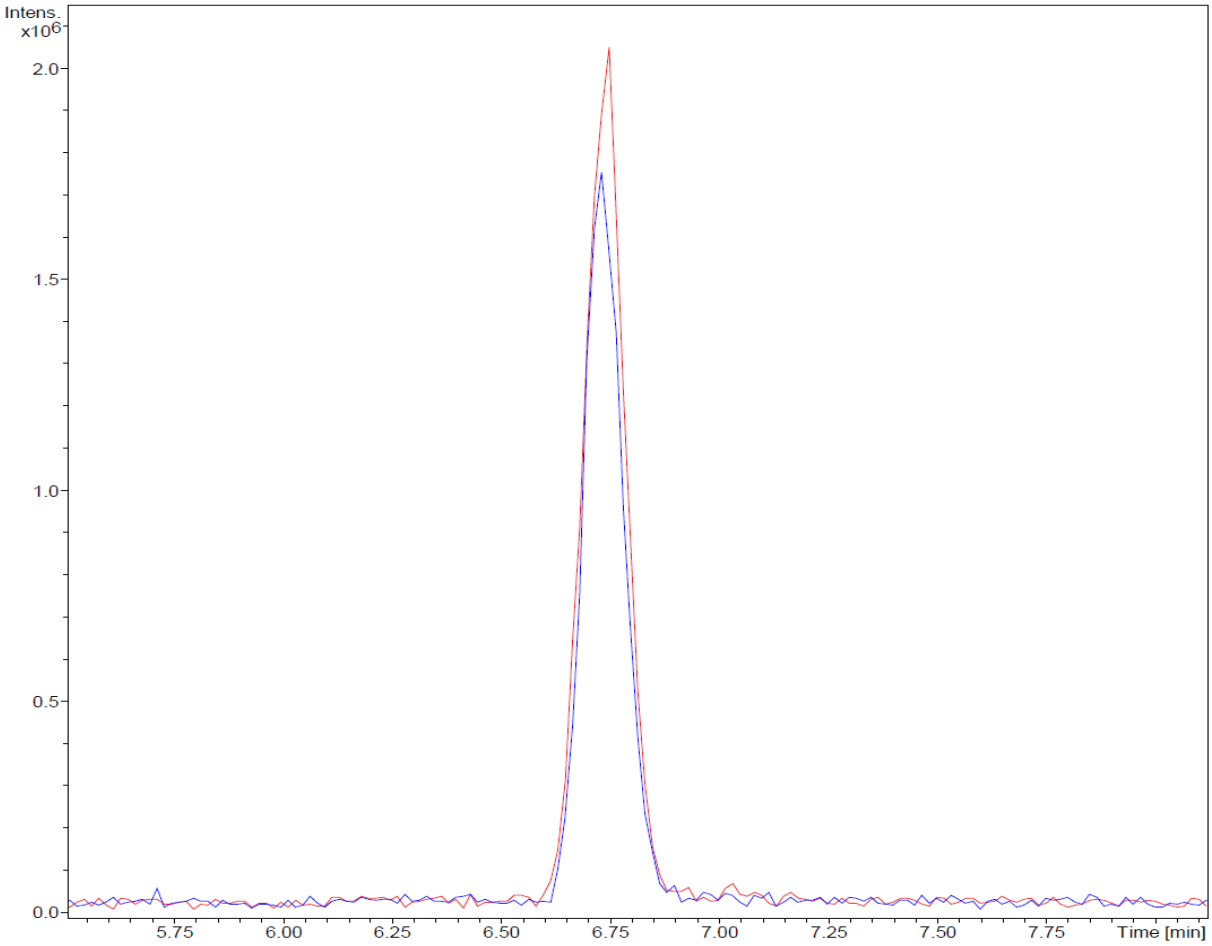

Figure 28. Chromatogram EIC m/z=167.08. Showing visible reduction of Modafinil concentration after an incubation period of 24 h (blue) in comparison with the blank sample (red).

5.1.4 Experiment 3

Table 9. Overview experimental settings Experiment 3

Cell line	Cell count	Substance	Concentration	Time points [h]	Well plates	Comment
HepG2	1 x 10^6	CE-123	0.1 µg/mL 0.5 µg/mL 1 µg/mL 4 µg/mL	0, 24	6 wells, flat bottom	Cut out filtration step

An overview of the experimental setting is provided in *Table 9*. The relevant data is provided in *point 7.1.3*

As reductions in concentrations where apparent in all experiments, but no metabolites could be determined, it was considered that the metabolic products may adsorb on the surface of the utilized material. The mass spectra also showed high levels of impurity. To minimize material, to decrease interferences and increase purity of the sample, the filtration step was cut out and replaced by extensive centrifugation (5000 rpm, 30 min). Centrifugation was considered the ideal option as interactions between the sample and the filter or other additional materials could be precluded, it also facilitates handling. The extensive centrifugation led to a phase separation. The two phases were measured separately to ascertain if there is an enrichment in either phase. The upper phase had higher concentrations of CE-123, than the lower phase. The CE-123 concentration, as well as the internal standard concentration of the lower phase was so low that the data evaluation was not possible. The elimination of the filtration step had however a positive effect on the sample's purity and reduced contaminations of the sample, as can be seen in *Figure 29*.

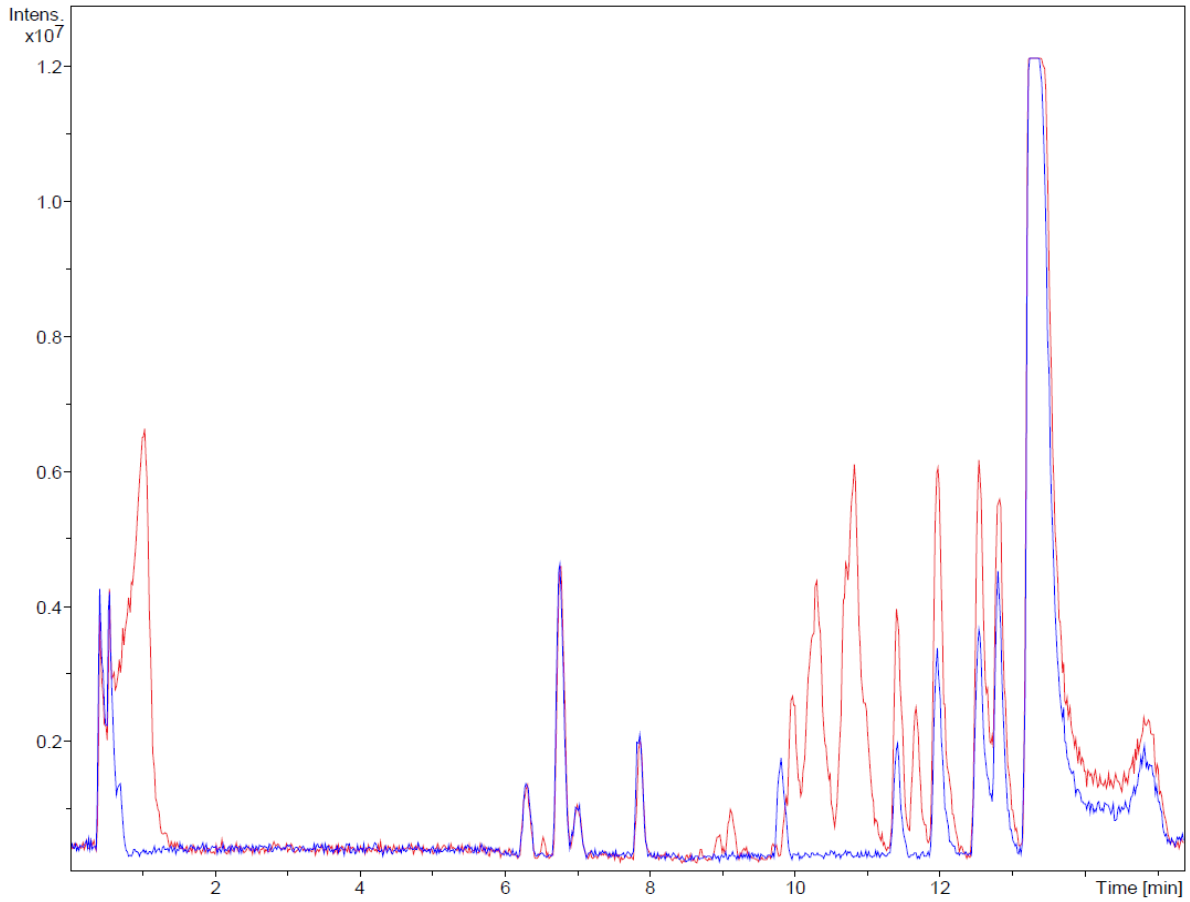

Figure 29. Comparison (BPC) of samples prepared through filtration or centrifugation. The sample purity depends on the sample preparation. The sample prepared through filtration (red) through a microfilter regenerated cellulose membrane (0.2 µm) presents more impurities than the sample prepared through centrifugation (blue).

