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Review

"*Commandeuring*" Xenobiotic Metabolism: Advances in Understanding Xenobiotic Metabolism

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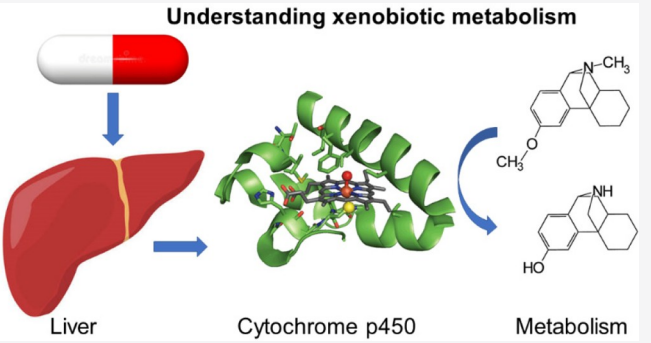
ABSTRACT: The understanding of how exogenous chemicals (xenobiotics) are metabolized, distributed, and eliminated is critical to determine the impact of the chemical and its metabolites to the (human) organism. This is part of the research and educational discipline ADMET (absorption, distribution, metabolism, elimination, and toxicity). Here, we review the work of Jan Commandeur and colleagues who have not only made a significant impact in understanding of phase I and phase II metabolism of several important compounds but also contributed greatly to the development of experimental techniques for the study of xenobiotic metabolism. Jan Commandeur's work has covered a broad area of research, such as the development of online screening methodologies, the use of a combination of enzyme mutagenesis and molecular modeling for structure−activity relationship (SAR) studies, and the development of novel probe substrates. This work is the bedrock of current activities and brings the field closer to personalized (cohort-based) pharmacology, toxicology, and hazard/risk assessment.



1. INTRODUCTION

Chemicals enter our bodies via our food, from the air we breathe, through the skin and other external barriers, or are directly injected into the bloodstream. This can be in the form of nutrition, pharmaceuticals, cosmetic ingredients, or environmental pollutants. They can have an effect at the site of exposure or somewhere else depending on the distribution. Mostly, pharmaceutical chemicals will have some beneficial properties at the exposed concentrations, although some can be toxic, in the parent form or as a metabolite. Determining the distribution and metabolic fate of a compound is thus essential for chemical safety evaluation and a major part of drug development and is important for physiological-based pharmacokinetic (PBPK) predictions.

Xenobiotic metabolism is generally achieved by phase I and phase II enzymes, either working together or separately depending on the chemical entity. Phase I enzymes are responsible for reactions involving oxidation, reduction, and hydrolysis to yield a polar water-soluble metabolite. Phase II enzymes transfer an endogenous hydrophilic group that often facilitates excretion of the molecule from the body; these reactions include glucuronidation, glutathione conjugation, methylation, acetylation, sulfation, and amino acid conjugation. Of the phase I enzymes, cytochrome P450s (CYP) are a large superfamily responsible for oxidation reactions and have approximately 57 members in humans. Of the phase II enzymes, UDP-glucuronosyltransferases (UGT) are responsible for glucuronidation reactions and have about 22 members. Glutathione-S-transferases (GSTs) catalyze the conjugation of the reduced form of glutathione (GSH); this family has approximately 18 members in humans. To make matters more complicated, several phase I and phase II enzymes show extensive genetic variability in human populations, which can affect efficacy and toxicity of pharmaceuticals and can dictate the toxicity of xenobiotics such as environmental toxins.[1−3]

The more we know about phase I and phase II metabolic enzymes, their interactions, inducibility, and polymorphisms, the better we can predict safe therapeutic dosing regimes and make better risk assessment analysis for chemicals. Associate Prof. Dr. Jan Commandeur has spent his professional life dedicated to understanding how biological systems break down chemicals and change them into different (potentially more

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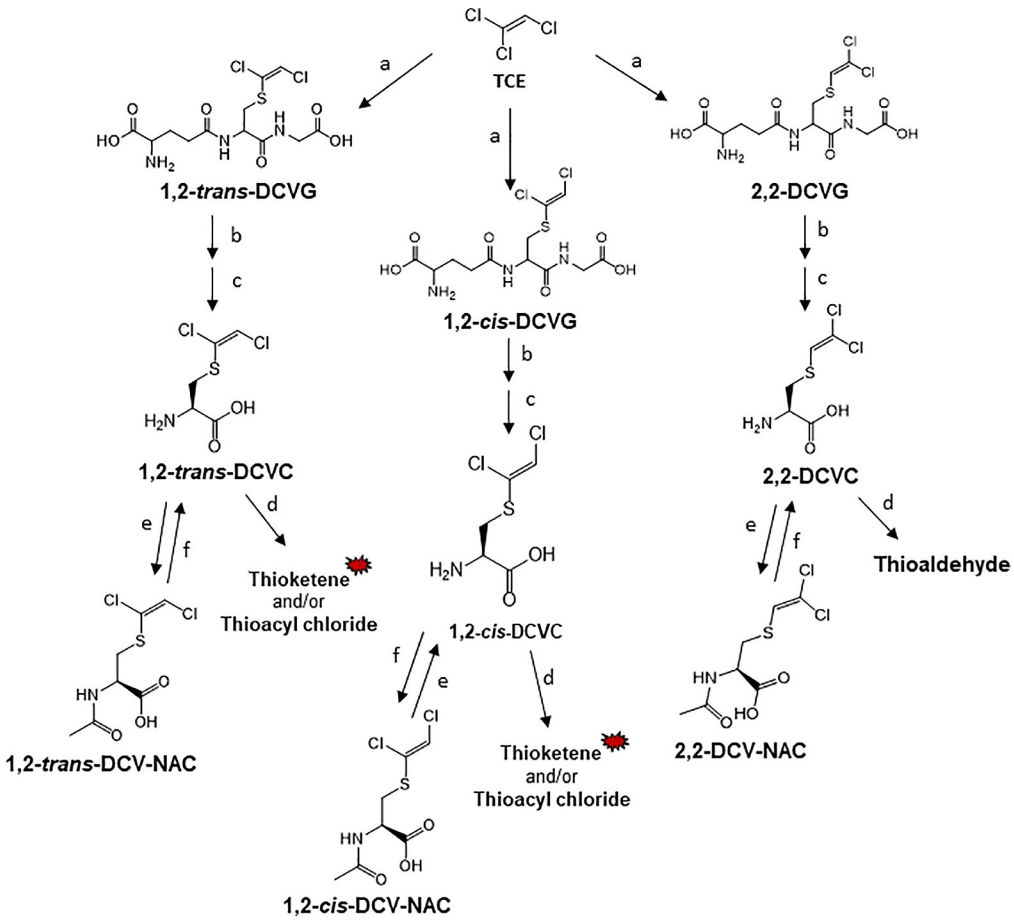

Figure 1. Reaction scheme representing regioisomer formation of GSH conjugation-dependent metabolism of TCE. Enzymes involved: (a) glutathione-S-transferases, (b) γ-glutamyltransferase, (c) cysteinyl−glycine dipeptidase, (d) β-lyase, (e) cysteine-conjugated *N*-acetyltransferase, (f) aminoacylase.

toxic) chemicals. His activities have led to a huge increase in knowledge and many Bachelors, Masters, and Ph.D. students and peers have very much benefited from direct interactions with him. Here, we will present some important findings and chemical case studies by Commandeur and his colleagues that led to a better understanding of xenobiotic metabolism.

2. HALOGENATED ALKENES (WHERE IT ALL BEGAN)

Jan Commandeur's Ph.D. thesis was entitled "Molecular mechanisms of chemically induced nephrotoxicity: Role of the mercapturic acid pathway in the bioactivation of halogenated hydrocarbons". This large family of chemicals has multiple industrial applications such as organic solvents, precursors for chemical synthesis, anesthetics, and dry cleaning.[4] At the time (late 1980s), halogenated hydrocarbons were extremely important from both industrial and economic standpoints despite the identified environmental and human risk concerns.[5] The work developed by Commandeur during this period helped to understand the nature of the reactive intermediates responsible for the nephrotoxicity of halogenated alkenes and determine their relative toxicities. The mercapturic acid pathway, known to be a major route of biotransformation of xenobiotics, was believed to play a key role in the metabolism of halogenated alkenes. In summary, this pathway consists of a series of multienzymatic reactions starting with glutathione (GSH) conjugation by glutathione-S-transferases (GSTs) followed by γ-glutamyl-transferase (GGT) and cysteinyl−glycine dipeptidase activities, leading to the formation of S-cysteine conjugates that can be either N-acetylated for excretion as mercapturic acids, reversibly deacetylated to S-cysteine conjugates, or bioactivated by β-lyase.[6]

In Commandeur's studies, the role of the mercapturic pathway in the bioactivation of nephrotoxic halogenated alkenes was investigated in structurally related fluorinated ethylenes and in the chlorinated hydrocarbon, trichloroethylene (TCE). Regarding fluorinated ethylenes, in vivo and/or in vitro rat studies were performed to evaluate the mode of action for toxicity of 1,1-dichloro-2,2-difluoroethylene (DCDFE), tetrafluoroethylene (TFE), chlorotrifluoroethylene (CTFE), and 1,1-dibromo-2,2-difluoroethylene (DBDFE). Here, DCDFE was administered in rats, leading to the identification of high levels of *N*-acetyl-S-(1,1-dichloro-2,2-difluoroethyl)-L-cysteine (DCDFE-NAc) in urine, suggesting GSH conjugation as a major route of biotransformation of DCDFE. Preadministration of aminooxyacetic acid (AOAA), a known β-lyase inhibitor in rats, reduced the nephrotoxicity of DCDFE, which supported the proposed role of β-lyase in the bioactivation mechanism of halogenated alkenes.[7] On the other hand, administering synthetic DCDFE-NAc in rats resulted in renal toxic effects similar to those seen by exposure of DCDFE itself. These results demonstrated how mercapturic acids could become a useful model compound to study β-lyase-mediated chemical bioactivation mechanisms of fluorinated

