Chapter 9

Summary, conclusions and perspectives

Summary

Humans are exposed throughout their lifetime to a large variety of compounds that are foreign to the human body. These so-called xenobiotics include industrial, consumer, and environmental chemicals and, in most cases, need to be converted into more water-soluble metabolites in order to be excreted from the body in the urine and/or feces. This process is known as biotransformation and usually results in the detoxification of the compounds metabolized. However, biotransformation may also lead to the formation of metabolites having increased therapeutic or toxic effects. This process is often referred to as bioactivation and may be explained either as enhanced activity or affinity of a metabolite towards an enzyme or receptor compared to the parent compound, or it may be seen as a process in which the metabolite becomes more toxic by for example its chemical reactivity which can lead to covalent binding to DNA, adduct formation to proteins or the formation of reactive oxygen species [1].

The endocrine system is one of the body's two major communication systems and has been shown to be susceptible to toxic effects of xenobiotics. Substances capable of disrupting the endocrine system are defined as endocrine disrupting chemicals (EDCs) and alterations of endocrine function caused by an EDC may be through interference with the synthesis, secretion, transport, binding, action or elimination of natural hormones in the body that are responsible for the maintenance of homeostasis, reproduction, development and/or behaviour. Many environmental xenobiotics, such as plasticizers [2], flame retardants [3, 4], pesticides [5] and other persistent organic pollutants, have been described as EDCs while natural compounds in the diet, such as flavonoids [6, 7], are also known to have hormone-type activity.

The main aim of the research described in this thesis was the development and validation of methods to investigate the role of biotransformation in the estrogenicity of xenobiotics. The first two target proteins on which this research has been focused are the human estrogen receptor α (ERα) and the human sulfotransferase 1E1 (SULT1E1) enzyme since both proteins play a role in the regulation of the endocrine system in the human body and have been associated with toxic effects. The third protein investigated is the microbial cytochrome P450 BM3 (CYP102A) enzyme from *Bacillus megaterium*. P450 BM3 is one of the most active P450s so far identified [8], is a soluble highly stable enzyme, and its substrate selectivity and activity can be manipulated by various genetic engineering approaches [9-12]. This makes this enzyme a highly suitable candidate enzyme for biocatalysis thus generating xenobiotic metabolites with ED potential.

This thesis is divided in two parts and nine chapters. The first part (**Chapter 2** to **5**) is dedicated to the development and validation of strategies to measure the affinity of xenobiotics and their possible metabolites towards SULT1E1 and ERα. The majority of the research described in the first part was performed in context of the European project EDEN (Exploring novel endpoints, exposure, low-dose and mixture effects in human, aquatic wildlife and laboratory animals) which was designed as an interdisciplinary effort to address key issues that hampered sound hazard- and risk assessment for EDCs in the European Union. The second part of this thesis (**Chapter 6** to **8**) focused mainly on the screening and employment of P450 BM3 mutants designed to generate biologically active metabolites of drugs or other xenobiotics.

In **Chapter 1** the endocrine system, the human ERα and the human SULT1E1 are introduced. It is shown that SULT1E plays a critical role in the biotransformation of endogenous estrogen steroid hormones and thereby regulates the activity of these potent natural steroids [13, 14]. Inhibition of the SULT1E1-mediated biotransformation of biologically active estrogens by EDCs can lead to an increased *in situ* availability of these endogenous steroids, which has been related to various estrogen-dependant unwanted features [15-20]. For ERα, it is explained that this receptor is a ligand-activated transcription factor that mediates the effects of estrogen steroid hormones on the growth, development, and function of a diverse range of tissues [21]. EDCs can mimic, enhance or inhibit the actions of these estrogen steroid hormones and thereby affect their functions which can lead to toxic effects [22-24]. Examples from literature are used to illustrate that xenobiotics may have endocrine disruption (ED) potential and that EDCs have caused adverse health effects in both humans and wildlife. Due to the toxicity of EDCs through interference with both SULT1E1 and ERα, a range of *in vitro* assays have been developed to assess the effects of xenobiotics on these two target proteins. A brief overview of the available screening assays is presented and the importance of EDC mixture screening is discussed. The involvement of cytochrome P450 monooxygenases (P450s) in the biotransformation of xenobiotics is illustrated and it is explained that these processes can lead to bioactivation of EDCs through the formation of metabolites with estrogenic activities. To get more insight into the bioactivation of xenobiotics, P450s can be used for the facile biosynthesis of sufficient quantities of metabolites for structural elucidation and pharmacological and toxicological evaluation [25]. In general human P450s display low activities and poor stabilities [26] while the microbial P450 BM3 (CYP102A) from *Bacillus megaterium* is a soluble highly stable enzyme and is one of the most active P450s so far identified [8]. This makes P450 BM3 a highly suitable candidate enzyme for biocatalytic purposes, especially since it is known that this enzyme can be manipulated by various genetic engineering approaches in order to alter its substrate selectivity and activity [9-12, 27]. Screening approaches to identify novel P450 BM3 mutants with improved properties are briefly discussed and it is explained that P450 BM3 mutants may be used to investigate the role of P450-mediated biotransformation in the estrogenicity of xenobiotics.

