CHAPTER 1

GENERAL INTRODUCTION
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Introduction

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1. **Role of drug metabolism in drug discovery and development**

Most xenobiotic substances such as drugs undergo an array of biotransformation reactions after internal exposure to humans. Drug metabolism by the host system is one of the most important determinants of the pharmacokinetic profile of a drug. Biotransformation of drugs can have different effects, such as the formation of chemically stable metabolites, which are devoid of pharmacological or toxicological activities, or the generation of short lived chemically reactive metabolites, which can lead to toxicological side effects (1). Furthermore, drug metabolism can also lead to formation of pharmacologically active metabolites which might contribute significantly to drug action in vivo, or may be responsible for pharmacologically-based adverse drug reactions.

A relatively novel approach in modern drug development is the use of pharmacologically active metabolites as potential resources for drug discovery and development. There are several advantages for screening drug candidates for active metabolites during drug discovery. For example drug metabolites can show superior properties compared to the lead itself, such as improved pharmacodynamics, improved pharmacokinetics, lower probability of drug-drug interactions, less variable pharmacokinetics and/or pharmacodynamics, improved overall safety profile and improved physicochemical properties (e.g. solubility) (1).

Examples of active metabolites of previously marketed drugs that have been developed into drugs are listed in Table 1.

<table>
<thead>
<tr>
<th>Parent drugs</th>
<th>Metabolite drugs</th>
<th>Biotransformation</th>
<th>Brand name</th>
</tr>
</thead>
<tbody>
<tr>
<td>Allopurinol</td>
<td>Oxyipurinol</td>
<td>Oxidation of xanthine</td>
<td>Oxyprim</td>
</tr>
<tr>
<td>Amitriptyline</td>
<td>Nortryptyline</td>
<td>N-Demethylation</td>
<td>Aventyl</td>
</tr>
<tr>
<td>Bromhexine</td>
<td>Ambroxol</td>
<td>N-Demethylation &amp; Hydroxylation</td>
<td>Mucosovan</td>
</tr>
<tr>
<td>Diazepam</td>
<td>Oxazepam</td>
<td>N-demethylation &amp; Hydroxylation</td>
<td>Serax</td>
</tr>
<tr>
<td>Etretinate</td>
<td>Acitretin</td>
<td>Deesterification</td>
<td>Soriatane</td>
</tr>
<tr>
<td>Hydroxyzine</td>
<td>Cetirizine</td>
<td>Carboxylation</td>
<td>Zyrtec</td>
</tr>
<tr>
<td>Imipramine</td>
<td>Desimipramine</td>
<td>N-Demethylation</td>
<td>Norpramin</td>
</tr>
<tr>
<td>Loratadine</td>
<td>Desloratadine</td>
<td>Descarboethoxylation</td>
<td>Clarinex</td>
</tr>
<tr>
<td>Loxapine</td>
<td>Amoxapine</td>
<td>N-Demethylation</td>
<td>Asendin</td>
</tr>
<tr>
<td>Phenacetin</td>
<td>Acetaminophen</td>
<td>O-Deethylation</td>
<td>Tylenol</td>
</tr>
<tr>
<td>Terfenadine</td>
<td>Fexofenadine</td>
<td>Carboxylation</td>
<td>Allegra</td>
</tr>
<tr>
<td>Thioridazine</td>
<td>Mesoridazine</td>
<td>S-Oxidation</td>
<td>Serentil</td>
</tr>
</tbody>
</table>
For example, fexofenadine, the primary metabolite of terfenadine was developed as anti-histamine agent with selective peripheral histamine H1-receptor antagonist activity. Fexofenadine is a safer drug than terfenadine because it does not has the ability to cause cardiotoxicity by hERG inhibition.

In some instances, metabolic transformation can also produce reactive or toxic intermediates or metabolites, with potential toxicological implications (3). Hence, a good understanding of the metabolism of a new chemical entity is needed early in the drug discovery process.

1.1. **Metabolites in safety testing (MIST)**

Drug metabolism and pharmacokinetics are crucial factors for successful drug development. Inappropriate pharmacokinetics previously was one of the main reasons of attrition in drug development. Although inappropriate pharmacokinetics has become a less important reason for attrition, less progress has been made in decreasing the attrition due to drug toxicity. The preparative synthesis of drug metabolites is currently of primary importance in industry in order to assess potential toxicity, drug-drug interactions and to examine metabolic pathways (4). In fact, testing the toxicities and biological activities of human metabolites of drugs is important in drug development to assure effectiveness and safety (5).

The importance of metabolite identification and quantification is stressed by the guidelines published in 2008 by the US food and drug administration (FDA). The so-called Metabolites in Safety Testing (MIST) guidelines describe the studies recommended to perform on metabolites having more than 10% systemic exposure of the parent drug to support human safety. A decision diagram of this is depicted in Figure 1.

These guidelines challenged the development of biocatalytic systems for the generation of large amounts of drug metabolites as well as analytical technologies dealing with their quantification and identification of drug metabolites in an efficient and intelligent way (7).
Figure 1. Decision tree flow diagram of the MIST guidelines as determined by the FDA, based on (6).

1.2. Enzymes involved in drug metabolism

Drug metabolism is typically divided in two phases. Phase I metabolism includes many types of functionalization reactions, e.g. oxidation, reduction, hydrolysis, hydration and dehalogenation reactions (9). Cytochrome P450s represent the most important class of enzymes involved in phase I metabolism, being involved in 75-80% of metabolism of marketed drugs, Figure 2. Other phase I enzymes include monoamine oxidases, flavin-containing oxygenases, amidases and esterases (9). Phase II metabolism involves conjugation of polar groups (e.g. glucuronic acid, sulfate, and amino acids) to drugs and/or phase I drug metabolites to further increase their hydrophilicity and facilitate excretion. These biotransformation reactions involve glucuronidation, sulfation, GSH conjugation, acetylation, amino acid conjugation and methylation reaction (10). Phase II enzymes include UDP-glucuronosyltransferase (UGTs), sulfotransferases (STs), N-acetyl transferases (NATs), methyl transferases and glutathione S-transferases (GSTs) (10), Figure 2. Almost all phase I and phase II enzymes can catalyze the formation of stable metabolites, as well as chemically reactive intermediates that may lead to the generation of adverse drug reactions (11).
Figure 2. Phase I and phase II metabolism of currently marketed drugs (adapted from (8)). The percentage of phase I and phase II metabolism of drugs that each enzyme contributes is estimated by the relative size of each section of the corresponding chart. ADH, alcohol dehydrogenase; ALDH, aldehyde dehydrogenase; DPD, dihydropyrimidine dehydrogenase; NQO, NADPH quinine oxidoreductase. COMT, catechol O-methyltransferase; GST, glutathione S-transferase; HMT, histamine methyltransferase; NAT, N-acetyltransferase; TPMT, thiopurine methyltransferase; UGTs, uridine 5’-triphosphate glucuronosyltransferases.