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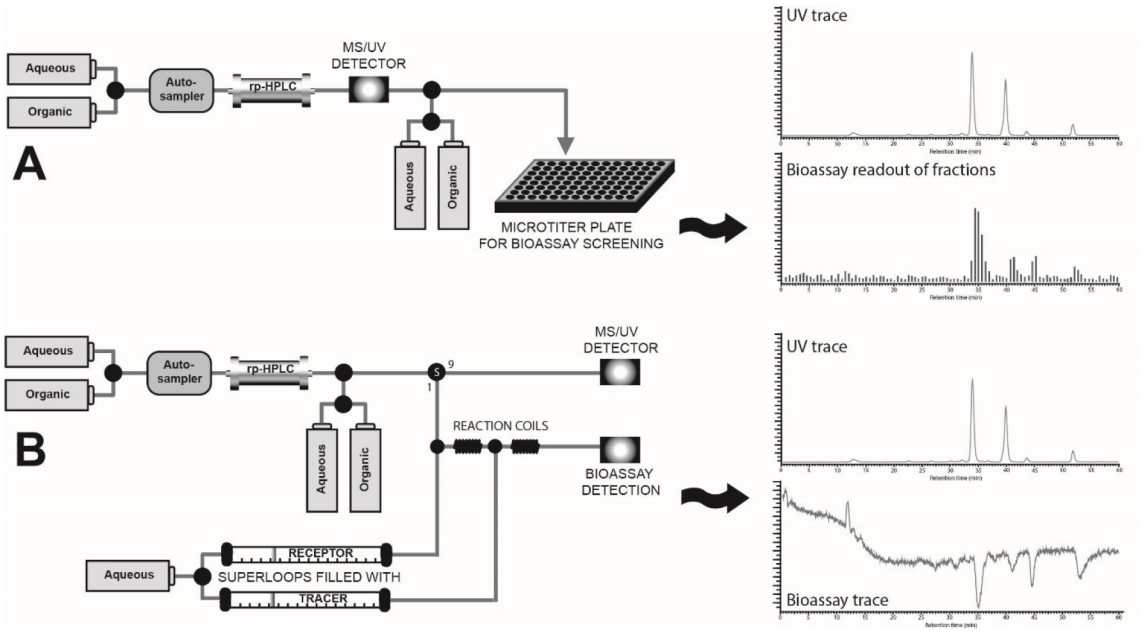

Figure 2. Different strategies for the screening of activity and/or affinity in complex mixtures. The upper portion (A) depicts a fractionation approach in which the gradient HPLC eluent after mixing with a postcolumn makeup gradient is collected in a microtiter plate, which is subsequently analyzed by the appropriate bioassays (e.g., receptor binding or enzyme inhibition assays). The lower portion (B) depicts the general scheme of a HRS setup. After gradient HPLC with postcolumn makeup gradient, the total flow is split to a bioassay and a parallel HPLC effluent readout. In the bioassay, HPLC effluent is first mixed in a reaction coil with the target protein (in this case a receptor), and subsequently, in the second reaction, coil mixed with a probe allows bioassay readout. When the system is operated in flow injection analysis (FIA) mode, the samples are introduced into the carrier solution by the autoinjector directly.

alkenes, as these have challenging chemical properties such as high volatility. Subsequently, in vivo and in vitro experiments with the mercapturic acids of TFE, CTFE, and DBDFE were performed. These studies demonstrated that the relative nephrotoxicity of the mercapturic acids of fluorinated alkenes was determined not only by the bioactivation of cysteine conjugates by β-lyase but also by the rates of acetylation/deacetylation and differences in the types of reactive intermediates formed from the different parent compounds.[8−10]

In addition, Commandeur investigated the GSH-dependent metabolism of TCE. In principle, three possible regioisomers can be formed: S-(1,2-*trans*-dichlorovinyl)glutathione (1,2-*trans*-DCVG), S-(1,2-*cis*-dichlorovinyl)glutathione (1,2-*cis*-DCVG), and S-(2,2-dichlorovinyl)glutathione (2,2-DCVG). After GGT and cysteinyl−glycine dipeptidase activity, these conjugates are converted into their corresponding cysteine-L-conjugates, which can be either bioactivated by β-lyase or reversibly N-acetylated into *N*-acetyl-S-(1,2-*trans*-dichlorovinyl)-L-cysteine (1,2-*trans*-DCV-NAC), *N*-acetyl-S-(1,2-*cis*-dichlorovinyl)-L-cysteine (1,2-*cis*-DCV-NAC), and *N*-acetyl-S-(2,2-dichlorovinyl)-L-cysteine (2,2-DCV-NAC) (Figure 1).

His studies demonstrated that two regioisomeric conjugates, 1,2-*trans*-DCV-NAC and 2,2-DCV-NAC,[11] could be detected by GC/MS in the urine of rats treated with TCE. However, a very low percentage of mercapturic acid excretion was found compared to the dose of TCE administered. Further experiments led to the conclusion that GSH conjugation could be a minor pathway in TCE biotransformation despite the demonstrated nephrocarcinogenicity observed in rat studies from TCE exposure. When comparing the cytotoxic and mutagenic effects of the different regioisomers, 1,2-*trans*-DCV-NAC presented a potency much higher than that of 2,2-DCVC and 2,2-DCV-NAC regioisomers. Incubations of the cysteine conjugates with a β-lyase mimetic model in the presence of a model nucleophile, 4-(*p*-nitrobenzyl)pyridine (NBP), confirmed the high reactivity of 1,2-*trans*-DCVC, whereas 2,2-DCVC incubations resulted in very weak alkylation of NBP.

Three decades later, Commandeur directed his attention to TCE, rejuvenated by new analytical methods and new human in vitro models. A new LC-MS method was developed, leading to the first identification of the third regioisomer from GSH conjugation of TCE—(1,2-*cis*-dichlorovinyl)glutathione (1,2-*cis*-DCVG). This analytical method allowed the detection of all regioisomers from GSH conjugation of TCE and their corresponding cysteine and mercapturic acids. Subsequently, a study regarding the GSH conjugation of TCE in different in vitro models was conducted. Different rates and profiles of the three regioisomers of TCE−GSH conjugation were found in rat and human liver fractions and human recombinant GSTs. The newly identified regioisomer seemed to be the major product from rat liver cytosolic incubations, whereas 2,2-DCVG was mostly produced in human material. From the human GST incubations, GSTA-1 and GSTA-2 were mostly responsible from the human liver cytosol TCE biotransformation. On the other hand, GSTP1-1, which showed 1,2-*trans*-DCVG as the major product, is likely to contribute to most of the extra hepatic GSH conjugation of TCE. In this study, the β-lyase mimetic model in combination with NBP was also used to evaluate the alkylating properties of 1,2-*cis*-DCVC. The results demonstrated the formation of similar products from NBP alkylation to 1,2-*trans*-DCVC incubations, which suggests that the two regioisomers have similar reactivities.

This study provides a new understanding regarding the bioactivation of TCE, reports differences in the species in the

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profile of conjugates formed, and highlights the importance of accurate and sensitive analytical methods in combination with toxicodynamics for accurate chemical risk assessment.

3. MEASURING METABOLIC ACTIVITY

In order to be able to put the knowledge gained from metabolic studies to practical use, it is essential to understand the biological activity of the metabolic products formed. To this end, a considerable amount of work was dedicated by Commandeur and co-workers to the implementation of screening methodologies that could be used to investigate biological activities present in a metabolic mixture. Examples of these biological activities are enzyme affinities and/or interaction with proteins. The basis of the assays to investigate enzymatic activities usually is the conversion of a specific model substrate into a product that can easily be detected fluorometrically or spectroscopically, whereas for the assays to investigate protein interactions, the bioaffinity detection is based on the competition of chemicals with a tracer compound that displays fluorescence enhancement upon interaction with the respective protein.

To determine these interactions, several experimental setups are available. Single reactions can be performed and continuously monitored spectroscopically or fluorometrically to determine the product formation. This setup is ideal for studying enzyme kinetics but can also be applied to determine, for example, enzyme inhibition. Alternatively, a microplate reader setup can be used which can measure product formation at selected time intervals; this allows a more high-throughput approach. This assay format is ideal for investigating the activity of multiple enzymes toward the same substrate, the activity of selected enzymes to multiple substrates, or enzyme inhibition at different concentrations to determine IC_{50} values. A third option is a setup in which an enzyme activity or receptor bioaffinity assay is transformed into an online assay in which enzymes or proteins are continuously mixed with a probe substrate or tracer to generate an online bioassay readout (Figure 2). Such an online assay can be coupled directly to an autoinjector to create a flow injection analysis (FIA) in which a series of compounds can be injected and their effect on the bioassay output can be evaluated. The online assay can also be coupled postcolumn to an analytical separation technique such as high-performance liquid chromatography (HPLC), the so-called high-resolution screening (HRS), to measure bioaffinity of individual components in complex mixtures such as natural extracts or metabolic incubations. HRS has been shown to be a very effective alternative for the traditional approach, which combines HPLC with fractionation techniques to enable the compounds in the mixture to be separated and allow screening of the individual components using the appropriate microplate reader assays. Both setups have been depicted in Figure 2.