In **Chapter 2**, the development and validation of an assay to measure the inhibition of human SULT1E1 is described. This fluorescence HPLC-based assay is suitable for screening of inhibitors and for determining activity of SULT1E1 and is also easy to use, rapid and sensitive. The assay makes use of the selective SULT1E1 substrate 1-hydroxypyrene (OHP) as a fluorescent probe, and represents a significant improvement over previous assays, which make use of radioactive [20, 28-32] or carcinogenic [33-35] compounds. It was shown that the method was suitable for obtaining rapid insights into the inhibitory properties of EDCs. The assay offers opportunities to investigate for example human tissue samples upon the presence of EDCs and possibly can be used to establish causative predictions between levels of EDCs and health problems concerning SULT1E1.

The aim of **Chapter 3** was to investigate the differences in inhibition by EDCs of murine and human estrogen sulfotransferase 1E1 and to study whether the observed differences could give suggestions as to reasons for differences in substrate inhibition [36-38] and quaternary structure of these two proteins. The inhibitory potential of 34 EDCs was investigated *in vitro* for both human and murine SULT1E1 and IC_{50} values were determined for 14 of the inhibitory EDCs. Significant differences in affinity between the human and murine SULT1E1 were only found for estrone (E1), dienestrol (DIS), and

enterolactone. Extensive molecular modeling was used to rationalize the experimental findings and to suggest possible explanations for substrate inhibition and the existence of an allosteric binding site. During the MD simulations the ligands moved away from the catalytically active position, something which was not observed when simulating the unit cell of the crystal structure. This finding suggests that catalytically inactive binding modes, other than the one observed in the crystal structures, are possible in SULT1E1. The ligands stayed longer in the catalytically active position in mSULT1E1, which is likely a result of simultaneous hydrogen bond formation on both sides of the binding pocket, which does not seem to be possible in hSULT1E1. The ligands in the human protein moved to a sub-pocket near the entrance of the active site, which offers hydrogen bond formation possibilities with Asp22 and Lys85 as well as favourable hydrophobic interactions. The ligands moved more randomly in mSULT1E1. These observations offer a possible explanation for the substrate inhibition only observed in hSULT1E1.

Chapter 4 describes the combination of on-line fluorescence polarization (FP) detection with high resolution screening (HRS) technology for the sensitive screening of ERα affinity of individual components in mixtures. The advantage of FP detection is that it limits the occurrence of interfering autofluorescence of test compounds in the bioassay since it allows detection at high wavelengths. A fluorescein labeled estradiol derivative was synthesized and successfully applied to develop an on-line FP-based receptor affinity detection (RAD) HRS bioassay. Proof of principle was demonstrated by separation of a mixture of five known estrogenic compounds, being 17β-estradiol (E2), 17α-ethinylestradiol (EE2) and the phytoestrogens coumestrol, coumarol and zearalenone (ZEN), followed by post-column bioaffinity screening for the individual affinities for ERα. Additionally, it was demonstrated that the ERα HRS FP system could be applied to screen affinities of off-line generated metabolites of ZEN for ERα. It was concluded that the HRS ERα FP system offers a novel technology to investigate the metabolic profile of drugs and other compounds such as phytoestrogens, which cannot be measured by the coumestrol-based HRS system [39] due to the occurrence of autofluorescence of test compounds.

Chapter 5 describes a systematic attempt to gain an impression of the spectrum of EDCs simultaneously present in wild bream (*Abramis brama*) from three Dutch freshwater locations and to establish whether tissue specimens with reproductive disorders show a spectrum of EDCs that is qualitatively and quantitatively different from that of controls free of symptoms. In addition, the usefulness of measures of total estrogenicity and SULT1E1 inhibition as predictors of negative effects in fish were investigated. The exposure of individual male bream to EDCs was determined by analysis of the adipose tissue for nearly 130 chemicals targeting different classes of EDCs. The estrogenic effects due to exposure to EDCs were assessed by examination of gonads for the formation of ovotestis (OT) [40] and measurement of vitellogenin (VTG) [41-43] concentrations. Bioassay-directed fractionation in combination with the recombinant yeast estrogen screen (YES) [44], the E-Screen bioassay [45], the SULT1E1 inhibition assay (**Chapter 2**), and the coumestrol-based ERα HRS platform [39] was used to determine if differences in estrogenicity could be observed between cases and controls. Although it was demonstrated that this combination of assays could be successfully used to analyze fish bile and adipose tissue samples and extracts, no differences could be observed between cases and controls free of symptoms while it was found that steroidal estrogens accounted for the majority of estrogenicity found in the samples. Full extracts and the corresponding fractions were also tested for human SULT1E1 inhibition and although significant inhibition was observed,

again no differences could be observed between cases and controls. Moreover, the identity of the causative inhibitory chemicals remained largely unknown. Further efforts are required for developing meaningful biomarkers of EDC exposure that can encapsulate its cumulative nature and more research is needed to establish a direct link between the occurrences of the observed reproductive disorders. The utilization of integrative bioassays sensitive to accumulations of certain classes of EDCs may be the way forward to resolve these issues.