1.3. Role of reactive metabolites in adverse drug reactions

Adverse drug reactions (ADRs), as defined by the World Health Organization, are noxious, unintended and undesirable effects of a drug, which occurs at therapeutical doses used in humans and which require hospitalization of patients to recover (11). Although much effort has been spent to the development of safer drugs, ADRs still constitute a major reason for attrition in drug development and withdrawal or restriction of marketed products. Therefore, prevention of ADRs constitutes a major challenge for the pharmaceutical industry (3).

Approximate 75% of ADR in patients are classified as Type A ADR which involve life-threatening exaggerated pharmacological activity (on-target and/or off-target) due to unanticipated increased plasma concentrations of the parent drug. This class of ADR often results from drug-drug interactions or genetic deficiency of an enzyme which is involved in the major pathway of metabolism. For the other classes of ADR formation of reactive drug metabolites are considered to play a crucial role (12).

The main difficulty for pharmaceutical industry is the fact that ADRs cannot always be predicted in preclinical animal studies. In particular idiosyncratic drug reactions (IDRs; Type B ADR) are difficult to predict as they normally have a very low incidence, have a
delayed time of onset, do not necessarily show classic dose-response relationships and are not predictable from the known pharmacology of the drug. No predictive animal model is currently available and IDRs therefore remain poorly understood.

Figure 3 shows a general scheme depicting the role of reactive metabolites in the development of toxicity. Drugs bioactivation can be catalyzed by both phase I and phase II enzymes. Different types of reactive metabolites can be distinguished such as electrophiles, radicals and reactive oxygen species. Electrophilic metabolites can react with nucleophilic sites in proteins and/or DNA. Free radicals possess unpaired electrons and can abstract hydrogen atoms from polyunsaturated fatty acids in membranes which will initiate the destructive process of lipid peroxidation, and can disrupt the cellular redox-state leading to oxidative stress and subsequent toxicity (12).

Table 2 gives examples of drugs whose toxicities are attributed to covalent binding of electrophilic metabolites to proteins. Bioactivation of xenobiotics which contain certain functional groups such as tertiary amine, furan ring or acetylene group, can also cause mechanism-based inactivation of P450, leading to adverse clinical drug-drug interactions (13-14).
### Table 2. Examples of drugs and reactive metabolites possibly involved in ADRs (12-15).

<table>
<thead>
<tr>
<th>Drugs</th>
<th>Reactive intermediate</th>
<th>Toxicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetaminophen</td>
<td>Quinone-imine</td>
<td>Hepatotoxicity</td>
</tr>
<tr>
<td>Carbamazepine</td>
<td>2-Hydroxy, Quinone-imine</td>
<td>Agranulocytosis, Aplastic anemia</td>
</tr>
<tr>
<td>Clarithromycin</td>
<td>Nitroalkane</td>
<td>Hypersensitivity</td>
</tr>
<tr>
<td>Clozapine</td>
<td>Nitrenium ion</td>
<td>Agranulocytosis</td>
</tr>
<tr>
<td>Dapsone</td>
<td>Hydroxylamine, Nitroso</td>
<td>Hemolysis, hypersensitivity</td>
</tr>
<tr>
<td>Diclofenac</td>
<td>Acylglucuronide, Benzoquinone imine</td>
<td>Hepatotoxicity</td>
</tr>
<tr>
<td>Halothane</td>
<td>Trifluoroacetyl</td>
<td>Hepatitis</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>Iminoquinone</td>
<td>Hypersensitivity, Agranulocytosis, Hepatotoxicity</td>
</tr>
<tr>
<td>Isonizaid</td>
<td>Isonicotinic acid, acetylating species</td>
<td>Hypersensitivity</td>
</tr>
<tr>
<td>Phenacetin</td>
<td>p-Nitrosophenetole</td>
<td>Hepatotoxicity</td>
</tr>
<tr>
<td>Procainamide</td>
<td>Hydroxylamine, Nitroso</td>
<td>Agranulocytosis</td>
</tr>
<tr>
<td>Tacrine</td>
<td>7-OH-tacrine</td>
<td>Hepatotoxicity</td>
</tr>
<tr>
<td>Tamoxifen</td>
<td>N-oxide, N-oxide-epoxide</td>
<td>Carcinogenesis</td>
</tr>
<tr>
<td>Ticlopidine</td>
<td>Keto, S-oxide</td>
<td>Agranulocytosis, aplastic anemia</td>
</tr>
<tr>
<td>Tielinic acid</td>
<td>Thiopene S-oxide</td>
<td>Hypersensitivity, hepatitis</td>
</tr>
<tr>
<td>Troglitazone</td>
<td>Conjugates, Benzoquinone, Quinone epoxide</td>
<td>Hepatotoxicity</td>
</tr>
<tr>
<td>Valproic acid</td>
<td>Acyl glucuronides</td>
<td>Hypersensitivity, Hepatotoxicity</td>
</tr>
</tbody>
</table>