3.1. Enzymatic Reactions: The Development of Versatile Colorimetric and Fluorescent Substrates.

Over the years, several of these experimental approaches have been used to study specific enzymatic and protein interaction. For example, to investigate the enzyme kinetics and substrate selectivities of four different rat GST isoenzymes and to determine if structure−activity relationships (SARs) could be used to get more insight into the GSH conjugation of CDNB, a cuvette-based enzyme activity assay was used in combination with a series of in-house synthesized 2- and 4-substituted 1-chloro-4-nitrobenzenes (CDNBs). The enzymatic activities of CDNB and its derivatives were measured colorimetrically to determine apparent Michaelis constants (K_m) and maximum velocities (V_{max}), which allowed calculation of the catalytic constants (k_{cat}) using total enzyme concentration. It was successfully demonstrated that, based on the obtained SARs, the k_{cat}/K_m values for the four GST isoenzymes and the k_s of the base-catalyzed reactions for the GSH conjugation of CDNB could be predicted, thereby enabling the possibility for rational substrate (and inhibitor) design.[12−15]

However, as fluorescence detection is often more sensitive and less prone to interference compared to colorimetric detection, fluorometrically detectable substrates are more suitable for the development of online HRS assays. For example, for CYP2D6, the highly selective substrate 7-methoxy-4-(aminomethyl)-coumarin (MAMC) was developed by a rational design.[16] Based on a small molecule model developed by De Groot et al.,[17] the probe substrate MAMC was synthesized, which could be converted into the O-demethylated metabolite 7-hydroxy-4-(aminomethyl)coumarin (HAMC). As the fluorescence properties of MAMC and HAMC differed significantly, metabolite formation could easily be monitored in time in a cuvette-based setup. The cuvette-based assay was subsequently successfully transformed into a high-throughput microplate reader assay which could be used to determine the inhibition and activity of CYP2D6 in heterologously expressed systems and human liver microsomes.[18]

Another series of promising fluorescent substrates constitute the family of 7-alkoxyresorufins. 7-Alkoxyresorufins can be O-dealkylated by CYPs to form the product resorufin, which has an extremely strong fluorescence at very high wavelengths.[19] The fact that the product formation can easily be monitored makes the 7-alkoxyresorufins ideal substrates for high-throughput screening assays, and as such, they are used routinely to determine CYP activities in various setups (e.g., CYP inhibition studies and ex vivo CYP induction studies).[20,22] In addition, it was demonstrated that the assay could also be used in whole *Escherichia coli* cells by making use of LPS-deficient cells.[21]

3.2. Application of the Developed Substrates in Online HRS Bioassay Systems.

To determine if GST inhibition could be analyzed in complex mixtures, an HRS system was developed which coupled reverse-phased HPLC to two parallel online bioassays with rat cytosolic GSTs and purified human GST Pi.[23] A microplate reader-based assay that made use of monochlorobimane as a substrate was transformed into an online assay in FIA mode. The performance of both assays was comparable, based on the similarity of the IC_{50} values for known GST inhibitors. Subsequently, the online assay was coupled to a gradient HPLC system, and this setup was used to analyze a complex mixture of GST inhibitors. In this setup, individual affinity for both rat cytosolic GSTs and human GST Pi could be determined. As such, it was demonstrated that the HRS setup could be used as a valuable new bioanalytical tool for the rapid and relatively sensitive screening of individual components in complex mixtures for their potential to selectively inhibit GSTs.[22]

The 7-alkoxyresorufin- and coumarin-based fluorescent substrates were applied in an online HRS setup in order to determine inhibition of selected CYPs by individual components in complex mixtures. First, an assay was implemented that made use of the rat CYP1A-mediated O-

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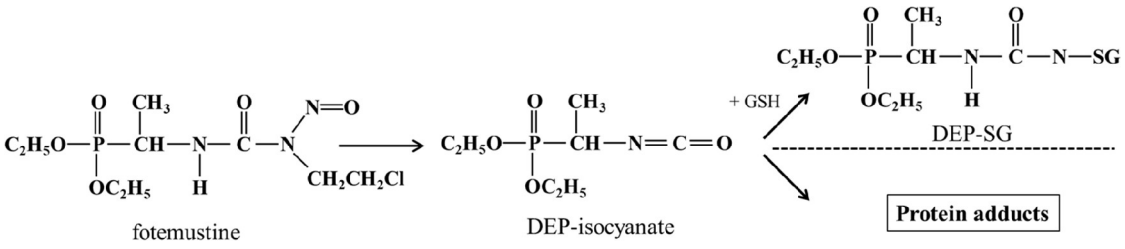

Figure 3. Reaction scheme of fotemustine metabolism.

dealkylation of 7-ethoxyresorufin to form resorufin.[24] Subsequently, additional assays were implemented for rat CYP2B and rat CYP3A that made use of the probe substrates pentoxyresorufin and 7-benzyloxy-4-trifluoromethylcoumarin (BTFC), respectively. It was shown that the rat CYP1A, rat CYP2B, and rat CYP3A assays could be incorporated online and in parallel with a gradient HPLC system to allow simultaneous screening of inhibitors for each of the three CYPs.[25] Two assays were successfully implemented that made use of heterologous human CYP1A2 and CYP2D6 in combination with the probe substrates 7-methoxyresorufin and MAMC, respectively.[26]

In a subsequent study, it was investigated if a similar setup could be used to screen CYP mutant libraries for diversity.[27] It was demonstrated that the setup could be used to determine activity of libraries of mutants toward different 7-alkoxyresorufins to determine enzyme kinetics for selected mutants and to generate affinity profiles for selected mutants toward a library of test compounds. As such, this relatively simple screening methodology could be a useful tool for research aiming at identification of mutants with improved activity and catalytic diversity with respect to substrate selectivity and regioselectivity. The developed FIA setup was further adapted to enable screening of the activity of mutants in the presence of organic solvents.[28] The assay setup not only allowed detection of the formed resorufin but also simultaneously monitored cofactor (nicotinamide adenine dinucleotide phosphate (NADPH)) depletion online. The methodology was shown to be a very powerful tool to screen for novel enzymes with increased tolerability toward organic solvents.

3.3. Screening Ligand−Protein Interactions Using an Online HRS Bioassay System. In the field of endocrine disruption, one of the main questions nowadays is how to deal with mixtures of endocrine disrupting chemicals (EDCs). The HRS technique is ideal to study complex mixtures, and therefore, in 1996, a HRS platform was developed based on the interaction of the fluorescent ligand coumestrol with the estrogen receptor. Coumestrol shows fluorescence enhancement when bound to the active site of the estrogen receptor compared to its unbound state.[29] This HRS setup was used to investigate the CYP-mediated metabolism of the nonsteroidal antiestrogen tamoxifen.[30] It was found that numerous metabolites were formed that displayed significant affinity for the estrogen receptor α (ERα). It was demonstrated that the combination of biosynthesis and HRS screening can be a very useful tool in early drug discovery as it allows the rapid generation, optimization, and, more importantly, toxicological and/or pharmacological evaluation of (novel) lead compounds and their metabolites.

The HRS method has enabled the simultaneous detection of the chemical nature and biological activity of compounds and their metabolites. This technique is sensitive enough to detect, identify, and characterize novel metabolites.

4. CHEMICAL CASE STUDIES

In order to understand the mechanism of toxicity of chemicals, it is crucial to understand their metabolism. This is relevant for understanding safety issues with xenobiotics, drug toxicity through bioactivation into toxic metabolites, and drug efficacy. The following examples were worked on by Commandeur and his colleagues.

4.1. Fotemustine. Fotemustine is a DNA-alkylating 2-chloroethyl-substituted *N*-nitrosourea with antineoplastic activity. Fotemustine alkylates guanine by forming chloroethyl adducts at the 6 position of guanine, resulting in N1-guanine and N3-cytosine cross-linkages. The compound is used for the treatment of various cancers, including melanoma and glioma;[31] however, its use can cause serious side effects including thrombocytopenia, hepatotoxicity, and nephrotoxicity.

In order to elucidate the mechanism of toxicity of fotemustine, various metabolites were synthesized.[32] Also, the metabolic and chemical stability of fotemustine was investigated with ^{31}P NMR and FAB-MS.[33] In the absence of glutathione (GSH), 95% of fotemustine decomposed rapidly into a reactive diethyl ethylphosphonate (DEP)-isocyanate, both in rat liver S9 fraction and in buffer. DEP-isocyanate, in turn, hydrolyzed rapidly into diethyl (1-aminoethyl)-phosphonate, which reacted subsequently with the parent DEP-isocyanate. In the presence of GSH, this hydrolysis of DEP-isocyanate was blocked, and a relatively stable glutathione conjugate (DEP-SG) was formed instead (Figure 3). Further work in rat hepatocytes demonstrated that fotemustine caused concentration- and time-dependent cytotoxic effects.[31,32] Extensive GSH depletion and formation of GSH disulfide (GSSG) were first observed, followed by lipid peroxidation and finally by cell death measured by LDH leakage. The cytotoxicity of fotemustine in rat hepatocytes was proposed to be caused by rapid and extensive depletion of GSH by DEP-isocyanate, consequently hampering the endogenous protection against its own toxicity. GSH conjugation of DEP-isocyanate was shown to protect against the cytotoxicity of fotemustine, however, only temporarily and not completely. It was also shown in rat hepatocytes that coadministration of sulfhydryl nucleophiles, in particular, NAC and GSH-IP, possibly in combination with antioxidants, such as vitamin E, are effective against the toxicity of fotemustine in vitro.[32]

4.2. Cimetidine and Other Thiourea-Containing Histamine Receptor Ligands. The metabolism and toxicity of thiourea-containing histamine receptor ligands, including burimamide and thioperamide, also received some interest from Commandeur and co-workers. Interest in the thiourea moiety as a toxicophore group was rekindled at the end of the

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1990s with the advent of combinatorial chemistry.[34] A variety of structural groups can easily and quantitatively be linked together with the help of the isothiocyanate moiety, which results in a thiourea as a spacer between the desired chemical structures. Furthermore, the thiourea moiety itself was shown to have pharmacological advantages in antibacterial and antiviral agents.[34,35] The toxicity of thiourea was already known since the 1940s when α-naphthylthiourea was developed as a rodenticide.[36] In the 1970s, the thiourea moiety in the first histamine H2-antagonists burimamide and metiamide was replaced with a cyanoguanidine to alleviate reversible agranulocytosis that developed in clinical trials[37] (Figure 4). Cimetidine became an extremely successful drug

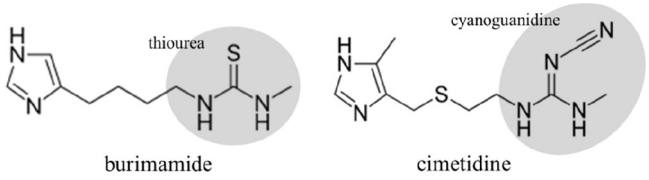

Figure 4. Chemical structures of burimamide (thiourea group) versus cimetidine (cyanoguanidine group).

targeting peptic ulcers and is still in use today. Replacing the thiourea moiety with an isostere group subsequently became common practice.