5.1.5 Experiment 4

Table 10. Overview experimental settings Experiment 4

Cell line	Cell count	Substance	Concentration	Time points [h]	Well plates	Comment
HepG2	1 x 10^6	Modafinil	10 µg/mL	0, 12, 24	6 wells, flat bottom	w/o Antibiotics
HepG2	1 x 10^6	CE-123	9.5 µg/mL	0, 12, 24	6 wells, flat bottom	w/o Antibiotics

An overview of the experimental setting is provided in *Table 10*. The corresponding data is provided in *point 7.1.4.*

As antibiotics may use up metabolic capacity of the already low metabolic activity of HepG2 cells, a media without Penicillin and Streptomycin was prepared. The goal was to determine if the antibiotics have a negative influence on the metabolization of CE-123 by occupying metabolic capacities. By omitting the antibiotics enhanced metabolization of either substances was desired.

The provided conditions did not promote the desired effects. As shown in *Figure 30*, there was a visible decrease of CE-123, but compared with prior experiments, the effect was less substantial. CE-123 showed a decrease of only about 13% in 24 h, compared with 36% in *Experiment* 1. The modafinil concentration also decreased by about 20% in 24 h, which is in accordance with previous results (Experiment 3).

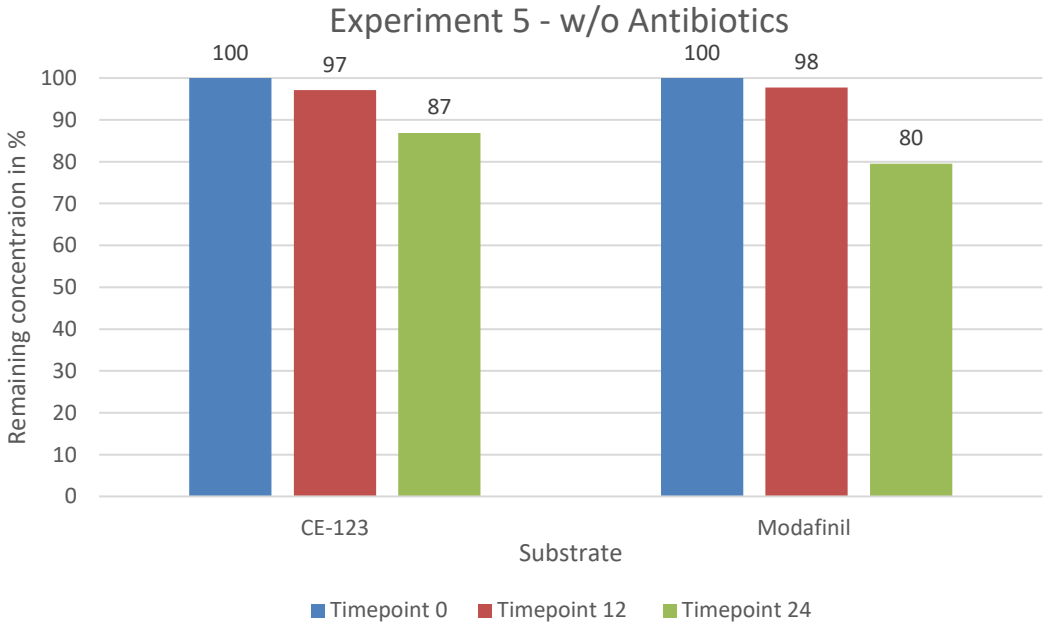

Figure 30. Presentation of the reduction of the CE-123/Modafinil concentration in an antibiotic free medium. The reduction of the substrate concentration is limited in comparison to a similar experiment conducted with antibiotics.

5.1.6 Experiment 5

Table 11. Overview experimental settings Experiment 5

Cell line	Cell count	Substance	Concentration	Time points [h]	Well plates
HepaRG	0.8 x 10^6	Modafinil	10 µg/mL	0, 20	12 wells, flat bottom
HepaRG	0.8 x 10^6	CE-123	9.5 µg/mL	0, 20	12 wells, flat bottom

Experiment 5, outlined in *Table 11* was conducted with HepaRG cells, according to the protocol developed and established for this thesis, with HepG2 cells. HepaRG cells were employed as a high-performance alternative to HepG2 cells, as the experiments conducted with HepG2 cells did not achieve the desired results. The aim was to see if distinguishable metabolization would be enabled by the high enzymatic activity of HepaRG cells and with that deliver measurable concentrations of one or more metabolites. The cells were treated as described in *point 4.2.3.2.*

The three most likely metabolites, shown in *Figure 18* predicted by Meteor™ nexus were traced as hydrogenic adducts via their molecular mass. The CE-123 thioether with a molecular mass of 298.08 g/mol, the para-hydroxylated CE-123 and the CE-123 sulfone, each with the identical exact molecular weight of 329.0544 g/mol. The analysis did not present any evidence of the CE-123 thioether. The extracted ion chromatogram for the molecular weight of 330.05 g/mol however presented two peaks, presented in *Figure 31.* One peak was identified as either the para-hydroxylated CE-123 or the CE-123 sulfone, with an intensity of about 2.8 x 10^4. The compound spectra revealed an intensity of 2.1 x 10^4 and an isotopic distribution, also shown in *Figure 31.* The result was confirmed via the “smartformula manually” tool (error tolerance of <10 ppm) and “simulated isotopic pattern” tool provided by the Compass DataAnalysis 4.2 (Bruker Corporation), presented in *Figure 32*. This result was present in all the three samples of the triplet. It was not detectable in neither blank nor other samples, rededicated from former experiments as control, to exclude the possibility of coincidence or mistake, as illustrated in *Figure 33*. It was however not possible to make the distinction, which proposed metabolite occurred, due to the lack of metabolite standard.