Detection of reactive metabolites is difficult due to the chemical reactivity of the short-lived intermediates and to the usually low amounts present in incubations. The most common way to screen for formation of reactive metabolites is to trap them with model nucleophiles such as GSH and to analyze the formed adducts with spectroscopic techniques. The endogenous tripeptide GSH can trap different types of RIs including quinones, quinoneimines, iminoquinone, methides, epoxides, areneoxides and nitrenium ions (15). The corresponding GSH adducts are typically analyzed by liquid-chromatography mass spectrometry (LC-MS). However, a major disadvantage is that structural information of the adducts obtained by LC-MS/MS experiments is limited, as it may be insufficient in identifying the exact position of oxidation, to differentiate stereoisomers, or to provide the exact structure of the metabolite (16). Complementing MS data with Nuclear Magnetic Resonance (NMR) experiments is required for complete structural elucidation of drug metabolites and/or resulting adducts. A limitation of NMR, however, is its relative insensitivity, as it requires several milligrams of pure metabolite.
The generation of pure metabolites can be achieved by organic synthesis, electrochemical oxidation of the parent drug or by biocatalysis using appropriate enzymes. The synthesis by organic chemistry is often complicated by the lack of appropriate synthetic routes for specific metabolites and may yield only low amounts of the desired product that has to be purified subsequently. Electrochemical oxidation has been used to generate drug metabolites and has the advantage that it can be easily scaled up. However, with this technique not all the relevant enzymatic metabolites can be formed. Electrochemistry has been previously used to generate GSH adducts of clozapine (17) and troglitazone (18).

Biocatalysis by human or bacterial P450s can also be used for the scaling up of metabolite production (19). For example, human relevant diclofenac metabolites were synthesized by microbial fermentation in large amount (up to 170 mg) and their structures were elucidated by MS and NMR (20).

More recently the use of highly active bacterial P450 BM3 mutants for the generation of reactive metabolites of drugs has been explored. Damsten et al. showed that BM3 mutants could be used for the generation of human relevant reactive metabolites of clozapine, acetaminophen, diclofenac (21), and trimethoprim (22). Boerma et al. showed that P450 BM3 mutants with high capacity to activate drugs (clozapine, acetaminophen and troglitazone) into relevant reactive metabolites can be employed to produce protein adducts to study the nucleophilic selectivity of highly reactive electrophiles (23). Dragovic et al. applied highly active BM3 mutant as bioactivation system to study the role of human GSTs in the inactivation of reactive intermediates of clozapine (24).

1.4. Biological screening for active metabolites

To assess the biological activity of drug metabolites there are several approaches available. The traditional approach is to isolate and purify all the metabolites from in vitro incubations with subcellular fractions such as liver microsomes or intact cellular systems (e.g. epatocytes) containing a full complement of drug metabolizing enzymes.

An alternative approach is to use bioassay-guided methods where biological samples containing biotransformation products are first evaluated for their pharmacological activity prior to isolation of the metabolites. The bioassay methods may be based on the assessment of the pharmacological activity using in vitro ligand binding (25, 26), cell based assays (1), or in vivo pharmacological assays (1).

One of the major bottlenecks in in vitro assays is the lack of possibility to screen affinity or activity of individual compounds in complex mixtures.

In general, two approaches can be used to measure the effect of individual compounds in a mixture. One strategy is to combine HPLC with fractionation techniques to enable the compounds in the mixture to be separated and allow screening of the individual components using plate reader assays.
The other available strategy is the on-line high resolution screening technique (HRS) depicted in Figure 4 (27-29).

HRS enables the screening of individual compounds in complex mixtures by coupling a separation technology, usually gradient HPLC, to post-column biochemical detection assays on-line. This technique has already been successfully used to develop a number of antibody- (30) and biotin- (31), receptor- (32), enzyme- (33) (34) (35), and MS-based (36) screening assays. The HRS platforms are especially attractive when complex mixtures such as metabolic incubations (37), combinatorial mixtures (38), or natural extracts (39) are screened. In contrast to conventional high-throughput screening (HTS), isolation of individual metabolites not showing affinity can be avoided. Thus, the laborious development of high-yield synthesis and isolation methods can be limited to compounds with the desired affinity.

Figure 4. Scheme of the online setup. The system enables the separation of mixtures and subsequent parallel detection of enzyme binding and accurate mass. The samples are injected into and separated by an HPLC system. The eluent is split between HR-MS and enzyme binding detection. The fraction which enters the enzyme binding detection is mixed with the target (e.g. p38α) and the tracer and incubated after each mixing step, 24 s for the target–ligand interaction and 11 s for the target–tracer interaction. Finally, the fluorescence is measured as readout of affinity towards the target. In parallel, the second part of the eluent is analyzed by HR-MS delivering structural information to identify the small molecules tested. Adapted from (28).

2. Microbial conversion of steroid compounds
Totally, about 300 approved steroid drugs are known to date, and this number tends to grow (40). Steroid pharmaceuticals are ranked among the most marketed pharmaceuticals and represent the second largest category next to antibiotics. The research on steroid drugs started in 1950, with the discovery of the pharmacological effects of cortisol and progesterone, two endogenous steroids, and with the identification of the 11α-hydroxylation activity of a Rhizopus species (41-42). Microbial steroid transformation is a powerful tool for generation of novel steroidal drugs, as well as for efficient production of steroid-containing active pharmaceutical ingredients (APIs) and key intermediates.

Steroid hydroxylation is a key tool to develop steroid drugs with improved potency, longer half-lives in the blood stream, simpler delivery methods and reduced side effects (43). The therapeutic area of hydroxy-steroids is very wide: they are used as anti inflammatory, immunosuppressive, prostagstonal, diuretic, anabolic and contraceptive agents (41, 44-47). Applications are found in the treatment of adrenal insufficiencies, in the prevention of coronary heart disease, as anti-fungal agents, as anti-obesity agents, and in the inhibition of HIV integrase, the prevention and treatment of infection by HIV and in the treatment of declared AIDS (43).

The complex structure of the steroid molecule requires complicated, multi-step schemes for the chemical synthesis of steroid compounds. The selective oxidation of an unactivated, aliphatic C-H bond to an alcohol is a challenging problem in synthesis (48). Chemical synthesis requires the use of reagents as pyridine, sulfur trioxide or selenium dioxide, whose disposal constitutes an environment issue (43). Moreover, the chemical catalysts used often show very low regioselectivity.

