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Chemical and biochemical transformation of bioactive compounds

Chemická a biochemická přeměna bioaktivních látek

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Statement

I hereby declare that I have written this thesis independently with the use of literature listed. Neither this work nor its substantial part was used to acquire different or same academic title.

In Prague, 04. 06. 2020

Prohlášení

Prohlašuji, že jsem závěrečnou práci zpracovala samostatně a že jsem uvedla všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

V Praze, 04. 06. 2020

Ema Šimášková

Aknowledgement

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Abstrakt

Xenobiotika, jako jsou léčiva, potravinářské přídatné látky, látky znečišťujicí prostředi a bioaktivní látky v potravě, jsou v organismu metabolizovány za pomoci mnoha enzymů, v důsledku čeho jsou bioaktivovány nebo detoxifikovány. Znalost struktury výsledných metabolitů je důležité pro jejich detekci v tělních tekutinách a tkáních, čehož se využívá v diagnostické a forenzní praxi. Tato práce se zabývá známými biochemickými procesy a enzymy, jenž se účastní metabolizmu xenobiotik, včetně cytochromů (CYP) a flavinových monooxygenas (FMO). S ohledem na to, že při biochemických reakcích se do velké míry účastní oxidačně-redukční reakce, tato práce zahrnuje praktické elektrochemické přístupy k objasnění oxidativního nebo redukčního mechanismu bioaktivních látek.

Klíčová slova:

Bioaktivní látky, Xenobiotika, Farmakokinetika, Oxidace, Redukce, CYP, FMO, SULT, UGT, Mikrozomy, Hepatocyty, Metabolizmus

Abstract

Xenobiotics, such as pharmaceuticals, food additives, environmental pollutants, and dietary bioactive compounds in organism are metabolized by various enzymes, resulting in their bioactivation or detoxification. Identification of structure of resulting metabolites is important for their detection in bodily fluids and tissues for diagnostic and forensic purposes. This thesis reviews known biochemical processes and enzymes involved in xenobiotic metabolism, including cytochromes 450 (CYP) and flavine monooxygenases (FMO). Given that biochemical reactions are to a major extent composed of electron-transfer reactions (i.e. oxidation and reduction), the thesis includes a section dealing with the practical approaches to determination of the oxidative or reductive mechanism of bioactive compounds.

Keywords*:*

Bioactive compounds, Xenobiotics, Pharmacokinetics, Oxidation, Reduction, CYP, FMO, SULT, UGT, Microsomes, Hepatocytes, Metabolism

Table of contents

Abbreviations

ABC - ATP-binding cassette

ADC - antibody-drug conjugates

AhR - aryl hydrocarbon receptor

AKR - aldo-keto reductase

APS - adenosine-5′-phosphosulfate

As3MT - S-adenosyl-L-methionine:arsenic(III) methyltransferase

ATP - adenosine triphosphate

ATPase - ATP synthase

AαC - 2-amino-9H-pyrido[2,3-b]indole

BCRP - breast cancer resistance protein

CAR - ponstitutive androstane receptor

COMT - catechol O-methyltransferase

CYP - cytochrome P450

CYP1A2 - Cytochrome P450 1A2

CYP3A4 - Cytochrome P450 3A4

CoA, CoASH - Coenzyme A

DAD - diode array detector

DMT - N, N-dimethyltryptamine

DNA - deoxyribonucleic acid

EC-MS - electrochemical mass spectroscopy

FAD - flavin adenine dinucleotide

FADH2 - dihydroflavine-adenine dinucleotide

FMO - flavin-containing monooxygenase

FMO3 – flavin-containing dimethylaniline monoxygenase 3

FMO5 – flavin-containing dimethylaniline monoxygenase 5

GRX – glutathione reductase

GSH - glutathione

GST - glutathione S-transferase

GSTP - glutathione S-transferase P

Glu - glutamic acid

Glu-P-2 - 2-aminodipyrido[l,2-a:3'2'd]- imidazole

HDMT - 5-hydroxy-DMT

His - histidine

HPLC -high-performance liquid chromatography

IARC - The International Agency for Research on Cancer

INMT - indolethylamine N–methyltransferase

IR - infrared

IQ - 2-amino-3-methylimidazo[4,5-f]quinoline

JNK - c-Jun N-terminal kinase

L-DOPA - 3,4-dihydroxy-L-phenylalanine

MB-COMT - membrane-bound COMT

MDR - medium-chain reductases

MRP - multidrug resistance protein

MS - mass spectrometry

MS/MS, MS2 - tandem mass spectrometry

MeAαC - 2-amino-3-methyl-9H-pyrido[2,3-b]indole

MeIQ - 2-amino-3,4-dimethyl-imidazo[4,5-f]quinoline

NAD - nicotineamide adenine dinucleotide

$NADP^+$ - nicotinamide adenine dinucleotide phosphate

NADPH - dihydronicotinamide-adenine dinucleotide phosphate

NAT - N-acetyltransferase

NAT2 - N-acetyltransferase 2

NNMT - nicotinamide N–methyltransferase

NQO - NAD(P)H dehydrogenase [quinone]

NQO1 - NAD(P)H dehydrogenase [quinone] 1

NQO2 - NAD(P)H dehydrogenase [quinone] 2

NTCP - sodium/bile acid cotransporter

OAT - organic anion transporter

OATP - organic anion-transporting polypeptide

PAP - 3'-phosphoadenosine 5'-phosphate

PAPS - 3′-phosphoadenosine 5′-phosphosulfate

PAPSS - bifunctional 3'-phosphoadenosine 5'-phosphosulfate synthase

PBD - pyrrolobenzodiazepine

PCR - polymerase chain reaction

POMT - phenol O–methyltransferase

PXR - pregnane X receptor

PhIP - 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine

S-COMT - soluble COMT

SAM - S–adenosylmethionine

SDR – short-chain dehydrogenases/reductase

Ser - serine

SLC - solute carrier transporter

SLC21, SLCO - solute carrier organic anion transporter

SULT - sulfotransferase

TMT - Thiol methyltransferase

TPMT - thiopurine S–methyltransferase

TRXR – thioredoxin reductase

UDP - uridine diphosphate

UDPGlc - UDP-glucose

UGT - UDP-glucuronosyltrasferase

UGT1A1 - UDP-glucuronosyltrasferase 1A1

UGT2B7 - UDP-glucuronosyltrasferase 2B7

UV-Vis - ultraviolet-visible light

VDR - vitamin D receptor

1. Introduction

Xenobiotics are chemical compounds that are found in an organism but are not normally present in that organism. The organism does not synthesize them and they do not enter the body as a part of its natural diet or exceed the amounts naturally present in the body.

To avoid reaching toxic concentrations of xenobiotics, organisms have developed metabolic pathways that help alter the compound in such a way that makes it utilizable or excretable.