The second part of this thesis focused mainly on the screening and employment of bacterial cytochrome P450 BM3 mutants designed to generate biologically active metabolites of drugs and other xenobiotics. **Chapter 6** describes the evaluation of the drug metabolizing potential of a library of P450 BM3 mutants by a LC-MS based screening technology. The goal of this evaluation was to select a minimal panel of stable BM3 mutants that are relatively easy to express and, more importantly, together are suitable to metabolize the largest fraction of drug chemistry space while still displaying differences in regio- and stereoselectivities. To achieve this goal, instead of screening a large number of mutants against a small set of substrates [46], it was decided to screen a limited number of mutants against in total 43 known drugs, which were selected to encompass a large diversity in drug chemical space. Based upon our screening, four mutants (M02, MT35, MT38, and MT43) could be selected that together were capable of metabolizing 77% of the 43 selected drugs by more than 20%. Additionally, it was shown that this panel of mutants was capable of producing P450-mediated metabolites for 41 of the 43 drugs tested although for some compounds the amounts of metabolite formed were low. This panel of BM3 mutants is highly suitable to be used in the drug-development process as general reagents for lead diversification. Furthermore, the panel can also be useful for the identification and rapid production of relevant quantities of human relevant drug(-like) metabolites for pharmacological and toxicological evaluation. The methods and experiments described are useful tools for future research to find better mutants for a selected structurally diverse compound library and the mutants described could be used as a starting point for further random or site-directed mutagenesis, to further increase their activity or to further broaden their substrate specificity or alter their metabolic profile.

In **Chapter 7**, a continuous-flow bioassay setup was used to screen a BM3 library for diversity. The screening strategy used was based on the inhibition of BM3-mediated *O*-dealkylation of the alkoxyresorufin 7-allyloxyresorufin to form the highly fluorescent resorufin. It was shown that the continuous-flow setup could be used to measure allyloxyresorufin *O*-dealkylation (AROD) activities of a library of BM3 mutants and that the setup also could be used to successfully determine enzyme kinetics on-line. The optimized BM3 enzyme affinity detection (EAD) assay was shown to be stable and reproducible and was used to determine affinities for six mutants (M02, M11, M11 V87A, MT35, MT43, and MT80) towards in total 30 xenobiotics. The tested BM3 mutants displayed significant differences in affinity profiles and these differences could be correlated to changes in metabolite distribution for the drug buspirone. It was shown that the developed flow-injection analysis (FIA) EAD approach is suitable to screen for diversity within BM3 mutants and this alternative screening technology offers new perspectives for rapid and sensitive screening of compound libraries towards BM3 mutants.

The aim of **Chapter 8** was to investigate the metabolism of ZEN by different P450s and to determine the estrogenic potencies of the *in vitro* P450-generated metabolites of ZEN. It was shown that mutants of the bacterial P450 BM3 could be used to produce

both human relevant and novel ZEN metabolites and that the HRS technology could be used to screen the metabolic mixtures for ERα affinity. In addition, it was shown that P450 3A4 is mainly involved in the oxidative metabolism of ZEN and that P450 3A4-mediated hydroxylation of ZEN leads to the formation of products with a reduced estrogenic activity. The experiments described were demonstrated to be useful to investigate the metabolism of the EDC ZEN and to identify and characterize its metabolites. In general, this approach could in the future be used for the elucidation of the metabolism of other EDCs and xenobiotics in order to get a better picture of the total effects of these compounds and their metabolites.

Conclusions and Perspectives

The main aim of the research described in this thesis was the development and validation of methods to investigate the role of biotransformation in the estrogenicity of xenobiotics. The human estrogen receptor α (ERα) and the human sulfotransferase 1E1 (SULT1E1) enzyme both play an important role in the regulation of the endocrine system in the human body and have been associated with toxic effects. For SULT1E1, the toxic effects of EDCs are mainly a result of inhibition of the biotransformation processes in which this enzyme is involved (see Fig 1A) since it is believed that SULT1E1 is involved in protecting peripheral tissues from excessive estrogenic effects and therefore plays an important role in the protection against several different carcinomas [47]. Consequently identification of EDCs which can inhibit SULT1E1 is very important. For ERα, it is known that in the cases of tamoxifen [39], methoxychlor [48], benzophenone-3 (BP3) [49], brominated biphenyls [32], bisphenol A (BPA) [50], and other xenobiotics, metabolites are formed by P450-mediated biotransformation which have a higher affinity for ERα than the parent compound. It is thus very important for the toxicological and pharmacological evaluation of such chemicals to get more insight into the possibility of P450-mediated bioactivation (see Fig 1B). The bacterial P450 BM3 obtained from *Bacillus megaterium* is a highly suitable candidate to be used for these purposes since the substrate selectivity, regiospecificity, and activity of this enzyme can be altered by protein engineering techniques and the resulting P450 BM3 mutants have been shown to bioactivate drugs and other xenobiotics [46, 51-53].