Steroid hydroxylation by microorganisms represents a powerful alternative to chemical synthesis. Current trends in microbial hydroxylation studies cover the search for novel biocatalysts capable of performing reactions targeting specific positions in the steroid molecule that are particularly important for the pharmaceutical industry (7α, 9α, 11α, 11β, 16α, 17α), production of novel hydroxysteroids with potent therapeutic properties, and the construction of novel biocatalysts by genetic engineering (40). Examples are given in Table 3.

Cytochrome P450s play a very important role in endogenous steroid metabolism. Testosterone hydroxylation in particular has been studied extensively with human liver microsomal P450s. For example, CYP3A4, the most abundant P450 in human liver and small intestine, catalyzes the monohydroxylation at 10 different positions of testosterone (8). Hence testosterone hydroxylation patterns have been utilized as probes for the presence and characterization of P450s (49) (50).

Hydroxylation of testosterone at 1β-, 2α-, 2β-, 6α-, 6β-, 7α-, 7β-, 15α-, 15β-, 16α-, 16β- and 17-positions has been reported in human and rat liver microsomes (Figure 5).

Table 3. Examples of steroid hydroxylation in microorganisms

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Product</th>
<th>Microrganism</th>
<th>Ref.</th>
</tr>
</thead>
</table>

...
<table>
<thead>
<tr>
<th>Hydroxylation</th>
<th>Compound</th>
<th>Organism/Strain</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>7α-Hydroxylation</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Testosterone</td>
<td>7α-OH-testosterone</td>
<td><em>Botrytis cinerea</em> AM235</td>
<td>(49)</td>
</tr>
<tr>
<td>Pregnenolone</td>
<td>7α-OH-pregnenolone</td>
<td><em>Fusarium oxysporum var.cubense</em></td>
<td>(50)</td>
</tr>
<tr>
<td>Epiandrosterone</td>
<td>7α-OH-epiandrostenone</td>
<td><em>Mortierella isabellina</em> AM212</td>
<td></td>
</tr>
<tr>
<td><strong>7β-Hydroxylation</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DHEA</td>
<td>7β- and 7β-derivatives</td>
<td><em>M.racemosus</em></td>
<td>(51)</td>
</tr>
<tr>
<td></td>
<td>7β-OH DHEA</td>
<td><em>Botryodiplodia malorum CBS 134.50</em></td>
<td>(52)</td>
</tr>
<tr>
<td><strong>7α-15α-Dihydroxylation</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DHEA</td>
<td>7α-15α-diOH-DHEA (3β,7α,15α-triOH-androst-5-ene-17-one)</td>
<td><em>C.lini CBS 112.21</em></td>
<td>(52)</td>
</tr>
<tr>
<td><strong>7β-15β-Dihydroxylation</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>17α-OH-progesterone</td>
<td>8β-OH-derivative, along with 15β-hydroxy derivative</td>
<td><em>Corynespora cassiicola CBS 161.60</em></td>
<td>(53)</td>
</tr>
<tr>
<td><strong>9α-hydroxylation</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Testosterone</td>
<td>9-OH-AD, 9α-OH-testosterone</td>
<td><em>R.equi ATCC 14887</em></td>
<td>(54)</td>
</tr>
<tr>
<td>Progesterone</td>
<td>9α-OH-progesterone</td>
<td>Expression of 9α-hydroxylase from <em>M.smegmatis</em> in <em>E.coli</em> BL21</td>
<td>(55)</td>
</tr>
<tr>
<td>Androsterone</td>
<td>9α-OH-adrenosterone, 9α-OH-11-keto-testosterone</td>
<td><em>Cunninghamella elegans TSY-0865</em></td>
<td>(56)</td>
</tr>
<tr>
<td><strong>11α-Hydroxylation</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Testosterone</td>
<td>11α-OH-testosterone</td>
<td><em>Rhizopus stolonifer ATCC 10404, Fusarium lini</em></td>
<td>(57)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NRRL 68751</td>
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</tr>
<tr>
<td><strong>11β-hydroxylation</strong></td>
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<tr>
<td>Progesterone</td>
<td>11β-OH-progesterone</td>
<td><em>T.harzianum, T.hamatum</em></td>
<td>(58)</td>
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<td><strong>14α-Hydroxylation</strong></td>
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<td>Testosterone</td>
<td>14α-OH-testosterone</td>
<td><em>A.wentii MRC 200316</em></td>
<td>(59)</td>
</tr>
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<td><strong>15α-Hydroxylation</strong></td>
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<tr>
<td>Testosterone</td>
<td>15α-OH testosterone</td>
<td><em>F.oxysporum var. cubense</em></td>
<td>(60)</td>
</tr>
<tr>
<td>Progesterone</td>
<td>15α-OH progesterone</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>15β-Hydroxylation</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Testosterone</td>
<td>15β-OH-testosterone</td>
<td>Solvent tolerant <em>Pseudomonas putida S12</em> expressing <em>B.megaterium</em> steroid hydroxyase CYP106A2</td>
<td>(61)</td>
</tr>
<tr>
<td><strong>16β-Hydroxylation</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Testosterone</td>
<td>2β,16β,17β-triOH-androst-4-en-3-one</td>
<td><em>Whetzelinia sclerotiorum ATCC 18687</em></td>
<td>(62)</td>
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<tr>
<td><strong>17α-Hydroxylation</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Progesterone</td>
<td>17α-OH-progesterone</td>
<td><em>B.sphaericus ATCC 245, B.sphaericus ATCC 7063, B.sphaericus ATCC 13805</em></td>
<td>(63)</td>
</tr>
</tbody>
</table>

*References:*
- (49) AM235
- (50) AM212
- (51) M.racemosus
- (52) Botryodiplodia malorum CBS 134.50
- (53) Corynespora cassiicola CBS 161.60
- (54) R.equi ATCC 14887
- (55) Expression of 9α-hydroxylase from *M.smegmatis* in *E.coli* BL21
- (56) Cunninghamella elegans TSY-0865
- (57) Rhizopus stolonifer ATCC 10404, Fusarium lini NRRL 68751
- (58) T.harzianum, T.hamatum
- (59) A.wentii MRC 200316
- (60) F.oxysporum var. cubense
- (61) Solvent tolerant *Pseudomonas putida S12* expressing *B.megaterium* steroid hydroxyase CYP106A2
- (62) Whetzelinia sclerotiorum ATCC 18687
- (63) B.sphaericus ATCC 245, B.sphaericus ATCC 7063, B.sphaericus ATCC 13805
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Figure 5. Hydroxylation of Testosterone by human P450s (adapted from (51)).