While the thiourea group as a pharmacophore remained interesting, studies also focused on the structure−toxicity relationships with a series of mono- and disubstituted thiourea-containing compounds in order to investigate whether the addition of various substituents around the moiety could alleviate the observed toxicity.[34] Several mechanisms of bioactivation had been proposed in the literature, notably the release of atomic singlet sulfur, the action of sulfenic acid, the first oxidation product of a thiourea group, and alkylation by subsequent oxidation products of the thiourea group. In the case of singlet sulfur, it was proposed that this would react with sulfhydryl groups of cysteine residues (R−S−H) of proteins, resulting in the formation of hydrodisulfide complexes (R−S−S−H), which would inhibit vital function of the proteins. Upon investigation of this mechanism, evidence for the formation of hydrodisulfides was found, but the amounts were minor and it could not be ruled out that they were produced in a secondary reaction. Studies in rat hepatocytes and precision-cut rat liver slices showed a clear relationship between cytotoxicity and the chemical structures around the thiourea moiety.[34,35,38,39] An aromatic structure linked to the thiourea was shown to increase cytotoxicity, and furthermore, it was found that toxicity increased with the electron-withdrawing capacity of its substituents. Since thiourea bioactivation is most likely due to the activity of flavin-containing monooxygenases (FMOs), the enzyme kinetics of thiourea oxidation were investigated. It was found that the ranking of cytotoxicity of the thiourea tested in rat hepatocytes and precision-cut rat liver slices was closely correlated to the turnover in microsomes containing human FMO1 and FMO3.[35] The metabolites of FMO-mediated oxidation of thiourea were shown to be active in a model for protein alkylation,[40] thus it was concluded that sulfinic acid is the reactive intermediary responsible for thiourea toxicity.

4.3. Acetaminophen. Acetaminophen (*N*-acetyl-*p*-aminophenol, APAP) has been one of the main over-the-counter painkillers since its market introduction in 1950. Although it is generally regarded as safe, it has been known for decades that it induces hepatotoxicity when taken in large doses and is unfortunately frequently used for self-harm, presumably due to the easy availability.[41,42] Hepatotoxicity can, however, also occur at much lower exposure, and thus research into acetaminophen toxicity has been a constant source of knowledge development, and new discoveries are still being made. One research avenue has been investigating several 3,5-disubstituted analogues.[43,44] The hepatotoxicity of APAP was proposed to be due to its quinone metabolite NAPQI, which is formed after oxidation by CYPs (Figure 5).[45] The quinone

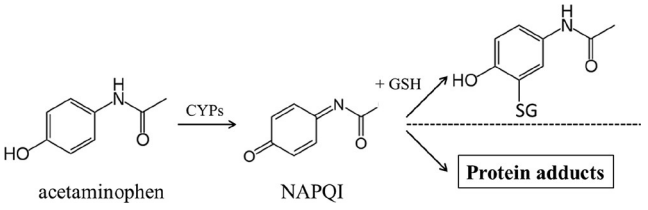

Figure 5. Reaction mechanism of the bioactivation of APAP to NAPQI and subsequent detoxification to GSH adducts.

intermediate would subsequently alkylate vital cellular proteins and cause GSH depletion, resulting in cell death. The 3,5-dihalogenated analogues were found to be subject to structure-related detoxification by glucuronidation and, similarly to APAP, undergo significant bioactivation by CYPs. The NAPQI reactive intermediates are detoxified by glutathione-S-transferase activity to GSH adducts. Similar to acetaminophen, for the dibromo and dichloro analogues, protein adducts were detected.[43,44,46] Since earlier studies had already shown that dialkylated analogues did not produce any cytotoxicity under similar conditions, these results confirmed that the reactivity toward vital cellular structures of the reactive intermediate (3,5-disubstituted NAPQI) was a determinant for the toxicity. Induction of CYPs of the 1A family by β-naphthoflavone (βNF) and depletion of glutathione in the hepatocytes were used to increase bioactivation and to investigate the preventative action of glutathione conjugation.[34]

Studies with βNF-induced rat liver microsomes using a series of 3,5-dialkyl and dihalogen acetaminophen analogues found that the K_m for bioactivation by CYPs of the 1A family decreased with an increase in electronegativity of the substituents.[44] The current consensus is, however, that while CYP1A2 is involved in the bioactivation of APAP, in human liver, the major isoform responsible for acetaminophen oxidation to NAPQI is CYP2E1 and to a lesser extent CYP3A4.[47] After working on the elucidation of the bioactivation of APAP, the protective effect of the antioxidative agent and antirheumatic drug lobenzarit was examined.[48,49] It was found that lobenzarit could protect against APAP toxicity in rat hepatocytes. The drug did not inhibit CYPs nor GSTs, and it was concluded that the protective effect was likely due to its antioxidant effect or its ability to stimulate glutathione reductase activity.[48,49] *N*-Acetyl-*meta*-aminophenol (AMAP), an acetaminophen regioisomer, was tested in precision-cut liver slices to investigate whether this compound could be used as a nontoxic alternative. AMAP was indeed found to be less toxic in mouse slices but not in rat or human slices, stressing the importance of the utilization of human enzymes.[50]

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4.4. 1,2-Dibromoethane. 1,2-Dibromoethane (1,2-DBE) is a category 1B carcinogen and classified as such under the EU CLP Regulation (EC 1272/2008). Exposure of 1,2-DBE to humans primarily occurred due to its use as leaded gasoline additive and as a fumigant. 1,2-DBE is metabolized by two routes: a conjugative route catalyzed by glutathione-S-transferases (GST) and an oxidative route catalyzed by CYP (Figure 6). The CYP-catalyzed oxidation of 1,2-DBE to 2-bromoacetaldehyde (2-BA) was studied using an optimized derivatization method to quantitate 2-BA formation.[51]

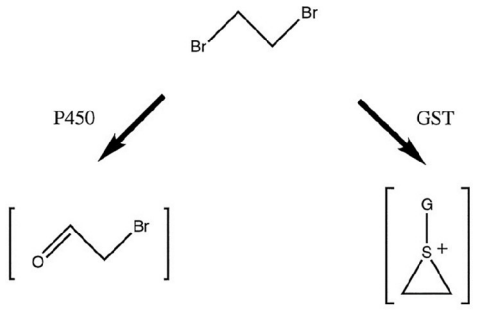

Figure 6. Oxidative (CYP) versus conjugative (GST) metabolic route for 1,2-DBE metabolism.

Using rat liver microsomes, it was found that 1,2-DBE metabolism was much more efficient in microsomes from pyrazole-induced rats, compared to phenobarbital- or β-naphthaflavone-induced liver microsomes. Upon pretreatment of rats with CYP2E1 inhibitors disulfiram and diallylsulfide, significant inhibition of the formation of thiodiacetic acid (TDA), a main urinary metabolite from 1,2-DBE, was observed, indicating that the CYP2E1-catalyzed oxidation of 1,2-DBE plays a major role in the TDA formation.

In humans, interindividual variability of 1,2-DBE oxidation was studied using heterologously expressed CYPs and human liver microsomes.[52] Out of 10 heterologously expressed human CYPs, only human CYP2A6, CYP2B6, and CYP2E1 metabolized 1,2-DBE, albeit with strongly differing catalytic efficiencies. The highest catalytic activity was seen for CYP2E1.[52] In human liver samples, a 46-fold interindividual variability in the 1,2-DBE metabolism was shown, which may have important consequences for the risk assessment for human exposure to 1,2-DBE. Regarding the GST-mediated conjugation reaction, the class theta GST was shown to be the principal GST class conjugating 1,2-DBE in erythrocyte cytosol.[53] This class of GSTs is known to be genetically polymorphic, resulting in interindividual differences in 1,2-DBE metabolism.

This combined information was used to develop a physiologically based pharmacokinetic model for 1,2-DBE metabolism in rats and human.[54] Initially, a liver-only model was built, but validation experiments in rats indicated that metabolism, which previously was assumed to be restricted to the liver, was underestimated. Therefore, the PBPK model was extended to include also the extrahepatic organs in which the enzymes involved in 1,2-DBE metabolism have been detected and quantified. With this extended model, the blood concentrations were much more accurately described, indicating the importance of the extrahepatic metabolism for 1,2-DBE. The saturation of the CYP route was predicted to occur faster in the rat than in human, and the rat was predicted to have a higher turnover of 1,2-DBE. Combining all data, it was recognized that the GST conjugation routes remain significantly active even at low 1,2-DBE concentrations.

This work is a good illustration on how metabolism and disposition information can be used to develop and validate PBPK models. The model also served to demonstrate the effect of genetic polymorphism of GST on individual exposure.