The impact of xenobiotic exposure on human organism is the subject of toxicology research, main areas of which are pharmaceutical toxicology (studying side-effects of pharmaceutical substances for treatment and prevention purposes), ecotoxicology (dealing with chronic exposure to xenobiotics in air, water and soil), food toxicology (dealing with xenobiotic residua in food and their chronic impact), industrial toxicology (mainly concerned with health safety and chronic impact of toxins in the work environment on the human organism), and military toxicology (concerned with the impact of chemical weapon agents on the human organism) (Balíková, 2017).

Along with the xenobiotics, which are taken in by humans mostly consciously (e.g. drugs, personal care products, food additives), an increase in air, soil and water pollution is a growing problem. Water gets contaminated by municipal effluents, which transport human waste along with the medication, personal care products used by people and inappropriately disposed of expired pharmaceuticals, as well as household chemicals. Surface effluents containing pesticides and animal waste with feed additives (such as veterinary medication) are another major source of contamination (FAO and WHO, 2019). This thesis overviews the xenobiotic-metabolizing pathways, as well as some recent advances made in their research.

2. Theoretical section

2.1. Metabolism phases

In an organism, xenobiotics are metabolized almost exclusively via reactions catalyzed by enzymes. Non-enzymatic transformation is an exception. Some of these enzymes are common for xenobiotics and endogenic substrates and nutrients if they have a degree of structural similarity. Others get metabolized by special biotransformation enzymes that lead them to detoxification and elimination.

Biotransformation of xenobiotics mostly occurs in liver cells and is divided into two phases. In Phase I, the compound is activated by the addition or uncovering of reactive groups. These groups allow polar interactions with Phase II enzymes. Phase I reactions include oxidation,

reduction, and hydrolysis. Most of these reactions do not run spontaneously and need to be catalyzed by enzymes such as cytochromes P450, flavin-containing monooxygenases, and other oxidases, reductases and hydrolases. Phase II reactions include glucuronidation, glutathione-conjugation, sulphonation, amino acid conjugation, acetylation, and methylation (Skálová, 2017).

Originally, only Phase I and Phase II had been considered regarding drug metabolism (*Döring & Petzinger, 2014; Williams, 1959), however the concept was lacking, as it did not take into account the polar compound's inaccessibility of the sites of metabolism due to their inability to pass through an amphiphilic plasmatic membrane.

Successful cloning of the first drug transporter (MDR1/P-glycoprotein (P-gp) from the ATP binding cassette (ABC) family) provided evidence for carrier-mediated drug transport (*Döring & Petzinger, 2014; Riordan et al., 1985; Roninson et al., 1986).

Since then, carrier proteins of Phase 0 and Phase III have been proven to play an important role in metabolism and in some cases they have been observed to interact with the enzymes of Phase I and Phase II (*Döring & Petzinger, 2014).

2.1.1. Phase 0

Phase 0 transport was only termed in 2006 as the carrier-mediated uptake from the blood or the gut lumen into the metabolizing cell (Petzinger & Geyer, 2006). The carrier proteins involved in drug uptake belong mainly to the SLC21/SLCO organic anion transporting family (genera OATPs) (*Roth et al., 2012; Hagenbuch & Stieger, 2013). Solute carrier proteins mainly act as uniporters passively transporting the compound down its concentration gradient via facilitated diffusion. SLC can also mediate symport during which the drug is transported uphill its electrochemical concentration gradient while a co-substrate molecule is transported downhill its electrochemical concentration gradient. The co-substrate gradient is established by energy expense (e.g., by the Na^+/K^+-ATPase if a Na^+ ion is co-transported or by the Na^+/H^+-antiporter if the co-transported ion is H^+). The substrate molecular structures for SLC carriers are, unlike for other enzymes, quite diverse (*Döring & Petzinger, 2014). This led to the introduction of the term "multispecific drug carriers" for drug the uptake transporters in liver cells (Hosoyamada et al., 1999; Petzinger et al., 1996; Zimmerli et al., 1989). Drug transport mediated by carrier proteins in polarized cells occurs in a strictly vectorial direction. The xenobiotic does not return into the cytoplasm of a liver cell across the canalicular membrane once it has been excreted. The uptake transporter pumps (OATPs, OATs, NTCP) are located at the blood-facing basolateral portion of hepatocyte membrane while the

excretion by MRP2, MDR1/P-gp and BCRP transporters into bile takes place at the luminal canalicular membrane. Under pathological conditions (e.g. extra-hepatic cholestasis), efflux transporters such as MRP3, 4 and 6 are recruited and inserted into the basolateral membrane to increase elimination back into the blood (*Döring & Petzinger, 2014). The main steps and proteins involved are illustrated in Figure 1.

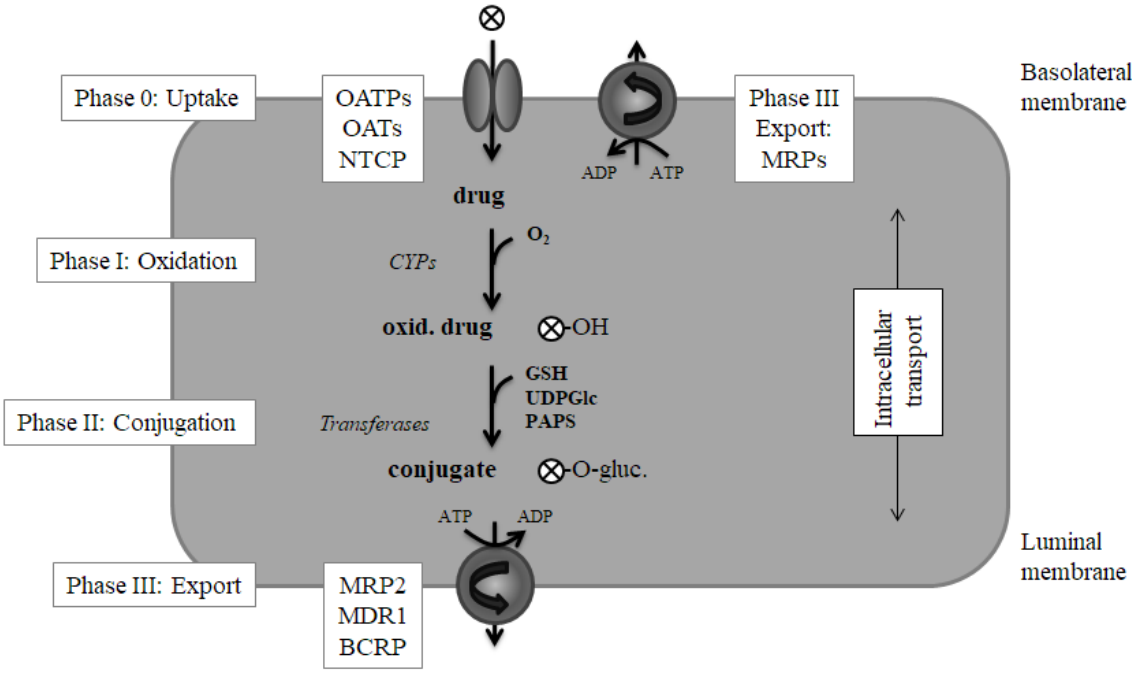

Figure 1 General scheme of drug metabolism according to (*Döring & Petzinger, 2014).