Figure 6. Testosterone hydroxylation by bacterial P450s (adapted from (40)).
Hydroxylation of Testosterone by bacterial P450s has also been extensively studied (Figure 6) (51). Profiling this hydroxylation provides useful information for characterizing bacterial P450s as monogenic traits and for comparing bacterial P450s with human P450s. The features and functionality of steroid hydroxylases have been recently reviewed by, e.g. Bernhardt (2006) (52), Hannemann et al (2007) (53), Novikova et al. (2009) (54) and Kristan and Lanisnik-Rizner (2012) (55).

3. Cytochrome P450s
Cytochrome P450s (P450s or CYPs) constitute a family of monoxygenases involved in the biotransformation of drugs, xenobiotics, alkanes, terpenes, and aromatic compounds, in the metabolism of chemical carcinogens, the biosynthesis of physiologically relevant compounds as steroids, fatty acids, eicosanoids, fat-soluble vitamins, bile acids and in the degradation of pesticides (52). They are able to catalyze a number of difficult oxidative reactions, such as C-H bond hydroxylation, N-dealkylation, N-hydroxylation, O-dealkylation, S-oxidation and epoxidation of numerous endogenous and exogenous compounds (3). An overview of the different reactions catalyzed by P450s is listed in Table 3. The broad substrate acceptance and this huge diversity in catalyzed reactions make the class of P450 very promising biocatalysts in industrial processes. In fact, in recent years there has been an increasing interest in the application of CYP biocatalysts for the industrial synthesis of bulk chemicals, pharmaceuticals, agrochemicals, and food ingredients, especially when a high grade of stereo and regioselectivity is required (57). Moreover P450s are of great interest in drug metabolism. They are ubiquitous enzymes often involved in the oxidation of drugs resulting in the generation of metabolites that are more easily excreted, thus modulating the toxicity of these compounds (57).

3.1. Human P450s
The human genome encodes for 57 functional P450s (58) of which approximately a dozen are involved in drug metabolism. The P450s mostly involved in metabolism of drugs in humans are listed in Table 5, where the main reaction and substrate specificities are indicated. Genetic polymorphisms that may alter the enzyme activity have been reported for a number of P450s (59). While for the P450s 1A2, 2A6, 2B6 and 2C8 the clinical effects due to these polymorphisms are minor, major effects are observed for 2C9, 2C19, and 2D6 (60). Polymorphisms in these isoenzymes affect metabolism of 20% to 30% of the clinically used drugs and this has become a major issue in the discovery and development of drugs and other NCEs (61).

<table>
<thead>
<tr>
<th>Reaction type</th>
<th>Example</th>
</tr>
</thead>
</table>
## Table 5. Substrate specificity of human CYPs

<table>
<thead>
<tr>
<th>CYP</th>
<th>Substrate properties</th>
<th>Drug/Substrates</th>
<th>Reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP1A2</td>
<td>Planar, polyaromatic compounds</td>
<td>Clozapine, Phenacetine, Olanzepine, Imipramine, Propranolol, Theophylline</td>
<td>Oxidation, O-deethylation, Oxidation, Demethylation, 4-hydroxylation, Hydroxylation</td>
</tr>
</tbody>
</table>
3.2. Structural organization of P450s
All P450s are heme-containing proteins containing an iron atom at the center of the heme which is also complexed by a cysteine residue as axial ligand. Based on their redox partner, P450s can be classified in different classes (Figure 7):

![Diagram of P450 catalytic cycle]

**Figure 7. Structural organization of P450s: (a) Class I (mostly bacterial P450s), (b) Class II (mostly microsomal P450s) and (c) P450 BM3. (Adapted from [62])**

Class I P450s (a) require two redox proteins: a small redox 2Fe-2S iron-sulfur ferredoxin and a FAD or FMN containing reductase. Mitochondrial P450s and most soluble bacterial P450s belong to this class (63).

In class II (b), electrons are transferred via only one reductase having a FAD and a FMN domain. Microsomal P450s that are attached to the endoplasmatic reticulum belong to this class (63).

Most bacterial P450s belong to class I. However, there are exceptions: in 1981 Cytochrome P450 BM3 (CYP102A1) from *Bacillus megaterium* was identified. P450 BM3 (c) is soluble and uses a Class II redox system. This soluble bacterial enzyme contains the P450 monoxygenase domain fused to the electron transfer flavin mononucleotide (FMN)/flavin adenine dinucleotide (FAD) reductase domain in a single polypeptide (63). In contrast, mammalian CYPs require additional redox partners as cytochrome P450 reductase (CPR) and often cytocrome b5 and lipids (64, 65).

### 3.3. The catalytic cycle

The general catalytic cycle for substrate hydroxylation by P450s is depicted in Figure 8.
In the resting state, in absence of substrate, the ferric iron atom is six-coordinated, complexed with a water molecule (I). Binding of a substrate displaces the water molecule (II). This causes the ferric spin state to convert from low spin to high spin, a transition that can be monitored spectrophotometrically since the Soret band undergoes a “type I” shift from ca. 418 nm to ca. 390 nm. A single electron supplied by a reduced pyridine nucleotide (NADPH or NADH) reduces the heme iron to the ferrous state (III). Subsequently, dioxygen binds to the ferrous iron generating the oxy-complex (IV). A second electron is then transferred, creating a ferric peroxy complex (V). Protonation of the terminal oxygen atom gives “Compound 0”, a hydroxyperoxy adduct (VI). Addition of a second hydrogen atom followed by loss of water gives “Compound I” (VII), a ferryl species thought to be the active entity in most P450 oxidations, which has been characterized recently (67). This complex abstracts hydrogen from the substrate (VIII) after which the hydroxyl group formed rebinds to the substrate radical, resulting in formation of the hydroxylated product which releases from the active site. NAD(P)H consumption is not necessarily fully coupled to product formation (68). If dioxygen binding is hindered, the second electron transfer will be too slow, resulting in the loss of superoxide from (IV) (superoxide uncoupling). If substrate fitting in the active site cannot prevent water encroachment, (VI) will be protonated at the iron-bound oxygen, resulting in the loss of
hydrogen peroxide (peroxide uncoupling). If a substrate binds with no hydrogen atom conveniently positioned for abstraction, the oxygen atom in (VII) could be reduced to water (oxidase uncoupling). Those alternative pathways can hamper the use of P450s in biocatalysis because the expensive electron donating cofactor is used in futile redox cycling instead of product formation. Moreover the peroxide and superoxide formed by uncoupling can damage the biocatalyst.