5. METABOLITES AS BIOMARKERS

Another area where Commandeur's studies on xenobiotic metabolism contributed is the field of noninvasive biomarkers. One example involves his research in the field of noninvasive urinary biomarkers of free radical damage, triggering lipid peroxidation (LPO). Degradation of lipid peroxides results in various products, including a variety of carbonyl compounds, and those were studied. The disposition of 4-hydroxy-2,3-nonenal (HNE), a cytotoxic aldehyde and end product of LPO, was investigated in rats.[55] Upon intraperitoneal injection of [2−3H]HNE, it was found that several metabolites were excreted in urine, among which were at least four mercapturic acids. From these, 1,4-dihydroxynonane mercapturic acid (DHN-MA) appeared to be the most abundant mercapturic acid excreted in urine (3.5% of the dose), and the excretion of the other three mercapturic acids amounted to 2% of the dose. Within 48 h, about 25% of the radioactivity was excreted in urine, whereas 18% of the radioactivity appeared in the feces. After 48 h, 7% of the radioactivity was still present in the liver and 0.2% in other organs, but this radioactivity appeared to not be covalently bound to cellular macromolecules. It was found

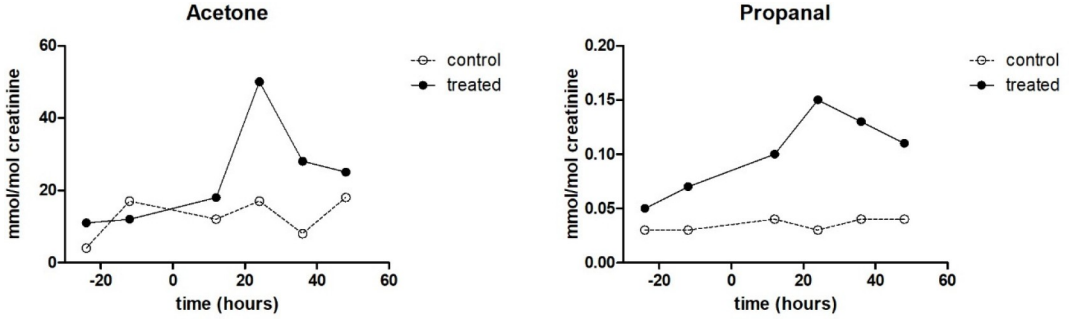

Figure 7. Urinary excretion of acetone and propanal determined every 12 h in urine fractions of four groups of male Wistar rats. Open circles, dotted line: control group. Closed circles, solid line: treated with 0.5 mL/kg body weight of CCl_4. Adapted with permission from De Zwart et al. 1998.

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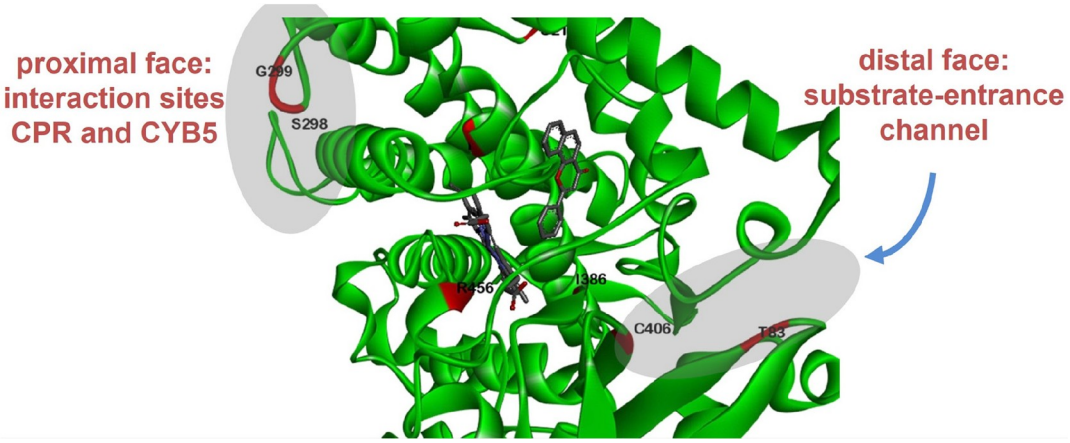

Figure 8. Computer model of the active site of CYP1A2, showing the location of residues 298/299 at the CPR/Cyt. B5 interaction site and residues 406/83 at the substrate entrance/product egress channel. The heme center is shown in gray stick mode. Residue I386 is located near the heme group in the substrate recognition site, and R456, which is involved in heme anchoring, is located below the heme group.

that only 0.13% of the radioactivity was covalently bound in the liver and even less in other organs.

A rapid, selective, and sensitive analytical method based on gas chromatography/electron capture detection (GC-ECD) was developed for the simultaneous determination of eight different lipid degradation products in urine of rats, i.e., for formaldehyde, acetaldehyde, acetone, propanal, butanal, pentanal, hexanal, and malondialdehyde.[56] These LPO-based degradation products were identified using GC-ECD after derivatization and extraction of rat urine samples. The developed analytical method was used to study noninvasive urinary biomarkers induced by free radical damage in the liver by carbon tetrachloride (CCl_4). In urine of CCl_4-treated rats, an increase in all eight lipid degradation products in urine was found 24 h following exposure. Acetone showed the most distinct increase, followed by propanal, butanal, and malondialdehyde. These products appeared to be useful as noninvasive biomarkers for in vivo oxidative stress induced in rats by CCl_4.

A more extensive in vivo study with CCl_4 was subsequently conducted in rats to evaluate the urinary biomarkers for radical-induced liver damage.[57] In this study, these eight degradation products of lipid peroxides were measured dose- and time-dependently in rats treated i.p. with three different single doses of CCl_4. A dose-dependent increase in the lipid degradation products alongside general measures of CCl_4 toxicity, i.e., clinical chemical parameters and histopathological damage, was found (Figure 7). An increase in these products was already found in the first 12 h after exposure. At the lowest dose, acetaldehyde and propanal already showed a statistically significant increase, as well.

In addition to CCl_4, the herbicide diquat and nitrosamine *N*-nitrosodimethylamine (NDMA) were studied in these models. The urinary excretion of seven aldehydes, acetone, coproporphyrin III, and 8-hydroxy-2′-deoxyguanosine (8-OH-dG) as noninvasive biomarkers of oxidative damage was measured in rats treated with diquat or NDMA, two compounds causing hepatic damage by different mechanisms.[58] In the rats treated with diquat, slight hepatotoxicity and nephrotoxicity were found, and the urinary excretion of several aldehydes was several-fold increased. An increase was also found in the urinary excretion of 8-OH-dG after the second dose of diquat.[58] Treatment of rats with calcium carbimide, an aldehyde dehydrogenase inhibitor, did not significantly influence the urinary excretion of aldehydes in control rats. However, in rats treated with diquat, calcium carbimide caused a potentiating effect on the excretion of acetaldehyde, hexanal, and malondialdehyde (MDA), indicating that oxidation of aldehydes to carbonylic acids by aldehyde dehydrogenases (ALDHs) might be an important route of metabolism of aldehydes. For the nitrosoamine NDMA, a substance mainly known for its carcinogenic effects and causing hepatic damage by a different mechanism than diquat, the developed urinary biomarkers were not affected, while slight hepatotoxicity was found. This is supported by data of Anundi and Lindros showing that glutathione (GSH)-dependent cytoprotective mechanisms and lipid peroxidation are not critical in NDMA toxicity.[59] Increased urinary excretion of various aldehydes, acetone, coproporphyrin III, and 8-OH-dG was observed after administration of diquat, probably reflecting oxidative damage induced by this compound. No such increases were found after NDMA administration, which is consistent with a different toxicity mechanism for NDMA. Therefore, excretion of aldehydes, acetone, coproporphyrin III, and 8-OH-dG might be used as easily accessible urinary biomarkers of free radical damage.

In collaboration with NV Organon, the noninvasive urinary biomarkers of free radical damage were also assessed in rats after acute exposure to three different nephrotoxic compounds, i.e., cisplatin, mercuric chloride ($HgCl_2$), and *N*-acetyl-S-(1,1,2,2-tetrafluoroethyl)-L-cysteine.[60] However, in this study, no convincing evidence of free radical damage could be found with the biomarkers for these substances. It was concluded that, in cases of acute severe nephrotoxicity, the use of urinary lipid peroxide degradation products as noninvasive biomarkers for free radical damage is complicated, which might, at least partly, be due to impaired kidney function. From the results, it seemed that there is no major role of free radical damage in the toxicity mechanism of the three nephrotoxic compounds used. For cisplatin, lipid peroxidation has been shown, but this may occur at a late stage in the development of toxicity, explaining the fact that no biomarker effects were seen in the acute study. For *N*-acetyl-S-(1,1,2,2-tetrafluoroethyl)-L-cysteine, the observed results were in agreement with earlier studies in which it was shown that free radical damage was an indirect effect. For $HgCl_2$, a substance which can cause acute renal

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failure and generates high amounts of H_2O_2, the explanation was given that it may not be able to induce radical formation by itself but only by indirect reactions, such as via GSH depletion.

6. SPECIFIC CYPs

A significant part of Commandeur's efforts focused on the elucidation of CYP function, for example, by studying the role of important residues and establishing QSAR models. His work on two of the most important CYPs for human drug metabolism, CYP1A2 and CYP2D6, will be discussed here.

6.1. CYP1A2. Human CYP1A2 is mainly expressed in the liver, where it mediates the metabolism of about 15% of therapeutic drugs and the bioactivation of approximately 20% of human carcinogens.[61] More than 30 CYP1A2 haplotypes are currently known, many of these contain mutations in the noncoding regions, but a substantial number carry non-synonymous mutations in the coding region, causing amino acid changes in the CYP1A2 protein.[62] Eight of these mutations were considered to be more informative, as they seem to be localized in the vicinity of the active center, heme moiety, entrance/exit channel, or the redox partner (NADPH-cytochrome P450 oxidoreductase (CPR), cytochrome b5 (CYB5)) interaction zone. Using a dedicated bacterial cell model, *Escherichia coli* BTC-CYP, each of these CYP1A2 variants was coexpressed with CPR, with or without additional coexpression of CYB5.[63,64] Variants were tested with eight different compounds, representing the different substrate classes with different types of CYP1A2-mediated reactions, namely, O-dealkylation, N-demethylation, and N-hydroxylation. Two variants, I386F and R456H, demonstrated reduced or absent holoprotein respectively, indicating instability or lack of incorporation of the heme moiety in the protein. Using the CYP1A2's crystal structure, the I386 residue could be identified to be part of the substrate recognition structure of the active site and located very close to the active center, near the heme (Figure 8). R456 is part of the protein region of importance for heme anchoring and has been shown to be strongly conserved among more than 6000 CYP sequences and could be assigned to interact with the 7′-propionate of the heme (Figure 8).