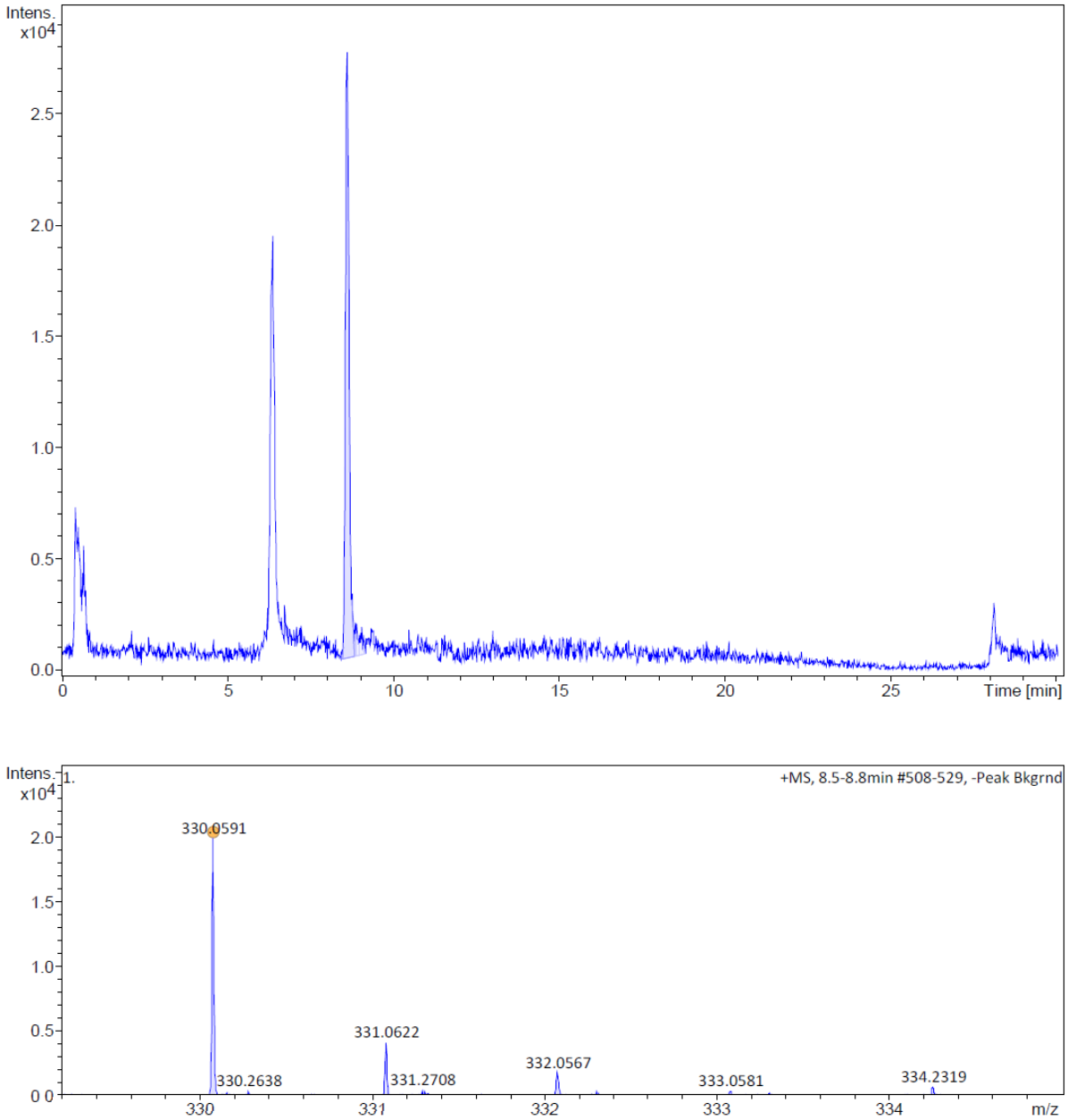

Figure 31. EIC and compound spectrum of m/z= 330.05. The mass of the hydrogenic adduct of a predicted metabolite. Ad EIC: Two visible peaks. The second peak represents a predicted metabolite of CE-123. Ad compound spectra of the second peak: showing m/z=330.0591 with an intensity of about 2.0 x 10^4 and also displaying the isotopic distribution of the peak.

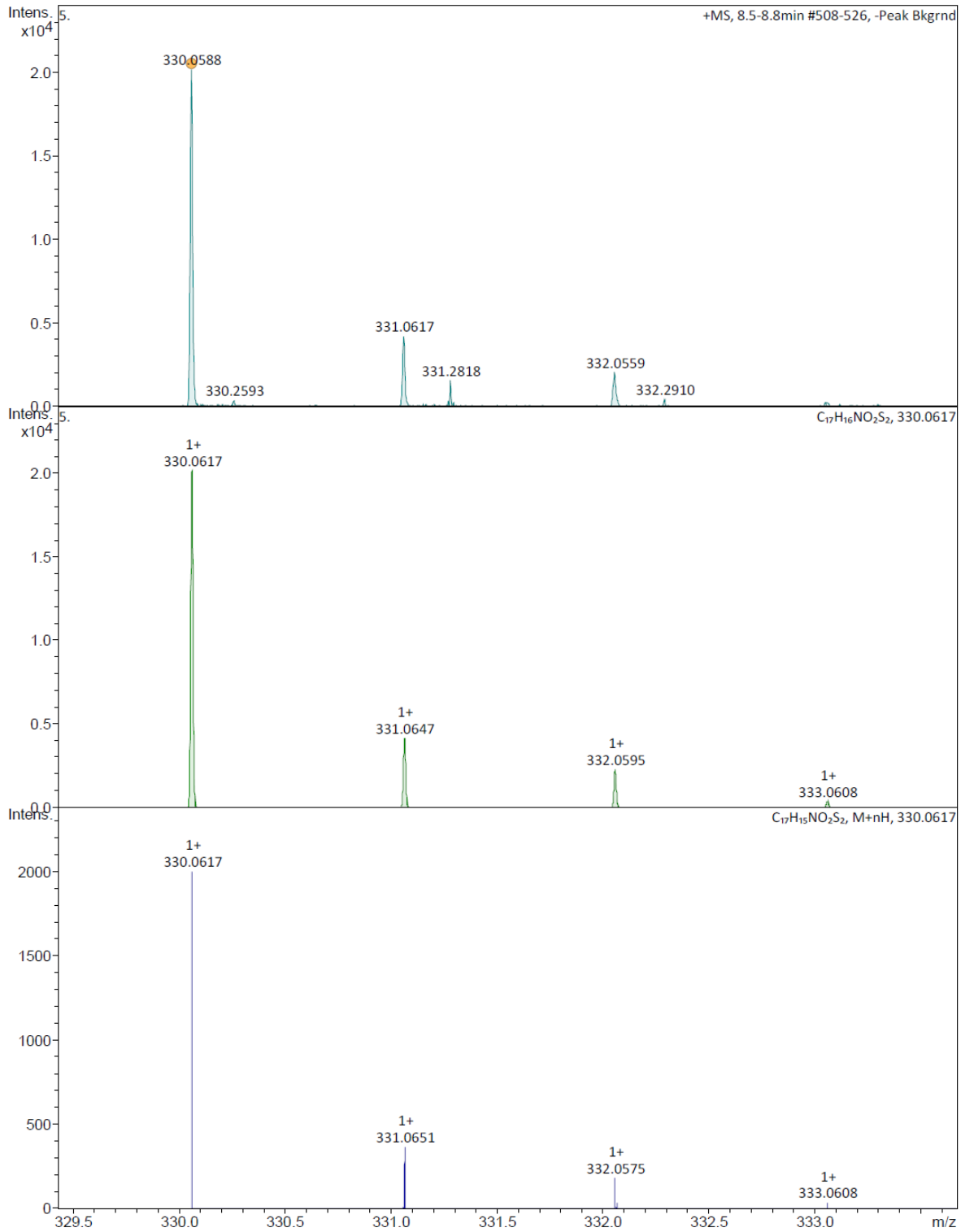

Figure 32. Comparison of results of analytic tools by Compass DataAnalysis 4.2 (Bruker Corporation). Top to bottom: the compound spectrum of the metabolite (m/z=330.0588), the analysis with smart formula and the simulated pattern analysis the at the bottom.

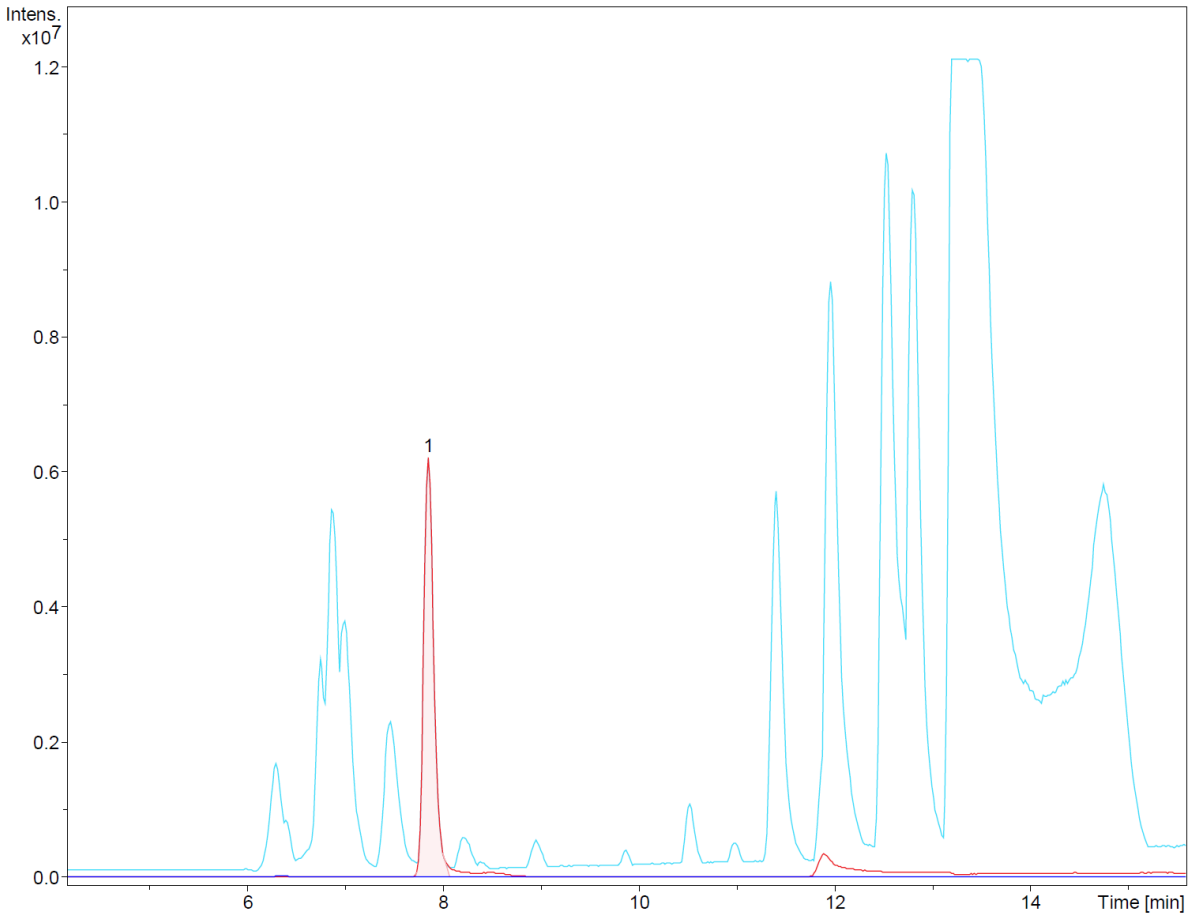

Figure 33. Chromatogram comparison of the blank sample illustrating the lack of m/z=330.05 fragments. Comparing the base peak chromatogram (BPC) (light blue), the EIC of 314.06 (red) and the EIC 330.05 (dark blue).