4. Bacterial Cytochrome P450 BM3

Bacterial P450 systems often exhibit much higher catalytic activities than membrane-bound eukariotic P450s and are easy to handle in the laboratory due to their solubility and high expression level in heterologous hosts (69). In particular Cytochrome P450 BM3 (BM3) is considered as one of the most promising monooxygenases for biotechnological applications as it possesses the highest activity ever recorded for a P450 (63, 70, 71) and can be easily expressed at high yield in Escherichia coli.

The natural substrates of BM3 are C12-C18 fatty acids which are hydroxylated at very high activity at subterminal positions (72). However, over the recent years, several research groups have succeeded in expanding the substrate selectivity of P450 BM3 and improving its catalytic properties by site-directed and/or random mutagenesis. Through rational redesign and directed evolution, BM3 mutants have been obtained that are able to oxidize aromatics (73), alkanes (74), hydrocarbons (75), carboxylic acids (76) and pharmaceuticals (21, 22, 77-84).

Recently a large body of research has been conducted to broaden and alter the substrate specificity of bacterial CYPs in an effort to generate “human like” P450 activities (57). The demand of “humanized” bacterial P450 with high activity is constantly growing to meet the needs of the fine-chemical synthesis, pharmaceutical production and bioremediation industries (85).

4.1. Engineering P450 BM3

The application of engineered bacterial P450s also have application in pharmacology and toxicology as it enable the generation of substantial amounts of drug metabolites as well as in optimization of lead compounds (86). Figure 9 shows examples for substrates of engineered cytochrome P450 BM3 variants.

In order to improve the selectivity of oxidation to a specific product, the substrate from which it is formed must be constrained to bind in a particular orientation (87). This can be obtained by rational redesign of the active site, for example introducing polar residues or bulky side-chains (88-90).
Directed evolution, in which mutations are introduced at random positions using error-prone PCR, is a valuable complementary tool to site-directed mutagenesis. This approach has the advantage of being able to generate unpredictable mutations with unanticipated beneficial properties. The benefits of the two strategies can be combined using rational evolution, in which selected residues are subjected to random or semi-random mutagenesis. Alternatively, directed evolution can be used to identify sites suitable for targeting, followed by site-saturation mutagenesis to establish which mutations are most effective in those positions.

For example, Dietrich et al. (91) developed a novel semi biosynthetic route for the production of artemisinin, a highly important antimalarian agent. By rational redesign of
the active site they introduced mutations F87A/A328L in the active site of substrate promiscuous P450 BM3: mutation F87A appeared to relieve the steric hindrance imposed upon the substrate amorphadiene and allowed an increased access to the heme group, while mutation A328L decreased the mobility of amorphadiene in the active site, promoting epoxidation.

A similar approach has been used by Seifert et al. (75), who focused on the same hotspots F87 and A328, creating a minimal and highly enriched P450 BM3 mutant library, by mutating those two positions to all possible combinations of five hydrophobic aminoacids. In this way, by systematically altering the size of the side chains in those two positions, a broad range of binding site shapes was generated to convert a range of differently sized and shaped terpenes (75). Recently, Seifert et al. (92) set up an iterative approach comprising successive rounds of modeling, designing of focused mutant libraries and screening, to identify a mutant for the production of the highly valuable product perillyl alcohol.

It is widely accepted that increased regio-, stereoselectivity is the result of a reduced number of substrate orientations close to the heme, as discussed in chapter 4 of the present thesis (82, 90, 93).

Considerable effort has been invested for improving the coupling efficiency (ratio of product formed to NAD(P)H cofactor consumed) of P450 BM3 by rational mutagenesis. However the efficiencies obtained are still far lower than with native enzyme-substrate. Detailed information on the structural determinants of coupling with novel substrates are missing, therefore it is difficult to rationalize mutations to improve coupling efficiencies. However, cost-effective, high-throughput screening methods will need to be developed to assess relative coupling efficiency among P450s in mutant libraries (86).

Properties that need to be improved to enable the application of P450s in industrial processes are: TTN (total turnover number), substrate acceptance, regio- and stereoselectivity, activity (\(k_{cat}\), \(K_M\)), inhibition and coupling efficiency (70). Also improvement of physical properties as thermostability, solvent tolerance, oxidative stability and substrate and product tolerance should be addressed (94).

4.2. P450 BM3 in biocatalysis

One emerging application of P450s for the pharmaceutical industry is the generation of large amount of drug metabolites, as discussed above (86). Several groups showed drug metabolite preparation using human P450s expressed in Escherichia coli and in insect cells (95). The Arnold group showed that human metabolites of propranolol could be produced by mutants of bacterial P450 BM3 heme domain in a reaction driven by hydrogen peroxide (5). Yields obtained were comparable to those produced by recombinant human P450s in bacterial or baculovirus bioreactors (96). Recently, Dubey et al. set up a biotechnological production of anticancer drug (colchicine derivatives) using P450 BM3 as biocatalyst obtaining a yield of 7.3 g/L in 70 L fermentor (97). Kim at al. applied BM3 wt
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and a set of mutants for the generation of the human metabolite piceatannol from the anticancer-preventive agent resveratrol (77), for the generation of human chiral metabolites of simvastatin and lovastatin (79) and for the generation of human metabolites of 7-ethoxycoumarin (78). The Arnold group compiled a panel of 120 mutants to prepare nearly all human metabolites and a number of novel hydroxylated derivatives for the drugs verapamil and astemizole (81). This panel of enzymes could also be applied for the generation of new chemical entities (NCEs) to diversify lead compounds. Within any catalyst panel both extremes of regioselectivity can be useful: enzymes selectively producing individual metabolites and less selective enzymes to survey metabolite possibilities (81).