In addition to transporter proteins, xenobiotic or xenobiotic-induced stress-recognizing transcription factors have been proposed to as a part of the Phase 0 (*Baldwin, 2019). These would notably include molecules, such as aryl hydrocarbon receptor (AhR), which upon xenobiotic-binding activate enzymes, such as CYP1A, CYP1B, NADPH:quinone oxidoreductase 1 (Bock & Köhle, 2005), and UDP-glucuronosyltransferase 1, nuclear receptors pregnane X-receptor (PXR) and constitutive androstane receptor (CAR), which among others regulate CYP3A, CYP2B, UDPGT, GSTA2, SULT2A, and MRP2 (Chen et al., 2003; Sonoda et al., 2002). This allows for the establishment of regulatory crosstalk of metabolism and transport with drugs (*Baldwin, 2019).

2.1.2. Phase I

2.1.2.1. Cytochromes P450

Cytochromes P450 (CYPs) constitute a superfamily of heme enzymes that emit the typical maximum in the UV-VIS spectrum at 450 nm when reduced and bonded to carbon dioxide.

Eukaryotic CYPs are membrane-bound triangular prisms localized at the cytosolic side of the smooth endoplasmic reticulum's membrane. They play a key role in the metabolism of xenobiotics (Skálová, 2017), and biosynthesis of low-molecular-weight compounds, such as prostacyclins, steroids, thromboxane A2, prostaglandins, fatty acid derivatives, and derivatives of retinoic acid. As such, they account for approximately two thirds of the total metabolism of all drugs. The conclusion was derived from a study on clearance mechanisms of top 200 drugs prescribed in the United States in 2002 (Williams et al., 2004). In the human organism, xenobiotics-metabolizing CYPs are mostly found in the liver, gastrointestinal tract, lungs, and kidneys, however other localizations such as brain, heart, placenta, lymphocytes, skin, spleen and endothelium are also relevant (P. *Anzenbacher & Anzenbacherová, 2001). The structure of apoprotein is one polypeptide chain (45 – 55 kDa) and a protoporphyrin IX coordinating a Fe^{3+} ion.

The ability to activate Fe-O-O moiety is universal to all CYPs and is determined by the nature of the heme-apoprotein bond. The heme iron is bound to the protein through the anionic thiolate sulfur of a cysteine residue. This allows the heme moiety to mediate the transfer of electron density to the dioxygen bound as the trans (sixth) ligand of the heme iron (Anzenbacher et al., 1989). The monooxidation reaction is summarized in Formula 1 (Skálová, 2017).

$$RH + O_2 + 2e^- + 2H^+ \rightarrow ROH + H_2O$$

Formula 1 Monooxidation

The oxygen in the monooxygenation reaction can also come from hydroperoxides (XOOH). The peroxidase activity is summarized in Formula 2 (Skálová, 2017).

$$RH + XOOH \rightarrow XOH + ROH$$

Formula 2 Peroxidation

In addition to oxidation reactions, CYPs can also act as reductases of both oxygen and xenobiotics. Under certain conditions, CYP Fe^{2+}-RH can provide an electron for substrate reduction instead of donating it to oxygen. These reactions can usually only run under anaerobic conditions as oxygen acts as a competitive inhibitor. The reaction is summarized in Formula 3 (Skálová, 2017).

$$RH + e^- + H^+ \rightarrow RH_2$$

Formula 3 Reduction

CYP reducible types of xenobiotics include N-oxides, imines, nitro-compounds, nitroso-compounds and azo dyes. In some cases, CYP's reduction activity can be toxic for the organism. Examples include the reduction of tetrachloromethane and other halogenderivates of alkanes, quinones, azo dyes, nitro-compounds, and N-oxides of tertiary amines (Skálová, 2017). CYP3A4 (Figure 2) is probably the most important CYP involved in drug metabolism in humans as it metabolizes more than 60% of all known drugs, however the actual extent to which CYP3A4 is responsible has been disputed as the other members of 3A CYP family might contribute to obtained results (Williams et al., 2004). A on bioactivation of a dual-tyrosine kinase inhibitor lapatinib concluded that the efficiency of metabolism of lapanitib by CYP3A4 was higher than that mediated by CYP3A5 and both of them have been shown to undergo mechanism-based inactivation by lapatinib (Teng et al., 2010; Towles et al., 2016).
CYP3A4 is found in the liver in great amounts a broad range of possible substrates leads to an undesired possibility of drug interactions. When a drug with a narrow therapeutic window (i.e. a narrow range of dosages to avoid health complications) such as terfenadine or cisapride, is administered simultaneously with a CYP3A4 blocking drug (for example anticancer drugs tamoxifen and irinotecan), The levels of the first drug can easily approach or exceed its safety limits and lead to fatal consequences (Zhou et al., 2005). On the other hand, when administering a drug that causes induction of CYP3A4 activity, the other drug's metabolism may become so efficient that it does not remain available in the amount or level desired. The upregulation of CYP3A4 expression is mediated by a PXR receptor that binds to CYP3A4 promoter region after activation by a xenobiotic ligand, such as an antibiotic rifampicin and the antimycotic clotrimazole (Lehmann et al., 1998).

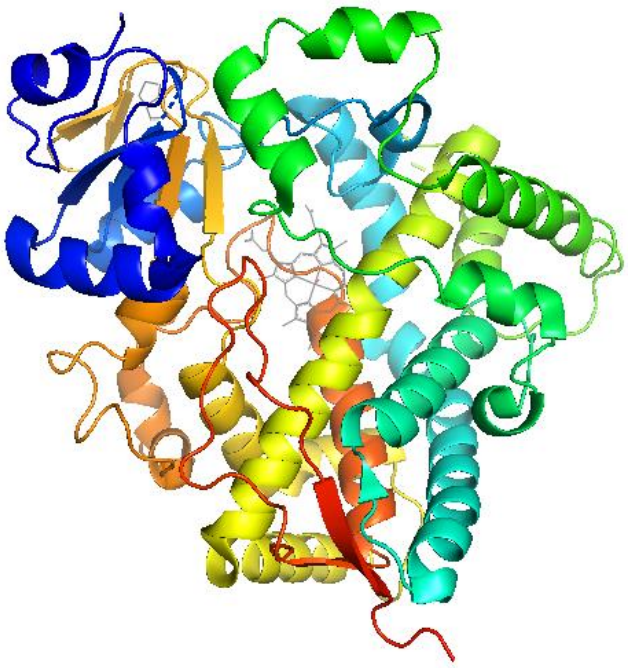

Figure 2 3D structure of CYP3A4. The color representation ranges from blue at the N terminus, to green, to yellow, to red at the C terminus. The overall structure is characteristic for the CYP superfamily and is comprised of a small beta-strand N terminal domain and a helical C terminal domain which contains the active site and the heme catalyst. Image of 1TQN (Yano, J.K., Wester, M.R., Schoch, G.A., Griffin, K.J., Stout, C.D., Johnson, 2004) created with PyMOL Molecular Graphics System (Schrödinger LLC, 2020)