5.1.7 Summary

The results imply that the concentration of CE-123, as well as modafinil decrease when incubated with HepG2 cells. The reduction of CE-123 in HepG2 amounts up to about 30%. The reduction of modafinil in HepG2 amounts up to 23%. The variations in concentration were utilized to determine if higher metabolization rates can be achieved due to induction of enzymatic capacities. A slight increase of CE-123 reduction was suggested, in the samples incubated with higher CE-123 concentration of 9.5 µg/mL in comparison with the lower concentration of 4 µg/mL.

To ensure that the reduction of the substance within a sample can be linked to metabolic activity, a blank sample was incubated in the incubator for the same amount of time as the sample itself, without cells. The data presented in *point 5.1.2* suggests that at least some of the reduction (up to 10%) may be due to instabilities under the experimental conditions.

All proposed structures (Meteor™ nexus) were searched with the Compass DataAnalysis 4.2 (Bruker Corporation) as hydrogenic and sodium adduct, but none were distinguishable within the samples incubated with HepG2 cells.

The samples incubated with HepaRG presented a distinct molecular weight linked to two proposed metabolites, when submitted into Compass DataAnalysis 4.2 (Bruker Corporation). CE-123 sulfone, as well as a para-hydroxylated CE-123 are possible metabolites with an identical exact molecular mass of 329.0544 g/mol and a sum formula of $C_{17}H_{15}NO_2S_2$. This result was reproduced in all three samples incubated with CE-123 of experiment 5 and was confirmed via the “smart formula manually” tool and the “simulate isotopic pattern” tool, available within the Compass DataAnalysis 4.2 (Bruker Corporation). “Smartformula manually” calculations were submitted with an error tolerance of <10 ppm.

The molecular weight in question was not traceable in the blank sample of experiment 5, nor in any samples incubated with modafinil. The latter samples were rededicated to ensure the exclusion of coincidence and respectively the confirmation of the result.

Cell toxicity was not observed at the concentration used.

6 Discussion and Conclusion

As already pointed out, the reduction of the CE-123 concentration, as well as modafinil was apparent in all samples, even though some of the reduction may be partly caused by experimental conditions.

As a source of possible metabolites, the software Meteor™ nexus was utilized. But none of the suggested metabolites could be found, neither as hydrogen- nor as a sodium adduct in the experiments conducted with HepG2 cells. The latter was suggested as modafinil and its fractions were primarily found as sodium adducts.

As HepG2 cells have an especially low expression of CYP 1A1 and 1A2, in comparison to hepatocytes, the metabolization of their substrates may be reduced. This might be of interest, as CYP 1A1 may be responsible for the transformation of benzene to phenol (Coleman, 2010; Richter et al., 2017; Westwick, 1976), and subsequently for the para-hydroxylation of the CE-123, as well as the para-hydroxylation of modafinil. This could be the reason why the experiments with HepG2 did not produce, or if, only produced very little of the desired metabolites.

Compared with HepG2, the HepaRG cells have a heightened activity of CYP 1A2, CYP 2C9, CYP 2D6 and CYP 3A4. As known modafinil has an inducing effect on CYP 3A4. That is notable as the formation of sulfone from sulfoxide is likely primarily caused by CYP3A4 and would therefore also be responsible for the formation of the CE-123 sulfone. The thiazole partial structure however constitutes a steric barrier that might hinder enzymatic activity at the sulfoxide group. These modifications limit access to the reactive partial structure of CE-123, which might be a reason for limitations in metabolization. These conjectures would imply that the most probable metabolite would be the para-hydroxylated CE-123, which would likely be formed by CYP 1A1.

The formation of further modafinil metabolites by other CYP enzymes could so far not be identified *in vitro* due to their slow formation and the limited time frames that *in vitro* studies provide (Robertson & Hellriegel, 2003), this might also be applicable to the metabolization of CE-123.

Modafinil is mainly eliminated through metabolization, taking place primarily in the liver. No more than 10% of modafinil is excreted renally. It's metabolites however are primarily excreted renally, modafinil acid for example accounts for up to 51% of a single dose (Robertson & Hellriegel, 2003). But the structural properties of CE-123 imply that urinary excretion plays an even less significant role for CE-123, than for modafinil. CE-123 lacks the amid partial structure that makes modafinil vulnerable for metabolic transformation, thus there is no corresponding path of metabolization for CE-123. This could indicate that CE-123, is hardly metabolized. Another possibility would be that CE-123 is transformed to the para-hydroxylated CE-123 in a phase I metabolization step. The para-hydroxylated CE-123 molecule presents a more reactive phenol partial structure that could facilitate further modifications through phase II enzymes and consequently allow renal elimination.

Due to the discrepancies between the decrease of concentration in the sample and the amount of identified metabolite, the following options should be considered:

1. CE-123 has a high plasma protein binding potential.
   As modafinil is bound up to 60% on plasma proteins (Robertson & Hellriegel, 2003), it should be considered that CE-123 might also has a high plasma protein binding potential. The degree of plasma protein binding is linked to the lipophilicity of a compound. The more lipophilic a compound, the greater the significance of plasma protein binding (Croom, 2012). As CE-123 is more lipophile than modafinil, it can be expected that the plasma protein binding is greater than 60%. The media used for cultivation and experimentation is supplemented with FBS, which contains albumin. Albumin is one of two key plasma proteins. However, if bound to albumin a xenobiotic is not available for metabolization. When however, the proteins are precipitated during the sample preparation the compound is released unchanged and can be measured. Unfortunately, there is no data available regarding the plasma protein binding of CE-123. Also, the composition provided for the HepaRG cells is not known, which makes it difficult to assess the impact of the media on the results.

2. Not all prospective metabolites have been included by Meteor™ nexus analysis and were therefore not taken into account. One other possible path of metabolization could take place similar to the metabolization of Leflunomide. Leflunomide is an immunosuppressive disease-modifying antirheumatic drug, its structure includes an iso-oxazole partial structure, which is metabolically cleaved. This is relevant as thiazole and oxazole have similar chemical characteristics. The aromaticity provides a certain stability to a thiazole due to its electron configuration, but the five ring heterocycles have a high negative charge density within their π system and are polarized due to the heteroatoms, which makes them susceptible for nucleophilic attacks (Latscha, Kazmaier, & Klein, 2016). The configuration of the heteroatoms however also plays a role in the reactivity of a heterocycle, which makes the iso-oxazole more vulnerable to nucleophilic attacks than the 1,3-thiazole. This would explain why the heterocycle of Leflunomide is more likely to be cleaved than the 1,3 thiazole of CE-123.

Further challenges included that the mass spectrometer is very sensitive, and the untargeted approach is difficult to conduct, as the cell culture media is a complex matrix to analyse and not all parameters can be fully controlled. The cell systems used are models and do not provide the entirety of metabolic enzymatic regiment and with that the metabolite concentration may fall beyond the limit of detection (LOD). The origin of some fragments could not be determined, e.g. the diphenylmethane structure, which is part of both modafinil as well as CE-123. It could either be a result of instabilities due to the experiments environment, in-source fragmentation, but is also suggested as a result of metabolic activity as the diphenylmethane was also presented as a possible metabolite by Meteor™ nexus.

The experiment conducted with HepaRG however present a molecular mass corresponding to the hydrogenic adduct of either the para hydroxylated CE-123 or the CE-123 sulfone.