Effects of xenobiotics on the biotransformation of estrogens by SULT1E1

One of the major pathways involved in the metabolism of endogenous estrogens is that of SULT1E1-mediated estrogen sulfonation. Compounds which affect the SULT1E1-mediated estrogen/estrogen sulfonate balance could therefore have indirect endocrine disruptin (ED) activity, acting to alter levels of endogenous estrogens rather than acting as direct agonists/antagonists at for example the estrogen or the androgen receptor. In this thesis the development and validation of a fluorescence-based HPLC assay for the determination of SULT1E1 inhibition is described (**Chapter 2**). This assay makes use of the non-carcinogenic 1-hydroxypyrene (OHP) [54], thus omitting the use of radioactive or carcinogenic compounds, and this method was demonstrated suitable for obtaining rapid insight into the inhibitory properties of xenobiotics. Furthermore, it was shown that the assay could be used to investigate the potential of fish tissue samples to inhibit the

SULT1E1 activity (**Chapter 5**) and that it could easily be adapted to investigate the effects of xenobiotics on the SULT1E1 activity *in vitro* in other species (**Chapter 3**). This assay thus offers opportunities to investigate environmental samples upon the presence of EDCs and possibly can be used to establish causative predictions between the levels of EDCs and health problems concerning SULT1E1.

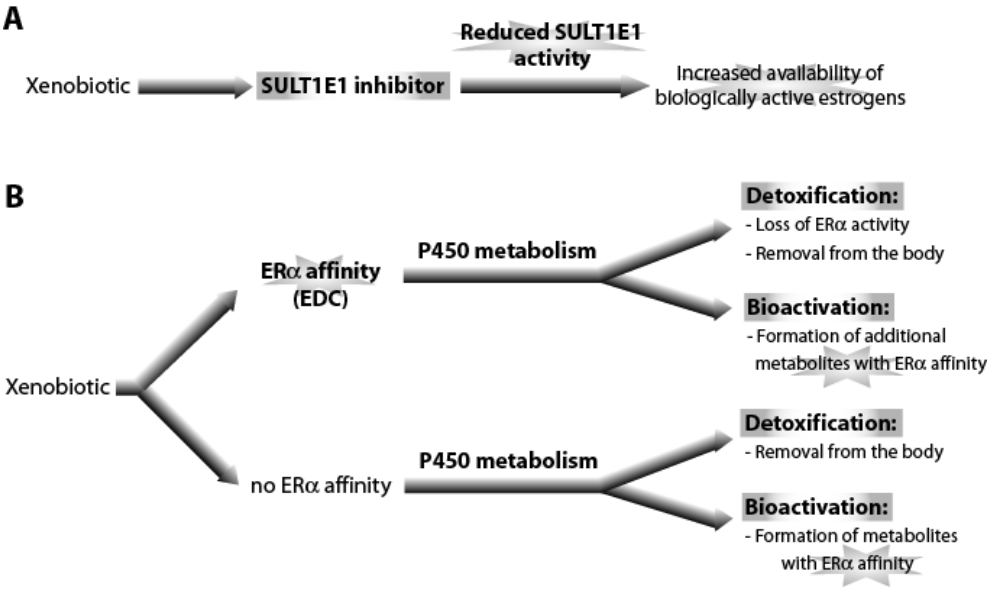

Figure 1 Possible roles of biotransformation in the estrogenicity of xenobiotics.
A) The xenobiotic can act as an inhibitor of the SULT1E1-mediated biotransformation of biologically active estrogens. **B**) When an EDC undergoes biotransformation, this can lead to detoxification (loss of ERα affinity) or to the formation of metabolites with a similar or even enhanced ERα affinity. The biotransformation of a compound with no ERα affinity can lead to detoxification or to the formation of products which display ERα affinity.

It has already been shown that alkylphenols and -phthalates, both known EDCs that are widely used as plasticizers and are common environmental contaminants, have relatively little effect on the estrogen receptors but are strong inhibitors of SULT1E1 [55]. In addition, it has been reported that several flavonoids, such as daidzein, equol, genistein and tricin that due to their estrogenic, antiviral, and antioxidant properties have been included in large amounts in many dietary supplements, are potent SULT1E1 inhibitors [55]. This means that the combined intake from the diet and from the environment could have ED potential through their action on SULT1E1 which stresses the importance of future investigation of the additive or even synergistic effects in mixtures of such compounds for accurate risk assessment. The SULT1E1 inhibition assay described in this thesis would be ideal to perform such studies. A possible approach to establish causative predictions between the levels of EDCs and health problems concerning SULT1E1 might be the use of the inhibition assay to investigate the potential of normal breast and breast cancer tissue samples to inhibit SULT1E1 activity. When differences exist it should be

investigated if the breast cancer tissue samples show a spectrum of EDCs that is qualitatively and quantitatively different from that of normal breast tissue samples.

The work described in this thesis regarding SULT1E1, mainly focused on the effects of unmetabolized xenobiotics on the SULT1E1-mediated biotransformation processes. However, it has been shown by van Lipzig *et al.* [32] and Kester *et al.* [30] that metabolites of polyhalogenated aromatic hydrocarbons (PHAHs) and biphenyls (PHBs), which are formed through P450 activity, inhibited SULT1E1 significantly. This means that for accurate risk assessment of exposure to environmental xenobiotics, it is very important to include the possibility of bioactivation through biotransformation which until now has not been done for SULT1E1 besides the examples mentioned above. It has been shown in this thesis (**Chapters 6**, **7**, and **8**) and by Damsten *et al.* [52, 53] that mutants of P450 BM3 can be used to generate large amounts of human relevant metabolites and this facilitates their isolation, structural elucidation, and toxicological evaluation. Therefore, it would be very interesting to investigate if a fractionation approach can be used in combination with our flourescence-based HPLC assay to investigate the inhibition of SULT1E1 activity by products of BM3-mediated metabolism of xenobiotics. Furthermore, the possibility to translate the fluorescence-based HPLC assay into a high resolution screening (HRS) technique should be investigated. Such a SULT1E1 platform will be highly suitable to investigate the SULT1E1-inhibitory potential of individual components of complex metabolic mixtures in a similar fashion as described for ERα in **Chapters 4** and **8** and would be very helpful for accurate risk assessment of xenobiotics which have the potential to affect SULT1E1 activity through bioactivation.