An interesting observation is the fact that the detrimental effect of several of the amino acid substitutions could be compensated by the presence of CYB5. This was the case of variant S121C, but particularly for variant G299S, which demonstrated the most altered activity profile in the absence of CYB5, becoming very much WT-like in the presence of CYB5. Superposition and alignment with several CYPs indicated that G299 is located at the surface of the heme domain at the proximal side near the CPR/CYB5 interaction interface together with the adjacent S298; the G299S replacement was hypothesized to disrupt the local fold by steric hindrance. The S298R replacement was determined to be less disruptive. Conversely, variants C406Y and T83M demonstrated more deviated activities when compared to the WT variant in the presence of CYB5. Residues C406 and T83 are located close to the substrate entrance/product egress channel.[65] The deviation of the activity profiles of the C406Y and T83M variants when in the presence of CYB5 indicated a substantial influence of CYB5 on the structure of this channel, located opposite of CYB5's interaction site (Figure 8).

Overall, the results indicated that several of the studied variants of CYP1A2 could have pharmacokinetic and/or toxicokinetic consequences for individuals who are carriers of these allelic forms. This underscores the value in studying polymorphic CYP activity as a driving tool for improvement on the molecular mechanism of the functioning of human CYP enzymes.

6.2. CYP2D6. When in the early 2000s crystal structures of mammalian CYPs became available, a new approach became possible: the site-directed introduction of mutations based on hypotheses derived from crystal structures. This approach can be used to aid the validation of protein homology models and elucidate the roles of active site residues in substrate binding and catalysis, in order to increase our understanding of the structure and mechanism of human CYPs. In the following section, this will be illustrated using the work on CYP2D6 performed in the early 2000s. The techniques used in the cited work range from metabolic studies on heterologously expressed CYP2D6 mutants to resonance Raman spectroscopy, spin relaxation NMR, automated docking, free energy calculations, and molecular dynamics simulations.

CYP2D6 is a particularly important isoenzyme because it plays a role in the metabolism of 30% of the drugs currently on the market.[66] Additionally, for this particular isoenzyme, a high level of genetic polymorphism is known, which results in large interindividual differences in CYP2D6 activity across the population. Approximately 2−21% of the Caucasian population is classified as "poor metabolizers", while another 1−5% has the "ultrarapid metabolizer" phenotype.[67] Since this can result in adverse drug reactions or ineffective drug therapies, all new clinical candidates are screened for P450 2D6 binding and/or metabolism in an early stage of drug development as a "negative selection criterion". Extensive structural knowledge of CYP2D6 to build and validate high-quality computational models could therefore be an asset in the development of safe and effective drugs.

Therefore, homology models of CYP2D6 have been constructed for some time. The first models were based on crystal structures of soluble bacterial cytochrome P450-cam and BM3.[68,69] Docking studies in an active site model of CYP2D6, based on crystal structures of several bacterial CYPs, showed how a range of substituted analogues of 7-methoxy-4-(aminomethyl)coumarin (MAMC) had increased hydrophobic interactions with active site residues with increasing alkyl chain length.[70] This was in perfect agreement with experimental findings, which showed an excellent correlation between the log IC_{50} values and calculated lipophilicities of the compounds.[70]

Despite the usefulness of these early homology models based on bacterial crystal structures, it was considered a great improvement when in 2003 the first CYP2D6 homology model based on a mammalian CYP template (rabbit CYP2C5) was developed.[71] With this model, the differences in IC_{50} values toward 11 CYP2D6 ligands could be rationalized. Additionally, several residues could be identified that were predicted to be part of the active site and play an important role in substrate binding and metabolism. This homology model was subsequently refined and validated.[72] Phenylalanine 120 (F120) was predicted to be involved in interactions with the aromatic moiety present in many typical CYP2D6 substrates. This prediction was validated experimentally using site-directed mutagenesis.[73] The phenylalanine residue was mutated to the smaller, nonaromatic alanine (F120A), and the effect of this mutation on the metabolism of several typical CYP2D6 substrates was studied. The effect of this mutation was shown

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to be strongly substrate-dependent; for example, MAMC metabolism was completely abolished, while bufuralol metabolism was hardly affected. For dextromethorphan and 3,4-methylenedioxymethylamphetamine (MDMA), the metabolic profile was altered by the mutation. The bulky F120 residue probably influences substrate orientation by steric interactions, thus affecting the metabolic profile. When automatically docking differently substituted 3,4-methylenedioxy-*N*-alkylamphetamines (MDAAs) in a CYP2D6 protein model of wild-type CYP2D6 versus the F120A mutant, followed by molecular dynamics (MD) simulations, the relative probabilities of different binding modes of the MDAAs were in good agreement with the altered metabolic profile observed experimentally.[74] In the wild-type enzyme, only O-demethylation was observed experimentally, whereas in the F120A mutant, N-demethylation and N-hydroxylation also occurred. The molecular modeling studies showed that N-demethylation/hydroxylation probabilities for neutral MDAAs were significantly higher in the F120A mutant than in the wild-type enzyme (Figure 9).

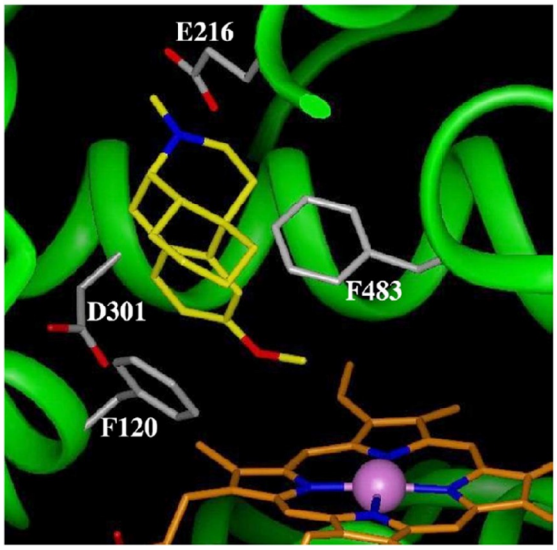

Figure 9. Active site of the homology model of CYP2D6 (Keizers et al. 2004), showing the active site residues F120, E216, D301 and F483. The pink ball at the bottom represents the heme iron atom. In yellow, the substrate dextromethorphan is depicted.

Another technique that was used to study the interaction between substrates and critical active site residues was resonance Raman spectroscopy.[75] With this technique, it could be shown that the F120A mutant allows for more substrate flexibility for dextromethorphan and MDMA compared to the wild-type CYP2D6, explaining the experimentally observed formation of different metabolites for these two substrates.

A second aromatic residue that was proposed by several homology models to be important for CYP2D6 affinity is F483.[76−79] Mutagenesis of F483 into a nonaromatic alanine again resulted in a strongly substrate-dependent effect on metabolism.[72] Similar to that observed for mutant F120A, MAMC metabolism was also completely absent in F483A. Bufuralol metabolism was severely hampered in F483A, whereas MDMA metabolism decreased only 2-fold. For dextromethorphan, the metabolite profile changed under the influence of the F483A mutation. Since binding affinities were hardly affected, it is likely that F483 undergoes steric interactions with ligands rather than aromatic−aromatic interactions between the aromatic moiety of the substrate and the aromatic phenyl ring (Figure 9).

Another interesting finding was that the F483A mutant has lower affinity for *R*-propranolol than for *S*-propranolol, whereas wild-type CYP2D6 does not show this stereospecificity.[80] MD simulations have been performed to reproduce and rationalize this experimental finding. Free energy calculations showed that the F483A mutation causes a loss of favorable hydrophobic interactions, which can be compensated by increased hydrogen bond formation by *S*-propranolol but not by *R*-propranolol.

With a range of MAMC analogues with increasing alkyl chain lengths, the active sites of wild-type CYP2D6 and the mutants F120A and F483A were probed.[81] Where MAMC was not metabolized at all by the mutants, the addition and elongation of an *N*-alkyl chain restored the ability of the mutants to metabolize this substrate. Results suggest that the elongated alkyl chains interact with a distal hydrophobic active site binding cleft. Automated docking of the MAMC analogues showed that F120 appeared to be more involved in aromatic−aromatic interaction, whereas F483 more sterically influenced substrate binding (Figure 9).

While residues involved in substrate binding are critical for the enzyme function, other critical residues include those that are involved in oxygen activation. Based on sequence alignments and homology modeling, threonine 309 in CYP2D6 is proposed to be the conserved I-helix threonine, which is supposed to be involved in dioxygen activation.[82] Experimental investigation of the site-directed mutant T309V showed that the binding affinity of typical CYP2D6 substrates to this mutant was unaffected, but the turnover rates and product ratios for MAMC, MDMA, and bufuralol were altered significantly. The experimental findings could be explained by the hypothesis that T309 is a key determinant in maintaining the balance of the three different oxygenating species that can be deployed by CYPs, which each have a preferred reaction and thus result in different products.

Iterative combination of homology modeling and site-directed mutagenesis has greatly advanced our understanding of the function and mechanism of CYP2D6.

7. GENERATION OF CYP MUTANTS FOR BIOCATALYSIS

Cytochromes P450 can be used as versatile, efficient, selective, and environmentally friendly biocatalysts. With mutagenesis techniques such as site-directed mutagenesis and directed evolution, it has now become possible to generate enzymes that are customized for biotechnology. Several applications of mutagenesis techniques on human and bacterial CYPs can be envisaged. For example, structure-guided site-directed mutagenesis or random mutagenesis ("directed evolution") techniques can be applied to generate CYP mutants with high activity, selectivity, and/or specificity.