The LC-MS analysis of the experiment conducted with HepaRG presented a hydrogen adduct of a predicted metabolite with a molecular mass of 330.05 g/mol, corresponding to the prediction of the Meteor™ nexus. The prediction shows two

possible metabolites with an identical exact molecular mass of 329.0544 g/mol. The metabolites in question could either be a para-hydroxy substituted CE-123 or a CE-123 sulfone. As pointed out earlier, the more likely candidate would be the para-hydroxy substituted CE-123, as the thiazole presents a steric barrier to enzymatic activity at the sulfoxide partial structure of CE-123. The suggested metabolite is found in a quantity of about 0.50% relative to the CE-123 AUC.

As Peters and Meyer (2011) pointed out, metabolite standards are essential to confirm the results of the mass spectrometric analysis. The structure of an implied metabolite or respectively its molecular weight can be affirmed by comparison with a metabolite standard. These standards are however in this case not available and might be difficult to synthesis and/or would pose high expenses, therefore the identification of the metabolite is at this point not possible. As there was no metabolite standard available that would allow a distinction between the two probable candidates and would have enabled the identification through comparison of e.g. a fragmentation pattern of the metabolite and the mass spectra itself, it is inferred that either a para-hydroxylated CE-123 or a CE-123 sulfone was detected.

7 Appendix

7.1 Data

7.1.1 Experiment 1

Table 12. Data Experiment 1, 4 µg/mL

C [µg/mL]	Sample	AUC CE-123	AUC IS	Ratio	Mean	Mean Reduction	Reduction %
4	**24h**						
	Blank	31635737	2472881	12.79			
	1	24903040	2873259	8.67	8.78	4.02	31.40
	2	20230520	2317387	8.73			
	3	18333844	2053188	8.93			
	48h						
	Blank	25560616	2253372	11.34			
	1	21938092	2895994	7.58	8.17	3.17	27.96
	2	24809142	2732318	9.08			
	3	21012020	2673795	7.86			

Table 13. Data Experiment 1, 9.5 µg/mL

C [µg/mL]	Sample	AUC CE-123	AUC IS	Ratio	Mean	Mean Reduction	Reduction %
9.5	**24h**						
	Blank	47072992	2260858	20.82			
	1	40657496	2535062	16.04	13.27	7.55	36.25
	2	25497824	2072806	12.30			
	3	24681312	2150066	11.48			
	48h						
	Blank	47072992	2260858	20.82			
	1	30590308	2698686	11.34	11.86	8.97	43.06
	2	32512272	2595415	12.53			
	3	28813414	2462033	11.70			

7.1.2 Experiment 2

Table 14. Data Experiment 2

c [µg/mL]	Sample	AUC Modafinil	AUC IS	Ratio	Mean	Mean Reduction	Reduction %
5	Blank	11236342	11168469	1.01	-		
	1	13135874	15059992	0.87	0.88	0.12	12.33
	2	13494919	14706271	0.92			
	3	13434639	15692639	0.86			
10	Blank	21724962	8560701	2.54	-		
	1	25205814	13893739	1.81	1.97	0.57	22.53
	2	24610936	11427339	2.15			
	3	24149270	12513616	1.93			

7.1.3 Experiment 3

Table 15. Data Experiment 3

Upper phase

C [µg/mL]	Sample	AUC Modafinil	AUC IS	Ratio	Mean	Mean Reduction	Reduction %
0.5	Blank	13528219	23116132	0.59	-	-	
	1	904826	13528219	0.07	0.07	0.51	87.51
	2	1260382	16897780	0.07			
	3	1712090	22015686	0.08			
1	Blank	3689287	22171630	0.17	-	-	
	1	2455546	16660037	0.15	0.14	0.02	14.06
	2	811492	13984975	0.13			
	3	3267038	18887608	0.15			
4	Blank	19200568	25830412	0.74	-	-	
	1	22600530	25895374	0.87	0.87		
	2	29108488	33573288	0.89			
	3	25848442	30689616	0.84			

Table 16. Data Filter/Centrifuge

	AUC CE-123	AUC IS	Ratio
Filter	15646545	35426736	0.44
Centrifuge	16687552	35849392	0.47

7.1.4 Experiment 4

7.1.4.1 Modafinil

Table 17. Data Experiment 4, Modafinil

Time Point [h]	Sample	AUC Modafinil	AUC IS	Ratio	Mean	Mean Reduction	Reduction %
0	Blank	26038340	9143880	2.85	-		
12	1	29431388	10138553	2.90*	2.78	0.06	2.17
	2	21718978	7078236	3.07*			
	3	25152513	9038292	2.78			
24	1	31596896	12200629	2.59	2.26	0.58	20.47
	2	25189144	14717906	1.71			
	3	30951120	12413544	2.49			

*outlier, not included in calculation

7.1.4.2 CE-123

Table 18. Data Experiment 4, CE-123

Time Point [h]	Sample	AUC CE-123	AUC IS	Ratio	Mean	Mean Reduction	Reduction %
0	Blank	4224018	9406820	0.45	-	-	
12	1	26974724	44090428	0.61	0.44	0.01	2.92
	2	27037388	48593816	0.56			
	3	9942227	71255920	0.14			
24	1	410345.9	1526634.8	0.27	0.39	0.06	13.12
	2	2021680	4724025.5	0.43			
	3	1904711	4021323.3	0.47			

7.1.5 Experiment 5

7.1.5.1 Modafinil

Table 19. Data Experiment 5, Modafinil

Time Point [h]	Sample	AUC Modafinil	AUC IS	Ratio	Mean	Mean Reduction	Reduction %
0	Blank	19457436	18720746	1.04	-		
24	1	17903560	16671742	1.07	1.04	0.00	-
	2	18012320	17797348	1.01			
	3	17628634	15907531	1.11*			

*outlier, not included in the calculation

7.1.5.2 CE-123

Table 20. Data Experiment 5, CE-123

Time Point [h]	Sample	AUC CE-123	AUC IS	Ratio	Mean	Mean Reduction	Reduction %
0	Blank	44627512	22974866	1.94	-		
24	1	38784100	21926038	1.77	1.79	0.15	7.89
	2	45349504	22251376	2.04*			
	3	40424624	22341230	1.81			

*outlier, not included in the calculation

Table 21. Suggested metabolite data evaluation

Sample	Substance	AUC	Ratio CE-123/IS	Ratio Metabolite /IS	Ratio Metabolite /CE-123	Metabolite % of CE-123
1	CE-123	38860216				
	Metabolite	206371.3	1.7699	0.0094	0.0053	0.53%
	IS	21955962				
2	CE-123	45438144				
	Metabolite	215784.5	2.0382	0.0097	0.0047	0.47%
	IS	22293652				
3	CE-123	40505848				
	Metabolite	202338.8	1.8092	0.0090	0.0050	0.50%
	IS	22388582				

7.2 Liquids and Chemicals

Acetonitrile (ACN), (LC-MS grade, Merck KGaA, Darmstadt, Germany)

DMEM high Glucose w/o Glutamine, w/o Sodium Pyruvate (Sigma Aldrich, Lonza)

Dimethylsulfoxid (DMSO), (≥99.5% BioScience-Grade, Carl RothGmbH + Co. KG, Karlsruhe, Germany)

Dubeccos Phosphate Buffer Solution

Fetal Bovine Serum (FBS) 10% (50 ml)

Formic acid (98%, Carl RothGmbH + Co. KG, Karlsruhe, Germany).

Glutamine 2% (10 ml)

Pen/Strep 1% (5 ml)

Trypsin Gibco

0.25% Trypsin diluted with 1:10 with EDTA 1 mM

Water (LC-MS grade, Merck KGaA, Darmstadt, Germany)

Water with 0.1% (v/v) Formic acid (Hypergrade for LC-MS LiChrosolv®, Merck KGaA, Darmstadt, Germany)

Williams E (provided by BIOPREDIC International)

7.3 Statistical Parameters

Mean: Calculated with MS Excel 365

SD: Calculated with MS Excel 365

Numeric data displayed is rounded to two decimals.