2.1.2.2. Flavin-containing monooxygenases and other xenobiotic oxidases

Flavin-containing monooxygenases (FMOs) are monomeric membrane-bound proteins localized mainly in smooth endoplasmic reticula of hepatic cells and to a lesser extent in cells of lungs and kidneys. They catalyze oxidations of organic compounds with a nucleophilic heterocycle (mainly N, S and P), as well as some inorganic ions, such as HS^-, I^-, CNS^- (Skálová, 2017). The catalytic mechanism requires FAD prosthetic group (which is a part of the enzyme), NADPH cofactor, and O_2 co-substrate. NADPH binds to the enzyme and then FAD is reduced to $FADH_2$. Next, molecular oxygen oxidizes the $FADH_2$, which results in the formation of 4a-hydroperoxyflavin, which is stabilized by $NADP^+$. The prosthetic group is now ready to oxygenate the substrate. After oxygenation, the remaining oxygen atom is reduced and released in the form of water and FAD is reformed. $NADP^+$ is released (Phillips & Shephard, 2019). The 4-hydroperoxyflavin formation is independent of the presence of a substrate but requires the presence of NADPH and oxygen. The catalytic cycle is shown below (Figure 3).

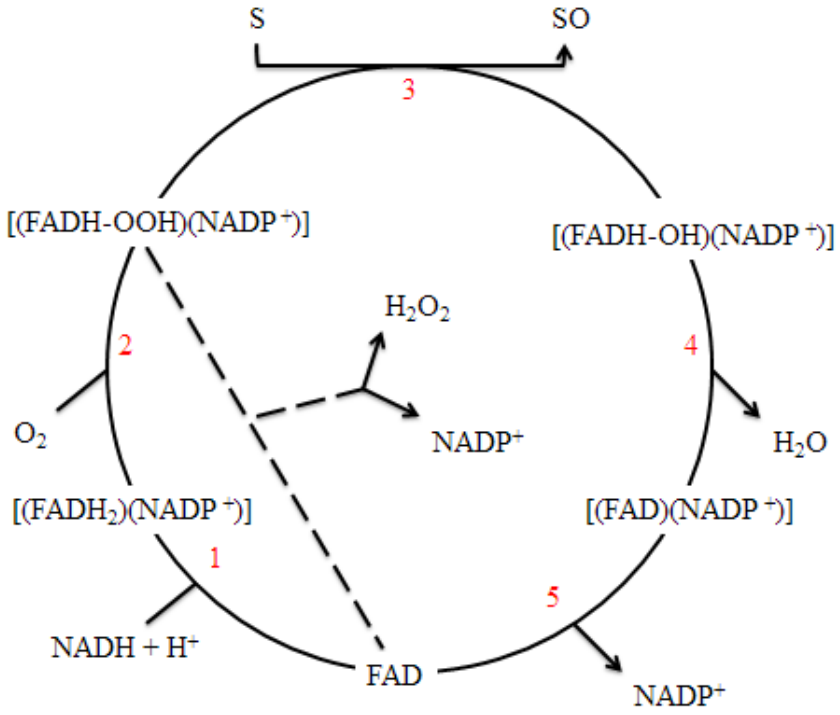

Figure 3 FMO's oxygenation mechanism according to (Phillips & Shephard, 2019)

The oxygenation of an insoluble substrate leads to the production of a readily excretable metabolite (Phillips & Shephard, 2019).

So far, five distinct genes, each encoding for one type of FMO enzymes, have been identified. Sequence analysis of the FMO gene family suggests divergence from a common ancestral form before speciation and a relatively high degree of conservation since that event took place (Hines et al., 2002). All of the FMO variants range in molecular weight from 60 to 63 kDa (Hines, 2007).

Substrate specificity and selectivity of FMOs, while being distinct for each of the five forms, tends to be quite broad and overlapping substrate specificities of CYPs. The main factor for specificity seems to be the structure-associated substrate's inability to access the enzyme's active site, which appears to be localized inside the enzyme (Hines, 2007). Both FMOs and CYPs, although working on the same xenobiotics, usually produce different metabolites (for example the oxidation of aliphatic tertiary amines results in the formation of N-oxides by FMOs and N-dealkylation products by CYPs) (*Krueger & Williams, 2005).

No human FMO crystal structure has so far been solved, however a recent study provided crystal structures of ancestral FMO enzymes (AncFMOs), some of which display high sequence identity with their human counterparts. For example, the sequences of AncFMO5 (Figure 4) and human FMO5 display a sequence identity of 92%. Both structures contain a

highly hydrophobic C terminal transmembrane anchoring helix, by means of which substrate is uptaken and released through the membrane. Following the uptake, the substrate is syphoned via a series of tunnels that differentiate the substrate profile based on the characteristics, such as the distribution of charged residues, the partition of hydrophobic versus hydrophilic residues at the entrance and inside the tunnel and the flexibility of the residues that gate tunnel access and substrate diffusion. The active site is buried within the molecule and exhibits no strict binding specificity (Nicoll et al., 2020).

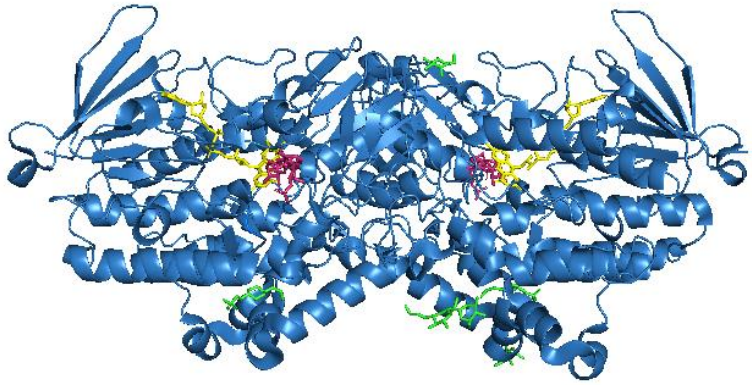

Figure 4 Structure of AncFMO5 listed by PDB code 6SEK (Nicoll et al., 2020) created with PyMOL Molecular Graphics System (Schrödinger LLC, 2020). FAD shown in yellow, NADP+ in magenta, dodecyl-β-d-maltoside detergent molecule in green.