7.1. Development of Drug-Metabolizing Bacterial CYP Mutants. Compared to mammalian CYPs, the catalytic activities of bacterial CYPs toward their natural substrates are generally much higher. Also, they are soluble instead of membrane-bound, and the catalytic domain is fused to the reductase domain. Unfortunately, these enzymes often have a narrow substrate specificity compared to the drug-metabolizing

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mammalian CYPs, which limits their use as a biocatalyst. This narrow substrate specificity, however, has been successfully addressed using site-directed and random mutagenesis. CYP102A1, also known as BM3, is one of the most studied bacterial CYPs, and it has the highest catalytic activity ever determined for a CYP for its natural substrate arachidonic acid.[83] Reaction rates of BM3 are generally several thousand-fold higher than those of mammalian CYPs, and 10−100-fold higher than those of other bacterial CYPs such as cytochrome P450-cam and cytochrome P450 eryF.[84−86] This makes BM3 an interesting candidate for the generation of highly active drug metabolizing enzymes. Such mutants could be interesting for the biosynthesis of pharmaceuticals or the formation of drug metabolites for toxicological and pharmacological characterization.

A first step in the direction of achieving this was the development of a fluorescent assay using alkoxyresorufins (see also section 3.1), which could be applied in permeabilized *E. coli* cells expressing (mutants of) BM3 in 96-well plate format.[87] First, a series of site-directed mutants of BM3 were generated, which contained mutations in several residues known to be involved in substrate binding and recognition. These mutants were screened using four alkoxyresorufins. A triple mutant was identified with a 900-fold higher benzyloxyresorufin O-dealkylation (BROD) activity than wild-type BM3. Subsequently, this BROD assay was used for inhibition screening of a library of 45 drug-like compounds. BROD activity by the BM3 triple mutant was inhibited by eight compounds in the library, including the fungicide and known CYP3A4 inhibitor ketoconazole. This suggests that ketoconazole is able to access the active site of the BM3 mutant, thus inhibiting BROD activity. Ketoconazole is known as a specific CYP3A4 inhibitor, which indicates that there are similarities between the active site of CYP3A4 and the active site of this particular BM3 triple mutant.[88]

The fact that inhibition was observed for eight out of 45 drug-like compounds suggests that these drug-like molecules are able to enter the active site; the next step was to investigate if the BM3 triple mutant was also able to metabolize them. To that end, the triple mutant was incubated with drug-like molecules with multiple sites of (potential) metabolism, which make good diagnostic substrates. The study showed that the BM3 triple mutant was able to metabolize amodiaquine, dextromethorphan, MDMA, acetaminophen, and several steroids (testosterone, nandrolone, progesterone, androstenedione). Another phenomenon regularly observed for CYP3A4, the simultaneous binding of multiple substrates and allosteric interactions, was also investigated for this particular BM3 triple mutant. Kinetic evaluation suggested the occurrence of both homotropic cooperativity (non-Michaelis Menten kinetics) and heterotropic cooperativity (up to 20-fold increased product formation in the presence of caffeine). This BM3 triple mutant was the first BM3 mutant described that is able to metabolize drug-like compounds. Overall, it appears to share several characteristics with CYP3A4; both enzymes have a similar substrate specificity and show allosteric kinetic behavior. It can be concluded from this research that BM3 is easy to manipulate and is active and stable; however, the described triple mutant was still considerably less active toward drug-like molecules than mammalian CYPs. Therefore, it was attempted to further improve this mutant using random mutagenesis.[89] A high-throughput screening assay was set up using four different fluorescent probe substrates. Promising mutants were further characterized using the drug-like substrates dextromethorphan and MDMA (Figure 10). The

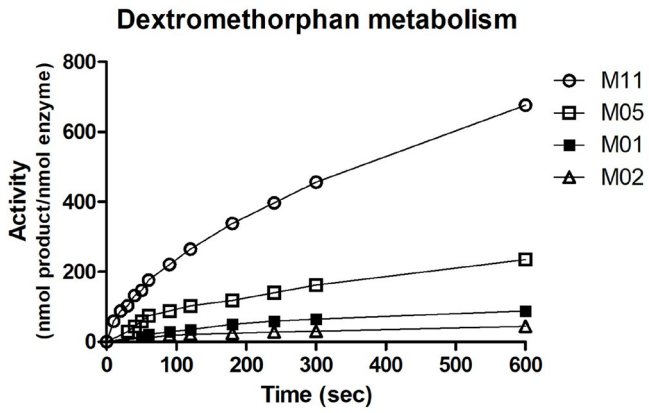

Figure 10. Time dependence of dextromethorphan metabolism by BM3 mutants M11 (open circles), M05 (open squares), M01 (closed squares), and M02 (open triangles). Enzymatic activity in nmol product/nmol enzyme is plotted against reaction time in seconds. Adapted with permission from van Vugt-Lussenburg et al. 2007.

two most promising mutants M01 and M02 were subjected to subsequent rounds of random mutagenesis. After three rounds of mutagenesis, a highly active mutant M11 was identified, which had a catalytic activity higher than mammalian CYPs and 200-fold higher than the triple mutant used as a starting point.

In order to gain more knowledge on the structure and function of BM3, the mutants were sequenced and the effects of the mutations were rationalized using a computer model based on a crystal structure of the substrate-free heme domain.[90] Various computer modeling techniques were used, such as docking, molecular dynamics simulations, and random acceleration molecular dynamics (RAMD) simulations to explore possible substrate entrance/product exit routes. Additionally, experiments were performed to determine coupling efficiency and spectral dissociation constants. Finally, resonance Raman spectroscopy was applied to study the effect of mutations and ligand binding on the heme properties. This technique can be used to detect changes in oxidation, spin, and coordination states of the heme, together with conformational changes of the heme's substituents. Interestingly, the residues responsible for the enhanced catalytic activity were not exclusively located in the active site. Several of these residues were located on the protein surface, where they could affect interaction with the reductase domain and the electron transport chain. Others were postulated to be involved in the formation or alteration of substrate entrance- or product exit channels.

7.2. Application of BM3 Mutants for Characterization and Structural Elucidation of Drug Metabolites. Several applications of these drug-metabolizing mutants have been explored. For example, the BM3 mutants were used to generate large enough quantities of metabolites of the mycotoxin zearalenone to allow toxicological characterization.[91] Zearalenone is a potent estrogen, but the estrogenicity of the major hepatic metabolites was still unknown. Using an online high-resolution screening ERα system, metabolites of zearalenone were generated using either human liver microsomes, recombinant human CYP isoforms, or BM3 mutants, followed by online detection of the estrogenicity of the metabolites

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formed. It was found that the amounts of the metabolites formed by microsomes or recombinant CYPs were too low for affinity detection in the screening ERα setup. The BM3 mutants, however, did generate sufficient quantities of human-relevant metabolites to allow affinity detection as well as structural elucidation. Also for the fenamate class of NSAIDs, BM3 mutants were identified that were able to regioselectively produce human relevant metabolites in sufficient quantities for structural elucidation and characterization, including several metabolites that are notoriously challenging to synthesize chemically.[92]

7.3. Application of BM3 Mutants for the Generation of Reactive Metabolites. Another example of an application where the highly active drug-metabolizing BM3 mutants could be effectively exploited is the generation of reactive metabolites. In some cases, metabolism of drugs by CYPs results in the formation of highly reactive electrophilic metabolites that can subsequently react with macromolecules, leading to covalent adducts to proteins. These events are thought to be related to serious adverse drug reactions. If relevant metabolites could be identified and generated in large quantities in an early stage in drug development, this would strongly facilitate pharmacological and toxicological characterization of the reactive metabolites, which is currently mandatory by the FDA and EMA. Several studies have been performed to demonstrate the applicability of BM3 mutants as biocatalysts in the production of reactive metabolites from the drugs clozapine, diclofenac, troglitazone, and acetaminophen as model compounds.[93−95] It was shown that several BM3 mutants were able to generate the same metabolites as human and rat liver microsomes, with an activity up to 70-fold higher compared to that of human enzymes. This led to the identification of three novel GSH adducts of diclofenac, which were also formed in incubations with human liver microsomes. Using site-saturation mutagenesis, a novel mutant was generated which was able to produce human-relevant GSH adducts of clozapine in sufficient quantities to enable structural characterization by NMR.[93] This demonstrates the applicability of drug-metabolizing mutants of BM3 for the biosynthesis of high amounts of toxicologically relevant reactive metabolites of drugs to enable structural elucidation and pharmacological and toxicological characterization.

As a follow-up, the reactive metabolites formed by BM3M11 from diclofenac and clozapine were incubated with glutathione-S-transferases (GSTs).[96−98] The formation of GSH adducts in the absence or presence of four recombinant GSTs was investigated. The studies showed that for both drugs, several-fold more GSH adducts were formed in the presence of GSTs than in their absence. GST P1-1 turned out to be the most active isoform, but GST A1-1 and GST M1-1 also showed significant activity. Several GSH adducts were only found upon incubation with GSTs but not by mere chemical conjugation with GSH. This illustrates the significant role of GSTs in drug detoxification; further studies are required to investigate whether genetic polymorphisms of hGST P1-1 and hGST M1-1 contribute to the interindividual differences in susceptibility to drug-induced adverse drug reactions. In a subsequent study, this was investigated for clozapine, diclofenac, and acetaminophen.[99] The ability of three allelic variants of GST P1-1 to catalyze the GSH conjugation of reactive metabolites was investigated. However, since the differences in total GSH conjugation activity catalyzed by these allelic variants were not higher than 30%, differences in inactivation of reactive intermediates by GST P1-1 were concluded unlikely to be a major factor in determining interindividual difference in susceptibility to adverse drug reactions induced by the drugs studied.