List of Tables

List of Figures

List of Abbreviations

ACN	Acetonitrile
cAMP	circular Amino-Mono-Phosphate
COMT	Catechol-O-methyltransferase
CNS	Central Nervous System
CYP	Cytochrome P450 monooxygenases
DA	Dopamine
DAT	Dopamine Active Transporter
DC	Direct current
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethylsulfoxid
EDS	Excessive daytime sleepiness
EDTA	Ethylenediaminetetraacetic acid
EEG	Electroencephalography
EIC	Extracted Ion Chromatogram
EMA	European Medicine Agency
ESI	electron spray ionization
eV	electron volt
FA	formic acid
FBS	Fetal bovine serum
FMO	flavin-monooxygenases
FT	Fournier transform
IP_3	Inositol-triphosphate
IS	Internal Standard
kV	kilo Volt
L-DOPA	L - 3,4 – dihydroxyphenylalanine

LC	liquid chromatography
LOD	Limit of Detection
LOQ	Limit of Quantification
MAO	Monoamine oxidases
Min	Minute
m/Q	mass to charge ratio
MS	mass spectrometry
MSLT	Multiple Sleep Latency Test
PBS	phosphate buffered saline
REM	Rapid Eye Movement
RF	radio frequency
Rpm	rounds per minute
RT	retention time
SD	standard deviation
SULT	Sulfotransferase
S/N	signal to noise ration
UDP	uridine-5-diphosphate
UGT	uridine-glucuronosyltransferases
Vpp	peak-to-peak Voltage
µL	microliters

8 References

Abad, V. C., & Guilleminault, C. [Christian] (2017). New developments in the management of narcolepsy. *Nature and Science of Sleep*, *9*, 39–57. https://doi.org/10.2147/NSS.S103467

Ahmed, I., & Thorpy, M. (2010). Clinical features, diagnosis and treatment of narcolepsy. *Clinics in Chest Medicine*, *31*(2), 371–381. https://doi.org/10.1016/j.ccm.2010.02.014

American Sleep Disorders Association (2001). *The international classification of sleep disorders, revised: Diagnostic and coding manual*. Rochester, MN: American Sleep Disorders Association.

Asanuma, M., Miyazaki, I., Diaz-Corrales, F. J., & Ogawa, N. (2004). Quinone formation as dopaminergic neuron-specific oxidative stress in the pathogenesis of sporadic Parkinson's disease and neurotoxin-induced parkinsonism. *Acta Medica Okayama*, *58*(5), 221–233. https://doi.org/10.18926/AMO/32105

Billiard, M. (2007). Diagnosis of narcolepsy and idiopathic hypersomnia. An update based on the International classification of sleep disorders, 2nd edition. *Sleep Medicine Reviews*, *11*(5), 377–388. https://doi.org/10.1016/j.smrv.2007.04.001

BIOPREDIC INTERNATIONAL (2011). ORIGINS of the HepaRG cell line. Retrieved from https://www.heparg.com/rubrique-origins-of-the-heparg-cell-line

Choi, H. J., Kim, S. W., Lee, S. Y., & Hwang, O. (2003). Dopamine-dependent cytotoxicity of tetrahydrobiopterin: A possible mechanism for selective neurodegeneration in Parkinson's disease. *Journal of Neurochemistry*, *86*(1), 143–152. https://doi.org/10.1046/j.1471-4159.2003.01808.x

CHROMacademy by Crawford Scientific (n.d.). Mass Spectrometry Fundamental LC-MS: Introduction. Retrieved from http://www.ecs.umass.edu/eve/background/methods/chemical/Openlit/Chromacademy%20LCMS%20Intro.pdf

Coleman, M. D. (2010). *Human drug metabolism: An introduction* (2nd ed.). Oxford: Wiley-Blackwell. Retrieved from http://site.ebrary.com/lib/alltitles/docDetail.action?docID=10381016 https://doi.org/10.1002/9780470689332

Croom, E. (2012). Metabolism of xenobiotics of human environments. *Progress in Molecular Biology and Translational Science*, *112*, 31–88. https://doi.org/10.1016/B978-0-12-415813-9.00003-9

Donato, M. T., Tolosa, L., & Gómez-Lechón, M. J. (2015). Culture and Functional Characterization of Human Hepatoma HepG2 Cells. *Methods in Molecular Biology (Clifton, N.J.)*, *1250*, 77–93. https://doi.org/10.1007/978-1-4939-2074-7_5

Dongiovanni, P., Valenti, L., Ludovica Fracanzani, A., Gatti, S., Cairo, G., & Fargion, S. (2008). Iron depletion by deferoxamine up-regulates glucose uptake and insulin signaling in hepatoma cells and in rat liver. *The American Journal of Pathology*, *172*(3), 738–747. https://doi.org/10.2353/ajpath.2008.070097

EMA (2010, July 22). Questions and answers on the review of medicines containing modafinil. London: European Medicines Agency. Retrieved from https://www.ema.europa.eu/en/documents/referral/questions-answers-review-medicines-containing-modafinil-initial_en.pdf

FDA (October, 2010). *Provigil (modafinil) Tablets [C-IV]*. Retrieved from https://www.accessdata.fda.gov/drugsatfda_docs/label/2007/020717s020s013s018lbl.pdf

Fearn, R. A., & Hirst, B. H. (2006). Predicting oral drug absorption and hepatobiliary clearance: Human intestinal and hepatic in vitro cell models. *Environmental Toxicology and Pharmacology*, *21*(2), 168–178. https://doi.org/10.1016/j.etap.2005.06.002

Ferraro, L. [L.] (1999). The Vigilance Promoting Drug Modafinil Increases Extracellular Glutamate Levels in the Medial Preoptic Area and the Posterior Hypothalamus of the Conscious Rat Prevention by Local GABAA Receptor Blockade. *Neuropsychopharmacology*, *20*(4), 346–356. https://doi.org/10.1016/S0893-133X(98)00085-2

Ferraro, L. [Luca], Fuxe, K. [Kjell], Tanganelli, S. [Sergio], Tomasini, M. C., Rambert, F. A., & Antonelli, T. (2002). Differential enhancement of dialysate serotonin levels in distinct brain regions of the awake rat by modafinil: Possible relevance for wakefulness and depression. *Journal of Neuroscience Research*, *68*(1), 107–112. https://doi.org/10.1002/jnr.10196

Fog, J. U., Khoshbouei, H., Holy, M., Owens, W. A., Vaegter, C. B., Sen, N., . . . Gether, U. (2006). Calmodulin kinase II interacts with the dopamine transporter C terminus to regulate amphetamine-induced reverse transport. *Neuron*, *51*(4), 417–429. https://doi.org/10.1016/j.neuron.2006.06.028

Forte, T. M., McCall, M. R., Knowles, B. B., & Shore, V. G. (1989). Isolation and characterization of lipoproteins produced by human hepatoma-derived cell lines other than HepG2. *Journal of Lipid Research*, *30*(6), 817–829.

Gerets, H. H. J., Tilmant, K., Gerin, B., Chanteux, H., Depelchin, B. O., Dhalluin, S., & Atienzar, F. A. (2012). Characterization of primary human hepatocytes, HepG2 cells, and HepaRG cells at the mRNA level and CYP activity in response to inducers and their predictivity for the detection of human hepatotoxins. *Cell Biology and Toxicology*, *28*(2), 69–87. https://doi.org/10.1007/s10565-011-9208-4

Gilhus, N. E., Barnes, M. P., & Brainin, M. (2011). *European handbook of neurological management*. Chapter 38; Management of narcolepsy in adults (2nd ed.). Chichester, West Sussex, UK, Hoboken, NJ: Wiley-Blackwell.

Giros, B., el Mestikawy, S., Bertrand, L., & Caron, M. G. (1991). Cloning and functional characterization of a cocaine-sensitive dopamine transporter. *FEBS Letters*, *295*(1-3), 149–154. https://doi.org/10.1016/0014-5793(91)81406-x

Giros, B., el Mestikawy, S., Godinot, N., Zheng, K., Han, H., Yang-Feng, T., & Caron, M. G. (1992). Cloning, pharmacological characterization, and chromosome assignment of the human dopamine transporter. *Molecular Pharmacology*, *42*(3), 383–390.

Graham, D. G. (1978). Oxidative pathways for catecholamines in the genesis of neuromelanin and cytotoxic quinones. *Molecular Pharmacology*, *14*(4), 633–643.