2.1.2.3. Reductases

The reduction is a process, during which the substrate gains one or more electrons which leads to a decrease in its oxidation state.

This process, although less prevalent, also plays a role in xenobiotic metabolism. Apart from the aforementioned reductive activity of CYP, the human organism is equipped with various reductive enzymes.

The majority of known enzymes belong to three superfamilies - aldo-keto reductases (AKR), short-chain dehydrogenases/reductases (SDR), and medium-chain dehydrogenases/reductases (MDR). These enzymes act on aldehydes and ketones by transforming their aldehyde and keto groups to hydroxyl groups (Skálová, 2017). These reactions transform ketones into secondary hydroxyl metabolites and aldehydes into primary alcohols. Since the carbonyl group is often a determining factor for the compound's biological activity, carbonyl reduction plays a key role in the deactivation of many drugs, such as doxorubicin and daunorubicin, non-steroidal anti-inflammatory drugs loxoprofen and nabumetone, antidepressant bupropion, antipsychotic agent haloperidol, neuroleptic agent timiperone and antiemetic dolasetron (*Barski et al.,

2008). Aromatic nitro-compounds and N-oxides are reduced to amines by xanthine oxidase and aldehyde oxidase.

Quinones, although structurally similar to ketones, are not reduced by AKR. Instead, their reduction is catalyzed by NAD(P)H:quinone oxidoreductases (NQO1 and NQO2). CYP-catalyzed reduction of quinones generates semi-quinone radicals and contributes to oxidative stress. NQO plays a role in protection against this effect by reducing the substrate in a one-step 2-electron reaction without a semi-quinone intermediate (Skálová, 2017). Various flavoenzymes with reductase activity, such as NADPH-cytochrome c reductase and NADH-cytochrome b5 reductase have been observed to metabolize xenobiotics, such as sulfoxides, hydroxylamines and amidoximes (Kitamura et al., 1981; Kurian et al., 2004). Some disulfide-containing compounds, such as pyrrolobenzodiazepine (PBD), have been demonstrated to be degraded by disulfide-bond cleaving cytosolic enzymes thioredoxin reductase (TRXR) and glutaredoxin (GRX). PBD is the DNA-binding agent of a new generation of antitumor antibody-drug conjugates (ADC). ADCs are tumor-antigen specific cytotoxic compounds that target cancer cells while simultaneously sparing healthy cells (Zhang et al., 2019).

2.1.2.4. Hydrolases

Hydrolases use water to break down chemical bonds in their substrates by binding an OH^- ion to one of the participating atoms and an H^+ ion to the other one. The most common substrates are esters, amides, and epoxides; however, generally, also thioesters, glycosides, amines, peptides, proteins, and nucleic acids can be hydrolyzed.

The reaction is schematized in Formula 4.

$$A\text{-}B + H_2O \rightarrow A\text{-}OH + B\text{-}H$$

Formula 4 Hydrolysis

The majority of hydrolytic activity takes place in the liver; however, some activity occurs also in other tissues, plasma, and gut microbiota. The enzymes involved are esterases, amidases, and peptidases. In their active center, all xenobiotic-hydrolyzing esterases share a common catalytic triad Ser-His-Glu. During the reaction, the hydroxyl group of Ser attacks ester bond of the substrate, while His and Glu transfer the charge.

Amidases and peptidases are enzymes widely-spread among all tissues and biological fluids. They catalyze the cleavage of amide and peptide bonds, mainly during physiological processes, such as protein maturation, food protein metabolism, and regulation of blood

pressure and blood clotting. Their role in the metabolism of xenobiotics has not yet been well researched (Skálová, 2017).

2.1.3. Phase II

Phase II enzymes are found mostly in the cytoplasm but also in endoplasmic reticulum and mitochondria (Gibson & Skett, 1986). All Phase II reactions involve conjugation of a foreign compound to an endogenic substance. The biotransformation by conjugation enzymes may follow a Phase I reaction, but not necessarily (Jančová & Šiller, 2012).

2.1.3.1. Glutathione conjugation

Glutathione (GSH) is a tripeptide consisting of amino acids Glutamic acid, Cysteine, and Glycine. Glutathione conjugation is catalyzed by the enzymatic activity of GSH S-transferases (GST) however, it can also occur non-enzymatically.
GST is a dimeric protein with the highest activity in the liver, but also present in the kidney, lung and intestine. In mammals, seven distinct classes of GST have been described, namely Alpha, Mu, Omega, Pi, Sigma, Theta, and Zeta. Via its characteristic sites (γ-Glu linkage and SH group), the protein is involved in protein and nucleic acid synthesis, as well as detoxification of peroxides and free radicals (*Hanna & Anders, 2019).
Prior to the GST reaction, GSH binds to a conserved portion of the protein located at the N-terminal G-site domain. The hydrophobic xenobiotic binds to an adjacent H-site domain. During the reaction, GSH is attached to the xenobiotic via its thiol group (Ruzza & Calderan, 2013).

Conjugation of some compounds to glutathione makes them susceptible to entering the mercapturic acid pathway, which generates highly reactive and toxic end products. Mercapturic acid pathway is a sequence of reactions, in which a mercapturic acid (N-acetyl-L-cysteine S-conjugate) is formed by the sequential hydrolysis of GST-produced S-conjugates to L-Cys-Gly S-conjugates and afterward to L-Cys S-conjugates, followed by their N-acetylation. The product of this pathway, mercapturic acid, is a polar compound that can be readily excreted by the kidney (*Hanna & Anders, 2019).
Elevated levels of GST have been a promising target site for GSH-conjugate anti-cancer drugs. These latent cytotoxic prodrugs' anticancer effect is released after either GSH-dependent or GSH-independent breakdown catalyzed by GST (*Ramsay & Dilda, 2014; Ruzza et al., 2009).

One particular enzyme of the GST family, GSTP1-1, has been observed to have an inhibitory effect on JNK1 a kinase involved in the apoptotic response to a cytotoxic stimulus. This observation has potential practical applications in the design of tumor chemosensitizers (Ruzza & Calderan, 2013).

2.1.3.2. Sulfation

Sulfation (or sulfurylation) is an enzymatic reaction catalyzed by a sulfotransferase (SULT). The reaction utilizes a so-called "active sulfate" or PAPS (3′-phosphoadenosine 5′-phosphosulfate) as a sulfuryl donor for a majority of sulfation reactions (Robbins & Lipmann, 1957).

The activation of sulfate occurs in two steps. First, the AMP moiety of ATP is transferred to the sulfate by an ATP sulfurylase releasing pyrophosphate and adenosine-5′-phosphosulfate (APS). Next, an APS kinase phosphorylates the ribose ring of APS, resulting in generation of PAPS. In humans, both of these steps are mediated by the same polypeptide called PAPS synthase (PAPSS) (*Mueller & Shafqat, 2013).