7.4. Potential of Drug-Metabolizing Mutants of BM3 as Selective and Specific Biocatalysts. In subsequent studies, it was demonstrated how several of the drug-metabolizing BM3 mutants could be further optimized for biotechnological applications. For example, the introduction of an additional mutation, A82W, to the mutants M01 and M11, dramatically increased their regioselectivity for steroid hydroxylation in the direction of 16β-hydroxylation by changing the orientation of the substrate in the active site.[100] Additionally, this mutation increased binding affinity and coupling efficiency, resulting in increased catalytic activity. In silico rationalization of these findings resulted in the identification of additional active site residues that may have an effect on the orientation of testosterone in the active site. A library of mutants, based on M01 and M11, was generated with substitutions at those positions.[101] Interestingly, one additional mutation (S72I) converted M01 A82W from an almost exclusive 16β-hydroxylase into an almost exclusive 16α-hydroxylase.

The anticancer prodrugs cyclophosphamide and ifosfamide require bioactivation by 4-hydroxylation to yield the active DNA-alkylating and cytotoxic metabolite. However, the additionally occurring N-dechloroethylation results in the formation of undesired neuro- and nephrotoxic metabolites.[102] Gene-directed enzyme prodrug therapies have been suggested to facilitate local bioactivation by expressing CYP enzymes within the tumor cells. For ifosfamide and cyclophosphamide, a clinical trial has already been described using angiographic implantation of encapsulated allogeneic cells, genetically modified to overexpress the enzyme cytochrome P450 2B1 into the vasculature leading to the tumor. Ideally, for such a purpose, CYP enzymes would be used that selectively perform the desired 4-hydroxylation reaction, but not the undesired N-dechloroethylation reaction, with high catalytic activity. Screening of a library of BM3 mutants resulted in the identification of two BM3 mutants that showed very rapid bioactivation of cyclophosphamide and ifosfamide, whereas N-dechloroethylation by these mutants was very low or even undetectable.[102]

In another study, the metabolism of a library of 43 drugs, covering a large part of the chemical space, by six BM3 mutants was analyzed using a rapid LC-MS method.[103] Thirty-four of these drugs were metabolized by at least one of the mutants for >20%, in most cases exceeding the activity of human liver microsomes (HLMs). With a subset of nine structurally diverse probe drugs, a larger library of BM3 mutants was also screened using the LC-MS method. This led to the development of a minimal panel of four BM3 mutants which were capable of producing both human-relevant and BM3-unique drug metabolites with high metabolic activities. This is an important step toward the application of bacterial CYPs for lead diversification in the drug development process and the biosynthesis of drug-like metabolites.

While the former method could be applied to study interactions between one mutant and one substrate, the screening process could be greatly enhanced using a cocktail approach. An example of such an approach was shown with the screening of 83 individual mutants against a cocktail of six drugs.[104] It was established that the presence of multiple

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substrates did not significantly alter the percentage of substrate depletion nor the metabolic profile of the enzymes. Each of the 83 mutants was incubated individually with the cocktail of six drugs. Using an LC-MS/MS-based screening method, metabolites could be measured for all drugs in the cocktail. This resulted in multidimensional data on 83 mutants, six substrates, and multiple metabolites per substrate. The data could be analyzed using several data visualization and analytical techniques, including principal component analysis and clustering techniques. This way, the mutants could be grouped or classified based on their substrate and metabolic specificity, which could be used as a basis for further optimization.

With directed evolution and targeted mutagenesis, BM3 was shown to be a fast, efficient, robust, and versatile enzyme which can easily be manipulated to perform specific reactions. Such an enzyme could play an important role in drug discovery, drug synthesis, lead diversification, and safety evaluation.

8. CONCLUDING REMARKS

The work of Jan Commandeur and his colleagues over the past four decades has made significant contributions to a wide variety of topics in the field of toxicology. From environmental monitoring and analysis of pesticides to bioactivation, screening methods, and molecular modeling, Commandeur has had an impact on all. However, the common theme in all of these endeavors was the innovative use of research tools and the scientific curiosity in discovering how chemicals behave in the body and why they have the negative effects we observe.

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Notes

The authors declare no competing financial interest.

Biographies

Dr. Barbara M. A. van Vugt-Lussenburg was a Ph.D. student in Dr. Commandeur's lab in the early 2000s. Her thesis was entitled "Towards novel biocatalysts for drugs and drug-like molecules". Currently, she holds a position as a project leader at BioDetection Systems, where her responsibilities include (but are not limited to) the development of modular metabolism in human cell based reporter gene assays.

Liliana Capinha is a Ph.D. candidate at the division of Molecular and Computational Toxicology of the Vrije Universiteit Amsterdam. Her work focused on combining kinetics and dynamics in vitro for human chemical risk assessment under the supervision of Prof. dr. Paul Jennings and Dr. Jan Commandeur.

Jelle Reinen first met Dr. Commandeur when he was his supervisor for the first practical course of his Pharmaceutical Sciences studies. He was supervised by Dr. Commandeur during his master internship, his Ph.D. research, and his postdoctoral research activities within the Molecular Toxicology department. Besides the valuable scientific input which Dr. Commandeur provided, he also appreciated his input during social activities with the group. Dr. Reinen currently holds a position as Group Leader with the In Vitro Drug Metabolism and Pharmacokinetics Section at Charles River Den Bosch.

Martijn Rooseboom worked with Dr. Commandeur during his Ph.D. training (1998−2002). and after this he has been from time to time interacting with Dr. Commandeur. From 2002−2014. he worked in the pharmaceutical industry at Organon, Schering-Plough, and Merck Sharp & Dohme in various positions in the area of drug metabolism & pharmacokinetics, toxicology, and clinical PKPD. He is currently Principal Toxicologist leading the toxicology team at Shell Plc. He is also a member of the Health Council of The Netherlands and a member of the Dutch Expert Committee on Occupational Safety (DECOS).

Michel Kranendonk first met Dr. Commandeur on his research visits to Amsterdam during his Ph.D. studies (1994−1998, Lisbon, Portugal). He got to know him as an excellent scientist, driven by his curiosity and ingenuity. A decade later, he and Dr. Commandeur shared supervision of a Ph.D. project between Lisbon and Amsterdam, performed by Dr. Kranendonk's student Bernardo Brito Palma. Dr. Commandeur's keenness and sharpness were of great importance for the success of this project. Dr. Kranendonk currently holds a position as a Principal Researcher at the NOVA Medical School, Universidade Nova de Lisboa.

Rob Onderwater studied chemistry at the Vrije Universiteit in Amsterdam. He performed his major in molecular toxicology on the topic of beta-lyase bioactivation, supervised by Dr. Commandeur. Subsequently, he obtained his Ph.D. in pharmacochemistry on the toxicity of thiourea-containing compounds, for which Dr. Commandeur was his copromotor. After his thesis, he worked in the pharmaceutical industry and at the Dutch research institute TNO in in vitro toxicology. In 2003, he moved to Belgium and started working for Wetland Engineering SPRL in Louvain-la-Neuve, and since 2011, he has been working as Scientific Leader of the Biotech unit of Materia Nova ASBL.

Paul Jennings is the current chair of Molecular and Computational Toxicology at the Vrije Universiteit Amsterdam. Prof. Jennings has a degree in Pharmacology from the University College Dublin, Ireland, and is habilitated in Physiology from the Medical University of Innsbruck, Austria.

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The authors of this review are direct beneficiaries of Jan's thirst for knowledge and passion for disseminating this knowledge. We acknowledge Jan Commandeur's contribution to the chemistry and toxicology field. Happy retirement Jan!

■ ABBREVIATIONS

1,2-DBE - 1,2-dibromoethane
2-BA - 2-bromoacetaldehyde
βNF - β-naphthoflavone
A - alanine
ALDH - aldehyde dehydrogenase
AMAP - *N*-acetyl-*meta*-aminophenol
AOAA - aminooxyacetic acid
APAP - acetaminophen
BROD - benzyloxyresorufin *o*-dealkylation
BTFC - 7-benzyloxy-4-trifluoromethylcoumarin
CCl4 - carbon tetrachloride
CDNBs - 1-chloro-4-nitrobenzenes
CPR - NADPH-cytochrome P450 oxidoreductase
CTFE - chlorotrifluoroethylene
CYB5 - cytochrome b5
CYP102A1 - BM3
CYP - cytochrome P450
DBDFE - 1,1-dibromo-2,2-difluoroethylene
DCDFE - 1,1-dichloro-2,2-difluoroehtylene
DCV - dichlorovinyl
DCVC - dichlorovinyl cysteine
DCVG - dichlorovinyl glutathione
DEP-isocyanate - diethyl ethylphosphonate isocyanate
DHN-MA - 1,4-dihydroxynonane mercapturic acid
EDC - endocrine disrupting chemical
ERα - estrogen receptor alpha
F - phenylalanine
FIA - flow injection analysis
FMO - flavin-containing monooxygenase
GC-ECD - gas chromatography/electron capture detection
GGT - γ-glutamyl-transferase
GSH - glutathione
GST - glutathione-S-transferase
HAMC - 7-hydroxy-4-(aminomethyl)coumarin
HLM - human liver microsomes
HNE - 4-hydroxy-2,3-nonenal
HPLC - high-performance liquid chromatography
HRS - high-resolution screening
LPO - lipid peroxidation
MAMC - 7-methoxy-4-(aminomethyl)coumarin
MD - molecular dynamics
MDAA - 3,4-methylenedioxy-*N*-alkylamphetamine
MDMA - 3,4-methylenedioxy methylamphetamine
NAC - *N*-acetyl-L-cysteine
NADPH - nicotinamide adenine dinucleotide phosphate
NBP - 4-(*p*-nitrobenzyl)pyridine
NDMA - nitrosamine *N*-nitrosodimethylamine
PB - phenobarbital
PBPK - physiologically based pharmacokinetic modeling
PCA - principal component analysis
PK - pharmacokinetics
PYR - pyrazole
QSAR - quantitative structure−activity relationship
RAMD - random acceleration molecular dynamics
SAR - structure−activity relationship
T - threonine
TCE - trichloroethylene
TDA - thiodiacetic acid
TFE - trifluoroethylene
UGT - UDP-glucuronosyltransferases
V - valine

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