Bioactivation of xenobiotics to form metabolites with ERα affinity

Exposure to environmental estrogens has been proposed as a risk factor for disruption of reproductive developments and tumorigenesis of humans and wildlife [56, 57]. For accurate risk assessment of estrogenic xenobiotics, the possibility of bioactivation through biotransformation processes needs therefore to be included since neglecting this effect may lead to underestimation of health risks. It has already been shown for the insecticide methoxychlor [48, 59] and the industrial chemical BPA [50] that P450-mediated metabolites have a higher ERα affinity than the parent compound. The on-line ERα HRS technology [39, 58] in principle enables the screening of individual compounds in (metabolic) mixtures by coupling on-line gradient HPLC to a post-column ERα affinity detection system. In **Chapter 8**, the coumestrol-based ERα HRS platform has been successfully used for the on-line profiling of ERα binding P450 BM3-generated metabolites of metabolites of ZEN. It was thereby shown that the combination of BM3-generated biosynthesis and ERα HRS screening can be highly valuable for this purpose since it can be used for the elucidation of the metabolism of environmental xenobiotics and new chemical entities (NCEs). Van Liempd *et al.* have previously developed a bioanalytical HRS platform in which an on-line rat liver P450-bioreactor is combined with on-line ERα affinity detection [59, 60]. Based on the results of the work described in this thesis, it would be very interesting to investigate if P450 BM3 mutants can be applied in a similar setup to create a tool for the analysis of potential estrogenic enhancement of EDCs by P450 activity.

In **Chapter 5**, the usefulness of the coumestrol-based ERα HRS system as a tool to screen environmental samples for the presence of xenobiotics with ERα affinity was evaluated. It was found, however, that the levels of EDCs were too low to detect ERα

affinity which implies that for these studies this platform is not yet suitable. However, it would be interesting to evaluate the usefulness of this setup in studies on exposure of fish to xenobiotics with ED potential during development where the levels of the investigated contaminants and their metabolites are expected to be present at much higher concentrations. In such studies, the HRS setup could be of added value, especially in combination with BM3-mediated biosynthesis of the metabolites to facilitate their detection, identification, and characterization.

The successful development and validation of an on-line HRS system using fluorescence polarization (FP) to screen the ERα affinity of individual compounds in complex mixtures is described in **Chapter 4**. This system offers new opportunities to investigate the metabolic and ERα affinity profile and of drugs and other xenobiotics, which can not be measured by the coumestrol-based HRS system due to the occurrence of autofluorescence of test compounds. More importantly, the novel on-line FP detector cell in principle can be applied to other bioaffinity assays which require FP detection [64]. Thereby it would offer new possibilities to develop HRS assays for other important ED protein targets, such as the androgen receptor, for which currently no HRS format is available.

In conclusion, the main aim of the research described in this thesis, the development and validation of methods to investigate the role of biotransformation in the estrogenicity, has been reached and the usefulness of these methods as tools to determine the ED potential of environmental samples was investigated in the context of the EDEN project. It was shown that the SULT1E1 inhibition assay could not only be used to get more insight in the inhibitory effects of EDCs in different species but also was successfully employed to measure the SULT1E1 inhibition by fish tissue samples. The biocatalytic potential of P450 BM3 mutants to generate human relevant and novel BM3 unique metabolites of xenobiotics was demonstrated and these mutants were successfully used to investigate the role of P450-mediated biotransformation in the ERα affinity of xenobiotics. It was thus shown that the employment of BM3 mutants in combination with the ERα HRS method can be a valuable tool for the ultimate and more accurate risk assessment of the estrogenicity of xenobiotics. The studies presented in this thesis can certainly be applied in environmental research and drug discovery and development and offer several interesting starting points for future research.

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Samenvatting

Mensen worden gedurende hun hele leven dagelijks blootgesteld aan een grote verscheidenheid lichaamsvreemde stoffen. Deze zogenaamde xenobiotica moeten, in de meeste gevallen, worden omgezet in meer wateroplosbare metabolieten om te kunnen worden uitgescheiden door het lichaam in de urine en/of de feces. Dit proces wordt biotransformatie genoemd en resulteert meestal in de ontgifting van de gemetaboliseerde stoffen. Echter, in sommige gevallen leidt biotransformatie tot de vorming van metabolieten met verhoogde therapeutische of toxische effecten. Dit proces wordt bioactivatie genoemd en kan in het geval van een verhoogde therapeutische werking verklaard worden door een verhoogde activiteit of affiniteit van de metaboliet vergeleken met de uitgangsstof ten opzichte van een enzym of receptor. Eventuele toxische effecten kunnen veroorzaakt worden door een toegenomen chemische reactiviteit wat kan leiden tot covalente binding aan DNA, eiwitadduct formatie of de vorming van zuurstofradicalen.

