The role of biotransformation in the estrogenicity of xenobiotics
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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 ER\(\alpha\) 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 \textit{in situ} availability of these endogenous steroids, which has been related to various estrogen-dependant unwanted features [15-20]. For ER\(\alpha\), 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 ER\(\alpha\), a range of \textit{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 \textit{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-hydroxypyrrene (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 \textit{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 alkoxyresorufin 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.