In the human organism, two isoforms of this enzyme are present (PAPSS1 a PAPSS2), each encoded by its respective gene. The mutations in a PAPSS2 gene have been linked to a connective tissue disorder called autosomal recessive brachyolmia (Miyake et al., 2012).

The main sites of localization of SULT are the liver, kidneys and the intestine. SULT is mostly regarded as a cytosolic enzyme, however many types of SULT are also present in Golgi membranes. The Golgi SULTs sulfate substrates of high molecular weight, such as carbohydrates, glycosaminoglycans, and proteins (*Habuchi, 2000).

Sulfation renders the substrate compounds highly anionic, increasing water-solubility. Additionally, the introduction of a sulfate moiety can alter the compound's physiological function in an organism. Certain hydrocarbons may become highly carcinogenic after sulfation (*Brockhausen & Kuhns, 1997).

2.1.3.3. Glucuronidation

Glucuronidation is a process, in which a UDP-glucuronosyltranferase (UGT) attaches a glucuronide moiety from UDP-glucuronic acid to a substrate. Approximately 1 in 10 drugs in the top 200 most prescribed in the US are metabolized by glucuronidation, the most prevalently responsible enzyme being UGT2B7, followed by UGT1A1 and UGT1A4 (Williams et al., 2004). UGT is, similarly to CYP and FMO, located in the smooth endoplasmic reticulum. The reaction generates glucuronides, which are usually much more

water-soluble than their parent compound but rely on membrane-bound efflux transporters for their exocytosis. The final amount of parent compound and its glucuronides reaching the intestinal lumen is therefore determined by both, UGT glucuronide generation and glucuronide distribution by efflux and influx transporters (*Yang et al., 2017).

Human UGTs are capable of conjugating the GSH moiety to a wide variety of compounds. This promiscuity is enabled by the composition of the active site, which consists of a serine hydrolase-like catalytic triad. A homology model of human UGT2B7 suggested a mechanism in which His35 deprotonates a suitable atom of the acceptor ligand, facilitating a nucleophilic attack at the C1 atom of glucuronic acid. A proposed catalytic mechanism of UGT2B7 is schematized Figure 5 (Miley et al., 2007).

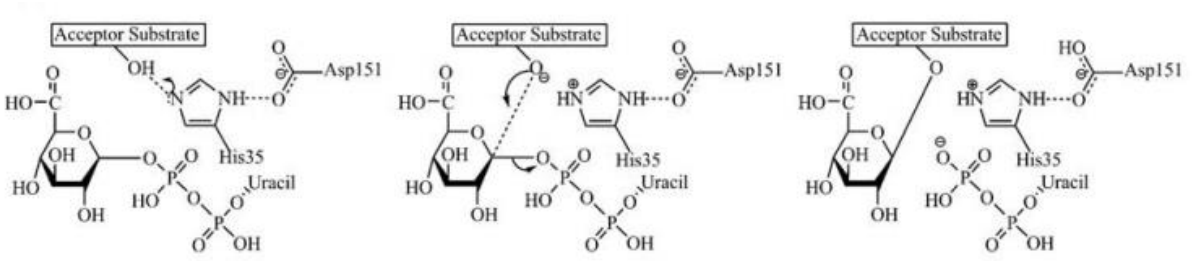

Figure 5 A proposed catalytic mechanism of UGT2B7 (Miley et al., 2007).

Common substrates include primary aromatic amines, hydroxylamines, amides, tertiary aliphatic amines, and aromatic N-heterocycles. These usually undergo 1st phase hydroxylation before glucuronidation (Kaivosaari et al., 2011).

Apart from the detoxification of xenobiotics glucuronidation also has helps to rid the body of some potentially toxic endogenous substances (e.g., bilirubin) (*Yang et al., 2017). Insufficient glucuronide formation has been observed to cause unconjugated hyperbilirubinemia known as Gilbert syndrome. It is an autosomal recessive disorder linked to a mutation in the promoter region of the UGT1A1 gene (Aono et al., 1995; Bosma et al., 1995; Chowdhury, Wolkoff, Chowdhury, Arias, 2001).

Additionally, a nonsense mutation in the same gene causes a more severe defect in the formation of glucuronides, Crigler–Najjar syndrome (Bosma et al., 1992).

2.1.3.4. Amino-acid conjugation

Amino-acid conjugation is catalyzed by enzymes residing in mitochondria. Amino-acid conjugation involves three steps. First, the carboxylic acid moiety is activated by ATP, generating an acyl adenylate and pyrophosphate. Next, the bound acyl adenylate reacts with a

molecule of coenzyme A (CoASH) to yield a 'high-energy' xenobiotic-CoA thioester intermediate. Finally, the activated acyl group is linked to the amino group of the acceptor amino acid and CoASH gets regenerated (*Knights et al., 2007).

In humans, two main amino-acid conjugating xenobiotic/medium-chain fatty acid CoA ligases have been discovered, 48 kDa HXM-A and 49 kDa HXM-B. The common substrates for these enzymes include benzoate, propionate, hexanoate, and octanoate (*Knights et al., 2007; Vessey et al., 1999). Other xenobiotics include hypoglycin A (*Knights et al., 2007; *Sherratt, 1986), pivalate (*Knights et al., 2007; Ruff & Brass, 1991), and anti-inflammatory zomepirac (*Knights et al., 2007; Olsen et al., 2005).

2.1.3.5. Acetylation

Although conjugation reactions generally result in increased solubility, acetylation and methylation represent an exception as their conjugates are often less soluble in water than the original compound.

Acetylation reactions participating in xenobiotic biotransformation can be grouped into three general types of reactions, namely N-acetylation, O-acetylation, and N, O-acetyltransfer, first one being the most prevalent type of reaction. All three types of reactions are catalyzed by Arylamine N-acetyltransferases (NATs) (Sim et al., 2012).

NATs utilize an acetyl-CoA to transfer its acetyl group to an aromatic amine or hydrazine group of a xenobiotic. In this reaction, acetyl-CoA acts as the cofactor. For example, the acetyl group of acetyl-CoA can bind to an N atom of an amine forming an acetyl conjugate. The reaction is schematized in Formula 5 (Chen, 2020).

$$R - NH_2 + Acetyl - CoA \rightarrow R - NH - COCH_3 + CoA$$

Formula 5 N-acetylation

O-acetylation reaction is similar, the difference being that the acetyl moiety binds to an oxygen atom of a hydroxyl in exchange for a hydrogen atom.

During N, O-acetyltransfer, the acetyl moiety is transferred from a nitrogen atom to an oxygen atom of the same compound. In contrast with the other reactions, N, O-acetyltransfer is not Acetyl-CoA dependent (Sim et al., 2012).