Het endocriene systeem is één van de belangrijkste communicatiesystemen van het menselijk lichaam en het is aangetoond dat dit systeem gevoelig is voor de toxische effecten van xenobiotica. Stoffen die in staat zijn om het endocriene systeem te ontregelen worden ‘endrocrine disrupting chemicals’ (EDCs) genoemd. De effecten van een EDC worden veroorzaakt door ontregeling van de synthese, de secretie, het transport, de binding, de actie of de eliminatie van natuurlijke hormonen in het lichaam die verantwoordelijk zijn voor het regelen van de homeostase, de voortplanting, de ontwikkeling en het menselijk gedrag. Van veel milieurelevante xenobiotica, zoals weekmakers, vlamvertragers, pesticiden en andere persistent aanwezige organische verontreinigingen, is bekend dat het EDCs zijn. Daarnaast is ook voor stoffen, die van nature in de voeding aanwezig zijn, aangetoond dat ze hormonale effecten kunnen hebben.

Het hoofddoel van het onderzoek, wat beschreven is in dit proefschrift, was de ontwikkeling en validatie van methodes om de rol van biotransformatie in de estrogeniciteit van xenobiotica te onderzoeken. De eerste twee eiwitten waar het onderzoek op werd gefocust waren de humane estrogeen receptor α (ERα) en het humane sulfotransferase 1E1 (SULT1E1) enzym aangezien beide eiwitten een rol spelen in de regulering van het endocriene systeem in het menselijk lichaam en gerelateerd zijn aan toxische effecten. Het derde eiwit dat onderzocht werd was het bacteriële cytochroom P450 BM3 (CYP102A) enzym afkomstig van *Bacillus megaterium*. P450 BM3 is één van de meest actieve P450s en is een oplosbaar en zeer stabiel enzym waarvoor aangetoond is dat de substraatselectiviteit en activiteit kunnen worden gemanipuleerd met behulp van diverse genetische modificatietechnieken. Hierdoor is dit enzym een uitgelezen kandidaat om gebruikt te worden voor biocatalytische applicaties om metabolieten te produceren van xenobiotica, welke mogelijkerwijs endocriene effecten kunnen hebben.

In **Hoofdstuk 1** worden het endocriene systeem, ERα en SULT1E1 geintroduceerd. Er wordt uitgelegd dat SULT1E1 een kritieke rol speelt in de biotransformatie van endogene estrogene steroïdes en dat remming van de SULT1E1 activiteit door EDCs kan leiden tot verschillende ongewenste verschijnselen. Er wordt verder uitgelegd dat ERα mede verantwoordelijk is voor de effecten van steroïdes op de groei, de ontwikkeling en het functioneren van een divers aantal weefsels en dat EDCs de werking van deze steroïdes kunnen nabootsen, verhogen of remmen wat kan leiden tot toxische effecten. Voorbeelden uit de literatuur zijn gebruikt om duidelijk te maken dat xenobiotica endocrien verstorende effecten kunnen hebben en dat dit geleid heeft tot

negatieve gezondheidseffecten voor zowel mensen als dieren in het wild. De rol van cytochroom P450 monooxygenases (P450s) in de biotransformatie van xenobiotica is besproken en er is uitgelegd dat deze biotransformatie kan leiden tot bioactivatie van EDCs. Dit betekent dat P450s in principe ook gebruikt kunnen worden om grotere hoeveelheden metaboliet te produceren die vervolgens gebruikt kunnen worden voor structuuropheldering en farmacologische en toxicologische studies. Er wordt uitgelegd dat P450 BM3 een zeer geschikte kandidaat is voor dit soort doeleinden en beschikbare methodes om nieuwe P450 BM3 mutanten te creeren en identificeren worden kort besproken. Tevens wordt uitgelegd dat deze BM3 mutanten gebruikt kunnen worden om de rol van biotransformatie door P450s in de estrogeniciteit van xenobiotica te onderzoeken.

In **Hoofdstuk 2** is de ontwikkeling en validatie van een methode om remming van de SULT1E1 activiteit te meten beschreven. Voor deze op fluorescentie gebaseerde HPLC-methode is het aangetoond dat hij geschikt is voor het screenen van remmers en het meten van SULT1E1 activiteit en dat hij snel, gevoelig en makkelijk toe te passen is. De methode maakt gebruik van het fluorescente en selectieve SULT1E1 substraat 1-hydroxypyrene (OHP) en is een verbetering ten opzichte van andere methodes welke gebruik maken van radioactieve of carcinogene stoffen. Het doel van **Hoofdstuk 3** was om te onderzoeken of er verschillen bestaan in de remming van de SULT1E1 activiteit in muizen en mensen door EDCs. Voor in totaal 34 EDCs is de remming van de SULT1E1 activiteit voor de mens en de muis in *in vitro* gemeten en computermodellen zijn gebruikt om de gevonden verschillen te rationaliseren. Hierbij kwamen verschillende zaken aan het licht die mogelijkerwijs een aantal bekende verschillen, waaronder het wel of niet optreden van substraatinhibitie, tussen beide enzymen kunnen verklaren. **Hoofdstuk 4** beschrijft de ontwikkeling van een hoge resolutie screenings (HRS) meetmethode die (metaboliet)mengsels kan scheiden en tegelijkertijd de ERα affiniteiten van de gescheiden componenten van het mengsel kan meten met behulp van een detectiemethode, die gebaseerd is op fluorescentie polarisatie. Het voordeel van deze detectiemethode is dat de kans dat autofluorescentie optreedt veel kleiner is dan in de reeds bestaande methodes het geval is en er is aangetoond dat de methode succesvol is toegepast om de affiniteit van afzonderlijke producten in metabolietmengsels te meten. **Hoofdstuk 5** beschrijft de toepassing van de ontwikkelde SULT1E1 inhibitie assay en een HRS methode, welke gebruik maakt van het substraat coumestrol, om te onderzoeken of deze methodes gebruikt kunnen worden om verschillen aan te identificeren tussen gezonde brasems en brasems die symptonen van endocriene disruptie vertonen.