A recent area of interest is the design and development of novel P450s as biocatalysts of reactions of commercial interest, such as in pharmaceutical or fine chemical synthesis (86) as well as for the optimization of lead compounds.

An example of the application of BM3 mutants in chemical synthesis is the chemoenzymatic elaboration of monosaccharides applied by the Arnold group (98). Regioselective deprotection of monosaccharide substrates using engineered P450 BM3 demethylases provides a highly efficient method to obtain valuable intermediates that can be converted to a wide range of substituted monosaccharides and polysaccharides (98). Engineered Cytochrome P450 BM3s were also applied for the enantioselective $\alpha$-hydroxylation of 2-arylacetic acid derivatives and could produce an authentic human metabolite of buspirone with high activity and selectivity (80).

Rentmeister et al. showed that products hydroxylated by BM3 can be subsequently fluorinated in a chemo-enzymatic process that greatly simplifies the insertion of fluorine into these structures at specific positions (99). Fluorination has become an important tool in drug discovery and development as it can modulate the pharmacokinetic and the pharmacological properties of drugs and lead compounds. In particular, fluorination can improve membrane permeability, metabolic stability and/or receptor-binding properties of bioactive molecules (99).

4.3. Key residues in the P450 BM3 active site

Control of regio- and stereoselectivity of biocatalysts is one of the major challenges in biotechnology, as a limited number of residues in the substrate pocket appear to possess significant selectivity-influencing powers. Ala82 and Phe87 are among those.

In Figure 10 key residues of the substrate channel and the active site of substrate-bound P450 BM3 are shown. P450 BM3 crystallizes in a “precatalytic conformation” in which the substrate is located too far to the heme iron for the oxidation to take place (87). Therefore it is unclear whether the residues located in the substrate access channel are also catalytically relevant. Also the spin relaxation NMR experiments of reduced P450 BM3 suggested that the substrates are 6 Å closer to the porphyrin ring than in the oxidized form, indicating that structural rearrangements occur in order to allow the substrate to
move closer to the heme iron \((100)\). In the present thesis mutagenesis studies at position 87 \((101)\) \((84)\) and position 82 \((89)\) will be presented.

---

**Figure 10.** The substrate access channel (A) and active site (B) of NPG-bound P450BM3 (Adapted from (66))

**Phe 87.** Phe 87 is one of the most studied residues of the active site of BM3 and it is object of chapter 2 and 3 of the present thesis. In the substrate-free crystal structure, this residue lies perpendicular to the porphyrin system, but in the substrate-bound crystal, the
orientation of the aromatic ring changes to parallel to the plane of the heme (102) (103) (63). It has been proposed that this motion influences the orientation of the substrate relative to the catalytic center and it is responsible for the displacement of the axial water ligand from its coordination site (104).

Phe87 forms with Phe81 a small hydrophobic pocket within the active site that sequesters the \( \omega \)-end of fatty acids. It seems likely that small hydrophobic substrates could preferentially bind in the pocket generating non-productive complexes (105).

Phe87 is the most commonly mutated residue of P450 BM3 (Table 6). Site-directed mutagenesis studies proved that Phe87 plays a key role in the control of reaction selectivity (106) (107).

Typically, Phe87 was mutated to aminoacids with less bulky side chains, in order to destroy the hydrophobic pocket, creating more space close to the heme, to accomodate larger substrates (73-75, 77, 80, 103, 105, 108-116). Such substitutions, however, lead to a decreased coupling efficiency of NADPH consumption to product formation due to a less efficient exclusion of water from the active site (105).

The F87V mutation is a common component of several drug metabolizing variants (69) (117). Moreover, it has been shown that mutation F87V significantly affected the stereoselectivity of arachidonic acid epoxidation (106) and the activity towards indoles (118). Li et al. also showed that mutation F87V is beneficial for oxidation of polycyclic aromatic hydrocarbons, such as naphthalene, fluorene, acenaphthene, acenaphthylene, and 9-methylnaphthalene (119).

In the present thesis saturation mutagenesis was applied to evaluate the effect of all possible amino acids in this position on the metabolism of alkoxyresorufins, testosterone and clozapine (84, 101).

**Ala 82.** The existence of a hydrophobic pocket between Phe81 and Phe87 clearly affects the attempts to engineer BM3 towards novel target substrates. Huang et al. (90) were the first to try to “fill” the hydrophobic pocket rather than destroying it, by mutating Ala82 to large hydrophobic residues like Phe and Trp.

The mutants A82F and A82W showed a remarkable increase in affinity (800-fold) for fatty acids. Moreover, the efficiency of indole hydroxylation increased, due both to increased \( K_{cat}/K_M \) values and to increased coupling efficiency, achieved by a more efficient exclusion of water from the active site. Mutation at this position will be object of chapter 4 of the present thesis.

**Table 6. Reported mutations at position Phe87:**

| ALA | (29 ) (73-75) (81) (98, 99) (107) (120-134) |
| ARG | (101, 135) |
| ASN | (101) |
| ASP | (101) |
5. **Spin lattice relaxation NMR (or T₁ paramagnetic relaxation NMR)**

In order to understand the structural basis that governs the regioselectivity of the metabolism of a certain substrate or the specificity of a given P450 for a particular drug, it is important to determine the structure of the substrate binding sites. For that purpose, $^1$H NMR appears as a powerful and attractive technique, since measurements of...
paramagnetic relaxation can be used to determine the distances between the heme iron and protons of the bound substrate in P450-substrate complexes (161).

The heme iron atom of a CYP is paramagnetic when in oxidized state. Hydrogen atoms in a magnetic field will re-align in the direction of this field after their orientation has been flipped with a radio-frequency pulse. The velocity of this re-alignment (relaxation rate) is dependent on the local strength of the magnetic field in the direct proximity of the hydrogen atom. So a hydrogen atom close to the heme in the active site of a CYP, will relax faster due to the magnetic moment of the heme iron atom.