Gripon, P., Rumin, S., Urban, S., Le Seyec, J., Glaise, D., Cannie, I., . . . Guguen-Guillouzo, C. (2002). Infection of a human hepatoma cell line by hepatitis B virus. *Proceedings of the National Academy of Sciences of the United States of America*, *99*(24), 15655–15660. https://doi.org/10.1073/pnas.232137699

Hart, S. N., Li, Y., Nakamoto, K., Subileau, E.-a., Steen, D., & Zhong, X.-b. (2010). A comparison of whole genome gene expression profiles of HepaRG cells and HepG2 cells to primary human hepatocytes and human liver tissues. *Drug Metabolism and Disposition: The Biological Fate of Chemicals*, *38*(6), 988–994. https://doi.org/10.1124/dmd.109.031831

Herdegen, T. (2013). Kurzlehrbuch Pharmakologie und Toxikologie. In T. Herdegen (Ed.), *Kurzlehrbuch Pharmakologie und Toxikologie* (3rd ed.). Georg Thieme Verlag. https://doi.org/10.1055/b-002-96280

Herdegen, T. (2020). *Kurzlehrbuch Pharmakologie und Toxikologie*. Stuttgart: Georg Thieme Verlag. https://doi.org/10.1055/b-006-163246

Huppelsberg, J., & Walter, K. (2013). Kurzlehrbuch Physiologie: Die interzelluläre Weitergabe einer Erregung. In J. Huppelsberg & K. Walter (Eds.), *Kurzlehrbuch Physiologie* (4th ed.). Georg Thieme Verlag. https://doi.org/10.1055/b-002-96275

Ishizuka, T., Sakamoto, Y., Sakurai, T., & Yamatodani, A. (2003). Modafinil increases histamine release in the anterior hypothalamus of rats. *Neuroscience Letters*, *339*(2), 143–146. https://doi.org/10.1016/S0304-3940(03)00006-5

Javitt, N. B. (1990). Hep G2 cells as a resource for metabolic studies: Lipoprotein, cholesterol, and bile acids. *FASEB Journal : Official Publication of the Federation of American Societies for Experimental Biology*, *4*(2), 161–168. https://doi.org/10.1096/fasebj.4.2.2153592

Jung, J.-C., Lee, Y., Son, J.-Y., Lim, E., Jung, M., & Oh, S. (2012). Simple synthesis of modafinil derivatives and their anti-inflammatory activity. *Molecules (Basel, Switzerland)*, *17*(9), 10446–10458. https://doi.org/10.3390/molecules170910446

Kalaba, P., Aher, N. Y., Ilić, M., Dragačević, V., Wieder, M., Miklosi, A. G., . . . Lubec, G. (2017). Heterocyclic Analogues of Modafinil as Novel, Atypical Dopamine Transporter Inhibitors. *Journal of Medicinal Chemistry*, *60*(22), 9330–9348. https://doi.org/10.1021/acs.jmedchem.7b01313

Kanamori, T., Kanda, K., Yamamuro, T., Kuwayama, K., Tsujikawa, K., Iwata, Y. T., & Inoue, H. (2015). Detection of main metabolites of XLR-11 and its thermal degradation product in human hepatoma HepaRG cells and human urine. *Drug Testing and Analysis*, *7*(4), 341–345. https://doi.org/10.1002/dta.1765

Kanbayashi, T., Abe, M., Fujimoto, S., Miyachi, T., Takahashi, T., Yano, T., . . . Shimizu, T. (2003). Hypocretin deficiency in niemann-pick type C with cataplexy. *Neuropediatrics*, *34*(1), 52–53. https://doi.org/10.1055/s-2003-38619

Kanbayashi, T., Sagawa, Y., Takemura, F., Ito, S.-U., Tsutsui, K., Hishikawa, Y., & Nishino, S. [S.] (2011). The pathophysiologic basis of secondary narcolepsy and hypersomnia. *Current Neurology and Neuroscience Reports*, *11*(2), 235–241. https://doi.org/10.1007/s11910-011-0178-y.

Kittler, K., Fessard, V., Maul, R., & Hurtaud-Pessel, D. (2014). Cyp3a4 activity reduces the cytotoxic effects of okadaic acid in HepaRG cells. *Archives of Toxicology*, *88*(8), 1519–1526. https://doi.org/10.1007/s00204-014-1206-x

Knowles, B. B., Howe, C. C., & Aden, D. P. (1980). Human hepatocellular carcinoma cell lines secrete the major plasma proteins and hepatitis B surface antigen. *Science (New York, N.Y.)*, *209*(4455), 497–499. https://doi.org/10.1126/science.6248960

Kristofova, M., Aher, Y. D., Ilic, M., Radoman, B., Kalaba, P., Dragacevic, V., . . . Aradska, J. (2018). A daily single dose of a novel modafinil analogue CE-123 improves memory acquisition and memory retrieval. *Behavioural Brain Research*, *343*, 83–94. https://doi.org/10.1016/j.bbr.2018.01.032

Latscha, H. P., Kazmaier, U., & Klein, H. A. (2016). Heterocyclen. In H. P. Latscha, U. Kazmaier, & H. Klein (Eds.), *Springer-Lehrbuch. Organische Chemie* (pp. 331–347). Berlin, Heidelberg: Springer Berlin Heidelberg. https://doi.org/10.1007/978-3-662-46180-8_22

Lavagnini, I. (2010). *Quantitative applications of mass spectrometry*. Chichester, England: Wiley.

LaVoie, M. J., Ostaszewski, B. L., Weihofen, A., Schlossmacher, M. G., & Selkoe, D. J. (2005). Dopamine covalently modifies and functionally inactivates parkin. *Nature Medicine*, *11*(11), 1214–1221. https://doi.org/10.1038/nm1314

Lee, D. Y., Bowen, B. P., & Northen, T. R. (2010). Mass spectrometry-based metabolomics, analysis of metabolite-protein interactions, and imaging. *BioTechniques*, *49*(2), 557–565. https://doi.org/10.2144/000113451.

Lee, M. S. (2002). *LC/MS Applications in Drug Development*: John Wiley & Sons.

Li, L.-B., Chen, N., Ramamoorthy, S., Chi, L., Cui, X.-N., Wang, L. C., & Reith, M. E. A. (2004). The role of N-glycosylation in function and surface trafficking of the human dopamine transporter. *The Journal of Biological Chemistry*, *279*(20), 21012–21020. https://doi.org/10.1074/jbc.M311972200

Lundanes, E., Reubsaet, L., & Greibrokk, T. (2014). *Chromatography: Basic principles, sample preparations and related methods*. Weinheim: Wiley-VCH.

Madras, B. K., Miller, G. M., & Fischman, A. J. (2005). The dopamine transporter and attention-deficit/hyperactivity disorder. *Biological Psychiatry*, *57*(11), 1397–1409. https://doi.org/10.1016/j.biopsych.2004.10.011

Manfred H. Gey (2008). *Instrumentelle Analytik und Bioanalytik*. Berlin, Heidelberg: Springer Berlin Heidelberg. https://doi.org/10.1007/978-3-540-73804-6

Meier, M., Klein, H. H., Kramer, J., Drenckhan, M., & Schütt, M. (2007). Calpain inhibition impairs glycogen syntheses in HepG2 hepatoma cells without altering insulin signaling. *The Journal of Endocrinology*, *193*(1), 45–51. https://doi.org/10.1677/joe.1.07087

Mignot, E. [E.], Nishino, S. [S.], Guilleminault, C. [C.], & Dement, W. C. (1994). Modafinil binds to the dopamine uptake carrier site with low affinity. *Sleep*, *17*(5), 436–437. https://doi.org/10.1093/sleep/17.5.436

Mignot, E. [Emmanuel], Lammers, G. J. [Gert Jan], Ripley, B., Okun, M., Nevsimalova, S., Overeem, S. [Sebastiaan], . . . Nishino, S. [Seiji] (2002). The role of cerebrospinal fluid hypocretin measurement in the diagnosis of narcolepsy and other hypersomnias. *Archives of Neurology*, *59*(10), 1553–1562. https://doi.org/10.1001/archneur.59.10.1553

Miyazaki, I., & Asanuma, M. (2009). Approaches to prevent dopamine quinone-induced neurotoxicity. *Neurochemical Research*, *34*(4), 698–706. https://doi.org/10.1007/s11064-008-9843-1

Nikiforuk, A., Kalaba, P., Ilic, M., Korz, V., Dragačević, V., Wackerlig, J., . . . Lubec, G. (2017). A Novel Dopamine Transporter Inhibitor CE-123 Improves Cognitive Flexibility and Maintains Impulsivity in Healthy Male Rats. *Frontiers in Behavioral Neuroscience*, *11*, 222. https://doi.org/10.3389/fnbeh.2017.00222

Oertel, W., Deuschl, G., & Poewe, W. (2012). *Parkinson-Syndrome und andere Bewegungsstörungen*. 24.3 Narkolepsie (1. Aufl.). s.l.: Georg Thieme Verlag KG. https://doi.org/10.1055/b-0034-63503

Overeem, S. [S.], Mignot, E. [E.], van Dijk, J. G., & Lammers, G. J. [G. J.] (2001). Narcolepsy: Clinical features, new pathophysiologic insights, and future perspectives. *Journal of Clinical Neurophysiology : Official Publication of the American Electroencephalographic Society*, *18*(2), 78–105.