In humans, NATs are present in two isoforms – NAT1, found mainly in the colon and NAT2, found in the liver as well as the intestine (Hickman et al., 1998; Zhou et al., 2013). Most drug metabolism is carried out by NAT2, including the acetylation of antibiotic therapeutic compounds, such as isoniazid, dapsone and the sulphonamides (Zhou et al., 2013).

Some dietary sources of xenobiotics, such as heterocyclic aromatic amines generated during thermal processing of meat are detoxified by NATs.

The International Agency for Research on Cancer (IARC) has recognized nine HAAs, including 2-amino-9H-pyrido[2,3-b]indole (AαC), 2-amino-3-methyl-9H-pyrido[2,3-b]indole (MeAαC), 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP), and 2-amino-3,4-dimethyl-imidazo[4,5-f]quinoline (MeIQ) (group 2B), as possible human carcinogens, and 2-amino-3-methylimidazo[4,5-f]quinoline (IQ) (group 2A) as a probable human carcinogens, and has recommended lower dietary exposure to these substances (Chen et al., 2020; IARC, 1993). NAT2 is a more important contributor to the genotoxic effects of IQ, f N-hydroxy-IQ, MeIQ, and MeIQx in humans than NAT1 (Chen et al., 2020). Polymorphism of NAT2 might be of importance as a study on Japanese and African-American populations showed that NAT2 could modify the correlation between red meat consumption and incidence of colorectal cancer (Chen et al., 2020; Wang et al., 2015).

The 12th Report on Carcinogens by U.S. National Toxicology Program listed aromatic amines and chemicals that can be converted into one to make up more than 12% of all known or suspected human carcinogens, making them a major class of human carcinogens (National Toxicology Program, 2011).

Acetylation has been linked to both, deactivation and activation of heterocyclic arylamine carcinogens, such as some compounds emerging in the thermal processing of meat, 2-amino-3-methylimidazo[4,5-f]quinoline (IQ) and 2-aminodipyrido[l,2-a:3'2'd]- imidazole (Glu-P-2), as well as 4-aminobiphenyl, β-naphthylamine and IQ which are found in cigarette smoke (Sugimura & Sato, 1983; Talaska et al., 1991; Yamashita et al., 1986). Some compounds, such as 4-aminobiphenyl or 2-aminofluorene, undergo N-hydroxylation catalyzed by CYP1A2 followed by their O-acetylation catalyzed by NAT2.

2.1.3.6. Methylation

Methylation is a minor metabolic pathway, only processing a few types of xenobiotics. Several ways of methyl-moiety conjugation occur (N-, O-, S-, As-), depending on the linking atom, however, all of them rely on the presence of an S–adenosylmethionine (SAM) cofactor, which is formed primarily by the condensation of ATP and L–methionine.

N-methylation can be catalyzed by several enzymes. One of these enzymes is indolethylamine N–methyltransferase (INMT), involved in the metabolism of tryptamine and structurally similar compounds (Jančová & Šiller, 2012). As such, it is suggested to be involved in the

transformation of tryptamine to a powerful endogenous psychedelic drugs N, N-dimethyltryptamine (DMT) (Dean et al., 2019) and 5-hydroxy-DMT (bufotenine, HDMT) (Dean, 2018) in human liver and lung, where high levels of the enzyme are present. On a subcellular level, INMT is localized at the Golgi apparatus and vesicles.(*Barker et al., 2012; Thompson et al., 1999). Additionally, the enzyme can be also found in adipose tissue, tumor cells, kidney, heart and muscle (Kannt et al., 2018). Recent research using PCR and in situ hybridization reported detectable amounts of INMT in the human brain, thus proposing neural activity and the possibility for DMT synthesis and release in concentrations similar to known monoamine neurotransmitters (e.g., serotonin) (Dean et al., 2019).

Another N-methylating enzyme is nicotinamide N–methyltransferase (NNMT). Its role in xenobiotic detoxification has been suggested because of its ability to N-methylate various small pyridine-containing molecules (Rini et al., 1990; Van Haren et al., 2016; Weinshilboum, 1989).

Additionally, some other xenobiotic-metabolizing N-methyltransferases include nonspecific arylamine N-methyltransferase, phenylethanolamine N-methyltransferase, and histamine N-methyltransferase.

O-methylation is mediated by two enzymes. Phenol O–methyltransferase (POMT) transfers the methyl group from SAM to phenol, anisole being the end product (Jančová & Šiller, 2012). POMT is mainly localized in the endoplasmic reticula of liver and lung cells but is also present in other tissues (Axelrod & Daly, 1968). Catechol O–methyltransferase (COMT) catalyzed the O-methylation of catechols, for which it depends on Mg^{2+}. COMT mostly deals with the inactivation of endogenous catechols, such as dopamine, epinephrine, and norepinephrine (Zhu, 2005). An example of a xenobiotic substrate is a neuroactive drug and dopamine precursor L-DOPA, which is used for the treatment of Parkinson's disease, and therefore COMT inhibitors are used as a combination treatment for enhancement of L-DOPA's effect. Additionally, L-DOPA might also be used in schizophrenia and depression treatments (Dorszewska et al., 2014; Lotta et al., 1995)..

Other xenobiotic substrates include triphenols and substituted catechols, such as dobutamine, isoprenaline, rimiterol, levodopa, benserazide, dihydroxyphenyl serine, and dihydroxy derivatives of tetrahydroxyisoquinolones (Axelrod, 1966; Lautala et al., 2001; Zhu, 2005). Two types of COMT exist – a soluble and a membrane-bound form. The soluble S-COMT is located in the cytosol, while the membrane-bound MB-COMT is bound to the membrane of the rough endoplasmic reticulum. The only distinction in the amino-acid sequence of the two types is a 50 residue long signal sequence of the membrane-bound form (*Ma et al., 2014;

Lotta et al., 1995). Except for the brain, S-COMT is the predominant form in most tissues (Tenhunen et al., 1994). S-methylation in the human organism is catalyzed by a membrane-bound Thiol methyltransferase (TMT) and a cytoplasmic thiopurine S–methyltransferase (TPMT).

TPMT methylates aliphatic sulfhydryl compounds such as captopril and D–penicillamine. It is localized in the red blood cell membrane. TPMT methylates aromatic and heterocyclic sulfhydryl compounds. This includes anticancer and immunosuppressive thiopurines, such as azathioprine, 6–thioguanine and 6–mercaptopurine. TPMT exhibits genetic polymorphism with about 1 person in 300 inheriting autosomal deficiency leading to TPMT deficiency (*McLeod et al., 2000). Due to a narrow therapeutic range (i.e., having little difference between therapeutic and toxic doses) of targeted drugs, TMPT genotyping and phenotyping have become standard pre-treatment procedures, genotyping found to be more reliable (Hindorf et al., 2006; Weitzel et al., 2018). Had their dosages not been lowered, TMPT deficient patients' hematopoietic tissues might accumulate toxic amounts of thioguanine nucleotides which could lead to death. 10-15 fold lower dosages are effective in the treatment of TPMT deficient patients (*McLeod et al., 2000).