Het tweede gedeelte van het proefschrift is vooral gericht op het screenen en het gebruik van bacteriele P450 BM3 mutanten, die ontwikkeld zijn om biologisch actieve metabolieten van geneesmiddelen en andere xenobiotica te produceren. **Hoofdstuk 6** beschrijft de ontwikkeling en evaluatie van een methode om het potentieel van BM3 mutanten om geneesmiddelen te metaboliseren te onderzoeken die gebaseerd is op een LC-MS gebaseerde scheidingstechnologie. In plaats van het screenen van een groot aantal mutanten voor de activiteit richting slechts één of twee substraten hebben wij ervoor gekozen om een klein aantal mutanten te testen tegen een groot aantal geneesmiddelen. Op deze manier hebben wij een viertal diverse mutanten kunnen selecteren die niet alleen van toegevoegde waarde kunnen zijn voor de productie van belangrijke metabolieten voor toxicologische studies, maar ook gebruikt kunnen worden in de zoektocht naar nieuwe geneesmiddelen aangezien ze mogelijkerwijs moleculen kunnen bioactiveren op nieuwe posities. In **Hoofdstuk 7** is de ontwikkeling en applicatie van een alternatieve on-line

screeningsmethode om de activiteit en diversiteit van BM3 mutanten te onderzoeken beschreven en deze op fluorescentie gebaseerde methode is gebruikt om aan te tonen dat affiniteitsprofielen gebruikt kunnen worden om de diversiteit van BM3 mutanten te onderzoeken. In **Hoofdstuk 8** zijn BM3 mutanten gebruikt om grote hoeveelheden metabolieten te produceren van de stof zearalenone (ZEN). Vervolgens is aangetoond dat HRS gebruikt kan worden om de ERα affiniteit van de gevormde metabolieten te meten. Hiermee wordt duidelijk aangetoond dat de combinatie van deze beide methodes van grote toegevoegde waarde kan zijn om in de toekomst de mogelijke bioactivering van xenobiotica tot estrogeen actieve producten te onderzoeken.

Het voornaamste doel van het onderzoek, de ontwikkeling en validatie van methodes om de rol van biotransformatie in de estrogeniciteit van xenobiotica te onderzoeken, is grotendeels bereikt. Het uitvoeren van de experimenten welke beschreven zijn in dit proefschrift heeft geleid tot meer inzicht in de effecten welke EDCs kunnen hebben op de biotransformatie processen welke gereguleerd worden door SULT1E1. Tevens zijn er nieuwe methodes ontwikkeld om de rol van door P450s gereguleerde biotransformaties van xenobiotica en de mogelijke ERα affiniteiten van de gevormde metabolieten te onderzoeken. Het grote potentieel van BM3 mutanten om geneesmiddelen te metaboliseren en toegepast te worden voor biocatalytische doeleinden om te helpen bij de risicoschatting van xenobiotica is ook aangetoond. De resultaten en experimenten welke beschreven zijn in dit proefschrift kunnen zodoende mogelijkerwijs gebruikt worden voor het onderzoeken van milieurelevante vraagstukken en bieden diverse mogelijkheden tot vervolgonderzoek.

List of publications

Sebastiaan M. van Liempd, Jeroen Kool, **Jelle Reinen**, Tim Schenk, John H.N. Meerman, Hubertus Irth and Nico P.E. Vermeulen (2005) *Development and validation of a microsomal on-line cytochrome P450 bioreactor coupled to solid-phase extraction and reversed-phase liquid chromatography*. J Chromatogr A **1075**(1-2): 205-12.

Jelle Reinen, Eveline Vriese, Hansruedi Glatt and Nico P.E. Vermeulen (2006) *Development and validation of a fluorescence HPLC-based screening assay for inhibition of human estrogen sulfotransferase 1E1*. Anal Biochem **357**(1): 85-92.

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Eva Stjernschantz*, **Jelle Reinen***, Walter Meinl, Beena J. George, Hansruedi Glatt, Nico P.E. Vermeulen and Chris Oostenbrink (2010) *Comparison of murine and human estrogen sulfotransferase 1E1 inhibition in vitro and in silico – Implications for differences in activity, subunit dimerization and substrate inhibition*. Mol Cell Endocrinol **317**(1-2): 127-40. *contributed equally

Jelle Reinen, Marc J.-F. Suter, Anja C. Vögeli, Mariana F. Fernandez, Hannu Kiviranta, Rik I.L. Eggen and Nico P.E. Vermeulen (2010) *Endocrine disrupting chemicals – Linking internal exposure to vitellogenin levels and ovotestis in Abramis brama from Dutch surface waters*. ETAP, in press.