The relationship between the iron atom to hydrogen atom distance and the rate of relaxation has been established by Solomon and Bloembergen (162) and can be used to measure substrate orientations in CYP active sites. A disadvantage of the technique is that high concentrations (~mM) of ligands need to be present to measure NMR spectra. Furthermore, average substrate active site orientations do not always match the metabolic profile (163).

An overview of reported spin lattice relaxation studies using CYPs is given in Table 6.

In chapter 4 of the present thesis this technique has been successfully applied to determine the orientation of testosterone in two BM3 mutants (M11 and M11 A82W) to rationalize the different regioselectivity obtained in the metabolic profile.

<table>
<thead>
<tr>
<th>CYP</th>
<th>Ligand</th>
<th>Distances (Å)*</th>
<th>Major reaction</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>102</td>
<td>12-Br-lauric acid</td>
<td>7.8 (C1H)-16.3 (C12H)</td>
<td>C2-OH</td>
<td>(163)</td>
</tr>
<tr>
<td>1A</td>
<td>Paracetamol</td>
<td>5.9 (phenylH)-6.7 (methylH)</td>
<td>Benzoquinone formation</td>
<td>(164)</td>
</tr>
<tr>
<td>1A2</td>
<td>Caffeine</td>
<td>6.5 (N1methylH)-6.7 (N3 and N7 methylH)</td>
<td>N1-demethylation</td>
<td>(163)</td>
</tr>
<tr>
<td>2B</td>
<td>Paracetamol</td>
<td>5.8 (methylH)-6.3 (phenylH)</td>
<td>Inactive</td>
<td>(164)</td>
</tr>
</tbody>
</table>
6. **Scope and objectives**

The research presented in this thesis was part of a wider interdisciplinary project “Metabolic stability assessment as a new tool in the Hit-to-Lead selection process and the generation of new lead compound libraries” financed by the Dutch Top-Institute Pharma consortium (grant D2-102). Several industrial (Merck/MSD (Oss, NL) and QPS (Groeningen, NL)) and academic (Vrije Universiteit Amsterdam and Radboud Universiteit
Nijmegen) partners participated in this project that led to several publications and four PhD theses.

The aim of the project was the application of new, more efficient and selective P450 BM3 mutants for the generation of biotransformation products, being new chemical starting points for lead optimization (lead libraries), followed by the determination of their binding affinity (e.g. competitive fluorescence detection assay) and chemical structural characterization (LC-MS/MS-NMR) in a hyphenated approach.

The general objectives of the present thesis are:

- Engineering drug metabolizing P450 BM3 mutants mimicking human P450s and mutants with unique catalytic properties based on regio- and stereoselective metabolism of diagnostic substrates.
- Utilization of “humanized” drug metabolizing P450 BM3 mutants for large scale production of physiologically relevant human metabolites of lead compounds.

Figure 11 shows the general strategy applied in this research: by site-directed, random or saturation mutagenesis BM3 mutants are engineered to obtain highly active mutants able to metabolize drugs and drug-like molecules. The metabolic mixtures obtained by incubation of diagnostic substrates with engineered BM3 mutants are then screened to verify the acquisition of certain properties: HRS enables the selection of mutants able to produce metabolites that show pharmacological activity, GSH trapping enables the identification of mutants able to produce reactive metabolites (subsequently trapped by GSH), screening by UPLC allows the identification of changes in regio- and stereoselectivity in a quick and informative way.

Selected mutants that acquired a certain property (e.g. production of high amounts of a pharmacologically active metabolite, high selectivity for the production of reactive metabolites, high selectivity of the production of a certain hydroxy-steroid) are then applied as biocatalysts for the large scale metabolite production in order to enable pharmacological and toxicological evaluation of drug metabolites and for their structural elucidation by NMR.
Figure 11. General strategy applied for the research presented in this thesis, leading to a “metabolite production and profiling platform”

7. Outline of this thesis

The work presented in this thesis shows application of BM3 mutants as biocatalyst for three purposes:

- For the regio- and stereo-selective hydroxylation of lead compounds (e.g. steroids)
- For the generation of reactive metabolites
- For the generation of pharmacologically active metabolites / lead optimization

Regio- and stereo-selective hydroxylation of unactivated C-H bonds of natural or synthetic compounds is one of the biggest challenges in synthetic organic chemistry. In particular steroid hydroxylation is extremely challenging due to the complexity of the steroid molecule that requires complicated, multi-step schemes involving protecting groups and subsequent regeneration.

In chapter 2 and 4 of the present thesis cytochrome P450 BM3 mutants are applied as biocatalysts for the regioselective hydroxylation of testosterone and norethisterone. In chapter 2 a site saturation mutagenesis library of BM3 mutants was applied to evaluate the effect of a single mutation in the active site of BM3 on the regioselective hydroxylation of testosterone and on the coupling efficiency in alkoxyresorufin oxidation. In chapter 4, a single active site substitution was applied in order to reduce the substrate mobility of testosterone, thus improving the regioselectivity...
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of hydroxylation. Spin lattice relaxation NMR was applied to determine how this single mutation affected the orientation of the substrate within the active site of two BM3 mutants, M11 and M11 A82W.

The generation of reactive metabolites is important from a toxicological point of view to be able to identify potentially toxic compounds that may be involved in ADRs or IDR.

**Chapter 3** of the present thesis focuses on the application of P450 BM3 mutants for the generation of reactive metabolites of clozapine, which is a drug known to be responsible of severe ADRs. By screening of a minimal library of BM3 mutants, a mutant was selected for the generation of large amounts of all major human relevant GSH-conjugates of clozapine to enable their structural elucidation by NMR.

The generation of pharmacologically active metabolites is important from a “lead optimization” point of view. Sometimes drug metabolites possess improved pharmacological activity and improved physico-chemical properties, compared to the lead itself.

In **chapter 5** of the present thesis a focused library of BM3 mutants has been applied for the generation of human relevant bioactive metabolites of p38α kinase inhibitor Tak-715. The HRS screening applied enabled the identification and pharmacological characterization of the drug metabolites in a quick and informative online mode. Large scale incubation with BM3 mutants and isolation of active metabolites by Prep-LC allowed their structural elucidation by NMR.

The last part of this thesis, **chapter 6** concerns an overall summary of the work described in the thesis including general conclusions and perspectives for future work.

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