Peever, J., & Fuller, P. M. (2017). The Biology of REM Sleep. *Current Biology : CB*, *27*(22), R1237-R1248. https://doi.org/10.1016/j.cub.2017.10.026

Peters, F. T., & Meyer, M. R. (2011). In vitro approaches to studying the metabolism of new psychoactive compounds. *Drug Testing and Analysis*, *3*(7-8), 483–495. https://doi.org/10.1002/dta.295

Rassow, J., Hauser, K., Netzker, R., & Deutzmann, R. (2016). *Biochemie*. Stuttgart: Georg Thieme Verlag. https://doi.org/10.1055/b-003-129341

Richter, L. H. J., Flockerzi, V., Maurer, H. H. [Hans H.], & Meyer, M. R. (2017). Pooled human liver preparations, HepaRG, or HepG2 cell lines for metabolism studies of new psychoactive substances? A study using MDMA, MDBD, butylone, MDPPP, MDPV, MDPB, 5-MAPB, and 5-API as examples. *Journal of Pharmaceutical and Biomedical Analysis*, *143*, 32–42. https://doi.org/10.1016/j.jpba.2017.05.028

Robertson, P., & Hellriegel, E. T. (2003). Clinical pharmacokinetic profile of modafinil. *Clin Pharmacokinet*, *42*(2), 123–137. https://doi.org/10.2165/00003088-200342020-00002

Romigi, A., Vitrani, G., Lo Giudice, T., Centonze, D., & Franco, V. (2018). Profile of pitolisant in the management of narcolepsy: Design, development, and place in therapy. *Drug Design, Development and Therapy*, *12*, 2665–2675. https://doi.org/10.2147/DDDT.S101145

Saint Hilaire, Z. de, Orosco, M., Rouch, C., Blanc, G., & Nicolaidis, S. (2001). Variations in extracellular monoamines in the prefrontal cortex and medial hypothalamus after modafinil administration: A microdialysis study in rats. *Neuroreport*, *12*(16), 3533–3537. https://doi.org/10.1097/00001756-200111160-00032

Siegel, J. M. (2005). REM Sleep. In *Principles and Practice of Sleep Medicine* (pp. 120–135). Elsevier. https://doi.org/10.1016/B0-72-160797-7/50017-3

Silber, M. H., Krahn, L. E., Olson, E. J., & Pankratz, V. S. (2002). The epidemiology of narcolepsy in Olmsted County, Minnesota: A population-based study. *Sleep*, *25*(2), 197–202. https://doi.org/10.1093/sleep/25.2.197

Springer, D., Staack, R. F., Paul, L. D., Kraemer, T., & Maurer, H. H. [H. H.] (2003). Identification of cytochrome P450 enzymes involved in the metabolism of 4'-methoxy-alpha-pyrrolidinopropiophenone (MOPPP), a designer drug, in human liver microsomes. *Xenobiotica; the Fate of Foreign Compounds in Biological Systems*, *33*(10), 989–998. https://doi.org/10.1080/00498250310001602775

Springer, D., Staack, R. F., Paul, L. D., Kraemer, T., & Maurer, H. H. [H. H.] (2005). Identification of cytochrome P450 enzymes involved in the metabolism of 3',4'-methylenedioxy-alpha-pyrrolidinopropiophenone (MDPPP), a designer drug, in human liver microsomes. *Xenobiotica; the Fate of Foreign Compounds in Biological Systems*, *35*(3), 227–237. https://doi.org/10.1080/00498250400028239

Staack, R. F., Theobald, D. S. [D. S.], Paul, L. D., Springer, D., Kraemer, T., & Maurer, H. H. [H. H.] (2004). In vivo metabolism of the new designer drug 1-(4-methoxyphenyl)piperazine (MeOPP) in rat and identification of the human cytochrome P450 enzymes responsible for the major metabolic step. *Xenobiotica; the Fate of Foreign Compounds in Biological Systems*, *34*(2), 179–192. https://doi.org/10.1080/00498250310001644544

Szabo, S. T., Thorpy, M. J., Mayer, G., Peever, J. H., & Kilduff, T. S. (2019). Neurobiological and immunogenetic aspects of narcolepsy: Implications for pharmacotherapy. *Sleep Medicine Reviews*, *43*, 23–36. https://doi.org/10.1016/j.smrv.2018.09.006

Tanganelli, S. [S.], Fuxe, K. [K.], Ferraro, L. [L.], Janson, A. M., & Bianchi, C. (1992). Inhibitory effects of the psychoactive drug modafinil on gamma-aminobutyric acid outflow from the cerebral cortex of the awake freely moving guinea-pig. Possible involvement of 5-hydroxytryptamine mechanisms. *Naunyn-Schmiedeberg's Archives of Pharmacology*, *345*(4), 461–465. https://doi.org/10.1007/BF00176625

Theobald, D. S. [Denis S.], & Maurer, H. H. [Hans H.] (2007). Identification of monoamine oxidase and cytochrome P450 isoenzymes involved in the deamination of phenethylamine-derived designer drugs (2C-series). *Biochemical Pharmacology*, *73*(2), 287–297. https://doi.org/10.1016/j.bcp.2006.09.022

Thompson, J. M. (2017). *Mass Spectrometry*: Jenny Stanford Publishing. https://doi.org/10.1201/9781351207157

Thorpy, M. J. (2012). Classification of sleep disorders. *Neurotherapeutics : The Journal of the American Society for Experimental NeuroTherapeutics*, *9*(4), 687–701. https://doi.org/10.1007/s13311-012-0145-6

Tse, D. C., McCreery, R. L., & Adams, R. N. (1976). Potential oxidative pathways of brain catecholamines. *Journal of Medicinal Chemistry*, *19*(1), 37–40. https://doi.org/10.1021/jm00223a008

U.S. Department of Health and Human Services, FDA, CDER (2016). Safety Testing of Drug Metabolites: Guidance for Industry. Revision 1.

Westwick, C. R. (1976). Item analysis. *The Journal of Nursing Education*, *15*(1), 27–32.

Willie, J. T., Renthal, W., Chemelli, R. M., Miller, M. S., Scammell, T. E., Yanagisawa, M., & Sinton, C. M. (2005). Modafinil more effectively induces

wakefulness in orexin-null mice than in wild-type littermates. *Neuroscience*, *130*(4), 983–995. https://doi.org/10.1016/j.neuroscience.2004.10.005

Wisor, J. P., Nishino, S. [Seiji], Sora, I., Uhl, G. H., Mignot, E. [Emmanuel], & Edgar, D. M. (2001). Dopaminergic Role in Stimulant-Induced Wakefulness. *The Journal of Neuroscience*, *21*(5), 1787–1794. https://doi.org/10.1523/JNEUROSCI.21-05-01787.2001

Wohlfarth, A., Castaneto, M. S., Zhu, M., Pang, S., Scheidweiler, K. B., Kronstrand, R., & Huestis, M. A. (2015). Pentylindole/pentylindazole Synthetic Cannabinoids and Their 5-Fluoro Analogs Produce Different Primary Metabolites: Metabolite Profiling for AB-PINACA and 5F-AB-PINACA. *The AAPS Journal*, *17*(3), 660–677. https://doi.org/10.1208/s12248-015-9721-0

Wohlfarth, A., Pang, S., Zhu, M., Gandhi, A. S., Scheidweiler, K. B., Liu, H.-f., & Huestis, M. A. (2013). First metabolic profile of XLR-11, a novel synthetic cannabinoid, obtained by using human hepatocytes and high-resolution mass spectrometry. *Clinical Chemistry*, *59*(11), 1638–1648. https://doi.org/10.1373/clinchem.2013.209965

Woods, A. G., & Darie, C. C. (2014). *Advancements of Mass Spectrometry in Biomedical Research* (Vol. 806). Cham: Springer International Publishing. https://doi.org/10.1007/978-3-319-06068-2

Zolkowska, D., Jain, R., Rothman, R. B., Partilla, J. S., Roth, B. L., Setola, V., . . . Baumann, M. H. (2009). Evidence for the involvement of dopamine transporters in behavioral stimulant effects of modafinil. *The Journal of Pharmacology and Experimental Therapeutics*, *329*(2), 738–746. https://doi.org/10.1124/jpet.108.146142