Jelle Reinen, Jolanda S. van Leeuwen, Yongmin Li, Lifang Sun, Peter D.J. Grootenhuis, Caroline J. Decker, John Saunders, Nico P.E. Vermeulen and Jan N.M. Commandeur. *Efficient screening of P450 BM3 mutants for their metabolic activity and diversity towards a wide set of drug-like molecules in chemical space*. Submitted

Jelle Reinen, Suilan Ferman, Eduardo Vottero, Nico P.E. Vermeulen and Jan N.M. Commandeur. *Application of a fluorescence-based continuous-flow bioassay to screen for diversity of cytochrome P450 BM3 mutant libraries*. Submitted

Jelle Reinen, Livia L. Kalma, Selina Begheijn, Ferry Heus, Jan N.M. Commandeur and Nico P.E. Vermeulen. *Application of drug metabolizing mutants of cytochrome P450 BM3 as biocatalysts for the on-line profiling of estrogen receptor binding metabolites of the mycotoxin zearalenone*. Submitted

Curriculum Vitae

Jelle Reinen was born on June 8th 1980 in Beverwijk. In 1998 he graduated from highschool at the Gymnasium Felisenum in Velsen-Zuid. In the same year he started studying Pharmacochemistry at the Vrije Universiteit in Amsterdam. He did his major in the division of Molecular Toxicology of the Leiden Amsterdam Center for Drug Research (LACDR), at the Department of Chemistry and Pharmaceutical Sciences of the Vrije Universiteit Amsterdam, under supervision of Prof. Dr. Nico P.E. Vermeulen and Dr. Sebastiaan van Liempd. The research involved the development and validation of a microsomal on-line cytochrome P450 bioreactor coupled on-line to solid-phase extraction and reversed-phase liquid chromatography. In December 2004 he received his MSc degree and initiated his PhD project in the same department of the Vrije Universiteit under supervision of Prof. Dr. Nico P.E. Vermeulen and Dr. Jan N.M. Commandeur. From June 2008 on he works on a project under the same supervision which targets the evaluation of the applicability of novel mutants of bacterial cytochrome P450 BM3 for large scale production of drug metabolites. From January 2009 until June 2009 part of this research project has been performed at the department of Drug Innovation at Vertex Pharmaceuticals (San Diego, CA, USA) under supervision of Prof. Dr. Peter D.J. Grootenhuis and Dr. Caroline J. Decker.

List of abbreviations

AROD	7-allyloxyresorufin *O*-dealkylation
BM3	*Bacillus megaterium*
BPA	bisphenol A
DIS	dienestrol
E1	estrone
E2	estradiol
E3	estriol
EAD	enzyme affinity detection
ED	endocrine disruption
EDC	endocrine disrupting chemical
EE2	17α-ethinylestradiol
EEQ	estradiol equivalents
ER	estrogen receptor
ERE	estrogen responsive element
FIA	flow-injection analysis
FP	fluorescence polarization
HCB	hexachlorobenzene
HLM	human liver microsomes
HPLC	high-performance liquid chromatography
HRS	high resolution screening
HTS	high throughput screening
LBD	ligand binding domain
LOD	limit of detection
LOQ	limit of quantification
NCE	new chemical entity
OHP	1-hydroxypyrene
OT	ovotestis
P450	cytochrome P450 monooxygenase
PAH	polyhalogenated aromatic hydrocarbon
PAP	adenosine-3',5'-diphosphate
PAPS	3'-phosphoadenosine 5'-phosphosulfate
PBB	polybrominated biphenyl
PBDE	polybrominated diphenyl ether
PCB	polychlorinated biphenyl
PCDD	polychlorinated dibenzo-*p*-dioxin
PCDF	polychlorinated dibenzofuran
PSA	polar surface area
RAD	receptor affinity detection
SULT	sulfotransferase
SULT1E1	human estrogen sulfotransferase 1E1
TEQ	toxic equivalent quantities
TES	testosterone
TEXB	total effective xenoestrogen burden
VTG	vitellogenin
YES	yeast estrogen screen
ZEL	zearalenol
ZEN	zearalenone

Nawoord

Aan alles komt een eind en godzijdank geldt hetzelfde voor de lijdensweg die komt kijken bij het succesvol afronden van een promotie. Ik moet eerlijk gezegd bekennen dat het mij achteraf gezien allemaal enorm is meegevallen en dat ik over het geheel genomen een erg leerzame, interessante en vooral prettige tijd heb mogen doormaken gedurende het hele proces. Natuurlijk kon ik op sommige momenten slechts met zeer veel moeite een miniscuul straaltje licht bespeuren aan het eind van een tunnel voorzien van een weg vol onneembaar ogende obstakels, maar ook deze hindernissen werden meestal overwonnen en leverden nuttige ervaringen voor de toekomst op. Uiteraard heeft dit alles niet kunnen plaatsvinden zonder de hulp van een groot aantal mensen en daarom wil ik een aantal van hen hiervoor dank betuigen.

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