As-methylation is important for biotransformation of arsenic, which is both, a carcinogenic groundwater contaminant and a promyelocytic leukemia drug (Wood et al., 2006). The entire arsenic metabolism pathway is a series of toxifying and detoxifying mechanisms, however, As-methylation catalyzed by a liver enzyme arsenic (+3 oxidation state) methyltransferase (As3MT) is considered to be an important step. Genetic polymorphism of As3MT might result in altered susceptibility to As-toxicity in different individuals (Fujihara et al., 2010).

2.1.4.Phase III

Phase III was first characterized by Ishikawa as a process of GSH-drug conjugates elimination from the cell via GS-X pumps (Ishikawa, 1992). Carrier gene cloning demonstrated that these pumps, including the multidrug resistance-associated protein 1 (MRP1) and P-gp, belong to the ABC family of carrier proteins (Riordan et al., 1985; Roninson et al., 1986). They are embedded into the luminal membrane of epithelial cells and thus mediate the excretion into the bile, milk, sweat and bronchial exudate. ABC transporters generate a ''primary active'' transport process, meaning the transport of the substrate is direct uphill transport against its concentration gradient. This process utilizes the hydrolysis of ATP (*Döring & Petzinger, 2014).

2.2. Chemical transformation of compounds

The development of new therapeutic compounds requires extensive research and evaluation before being approved for use in the treatment of humans. To ensure safe use, all newly discovered compounds must first be characterized by their physical, chemical and biological properties. Studies on their efficacy, stability, pharmacokinetic properties (i.e., absorption, distribution, metabolism, and excretion) and toxicity are conducted to enable the determination of correct salt form, drug formulation, dose level, administration route, and dosing regimen (*Álvarez-Lueje et al., 2012; *Beaumont et al., 2014).

2.2.1 Oxidation

Oxidation is the complete, net removal of one or more electrons from a molecular entity, as well as an increase in the oxidation number of any atom within any substrate (Muller, 1994). Oxidative reactions play a key role in the metabolism of many xenobiotics. In the early stages of drug discovery, electrochemical replication of drug metabolism is used as a faster, more cost-effective instrumental approach (*Álvarez-Lueje et al., 2012). Electrochemical oxidations can mimic biological processes, such as oxidative biotransformation of drugs (Alvarez-Lueje & Dragnic, 2010). Mechanism of Phase I reactions is initiated by a single-electron oxidation. Some reactions, such as aromatic hydroxylation, S-oxidation, P-oxidation, dehydrogenation, alcohol oxidation, and O- and N-dealkylation, can be emulated by application of specific positive potential for a specific amount of time to the compound in an electrochemical cell. The compound is oxidized and its metabolites identified, characterized and further studied. Some phase II reactions, such as conjugation with GSH or other thiols from electrochemically generated phase I metabolites, has successfully been mimicked by electrochemical oxidation after the phase I imitation from parent drugs (Lohmann & Karst, 2006; Madsen et al., 2008).

2.2.2 Reduction

Reduction is the complete transfer of one or more electrons to a molecular entity and more broadly the reverse process described under oxidation (Muller, 1994). Reduction in an electrochemical experiment can be achieved by the application of a sufficiently negative potential to a cell containing the compounds of interest (*Álvarez-Lueje et al., 2012).

3. Methods in xenobiotic metabolism research

3.1. Substrate depletion

Clearance of a compound is a pharmacological measurement which helps to determine the rate of drug elimination from the organism. It represents the net result of the amount of xenobiotic uptaken by hepatocyte, followed by its biliary excretion and potential return into blood circulation. The substrate depletion method measures the intrinsic clearance CLint of a compound, i.e. the ability of the liver to remove drug in the absence of flow limitations and binding to cells or proteins in the blood (Jones & Houston, 2004). The resulting values are then used in calculations for in vivo hepatic clearance CLH prediction (Formula 6).

$$CL_H = \frac{Q_H + \frac{in\ vitro\ CL_{int} \times SF}{f_{u\ inc}} \times f_{u\ b}}{Q_H + \frac{in\ vivo\ CL_{int} \times SF}{f_{u\ inc}} \times f_{u\ b}}$$

Formula 6 Q_H is the hepatic blood flow, SF represents the milligrams of microsomal protein or million cells per gram of liver multiplied by the grams of liver weight; $f_{u\ b}$ is the unbound fraction of drug in the blood, and $f_{u\ inc}$ is the unbound fraction in the incubation matrix (Jones & Houston, 2004).

3.1.1. Microsomal fraction

Microsomes are fractions of predominantly smooth endoplasmic reticulum membranes obtained after homogenization of the cell (Knights et al., 2016; Palade & Siekevitz, 1956). For experiments on metabolism, hepatic microsomes are used as they contain high concentrations of key metabolic enzymes, most notably CYPs and UGTs. Characterization of particular enzymes involved enables qualitative prediction of factors that might alter the clearance of the drug and its metabolites. Incubation with hepatic microsomes leads to substrate depletion, which can then be measured to determine metabolic stability. In the case of a known metabolite, the measurements of metabolite formation kinetics are used.

CYP and FMO-catalyzed metabolic pathways are NADPH-dependent. A negative control is performed in which the experiment is carried out without the addition of NADPH. This way a distinction in depletion of a negative control sample and the test sample can be drawn, indicating the influence of confounding factors such as compound instability or non-NADPH-dependent enzyme degradation. A general rule for NADPH-dependent reaction without a

grave influence of confounding factors is that 90% of the original compound concentration should be still present in the control sample after 30 minutes (Knights et al., 2016).

3.1.2. Cytosolic fraction

The determination of the involvement of cytosolic enzymes in the breakdown of a compound is performed by differential centrifugation of whole liver homogenate. During centrifugation, the cytosolic fraction is isolated. Clearance measurement in cytosolic fractions can help identify involvement of phase II enzymes, mainly N-acetyltransferases (NATs), Glutathione S-transferase (GST), sulfotransferases (SULTs), in lower concentrations also carboxylesterase, soluble epoxide hydrolase, diamine oxidase, xanthine oxidase, and alcohol dehydrogenase (*Sowjanya et al., 2019).

3.2. Working in inert atmosphere

To achieve accurate measurements, the oxidation-prone compound must be handled in solutions without oxygen. This can be achieved by the administration of inert gas (e.g., nitrogen, argon, helium) and only opening the cell for a brief moment when it is necessary (e.g., taking a sample mid-experiment). To minimize the chance of sample contamination by oxygen, a glove box can be used. Only non-aqueous solutions and properly dried solids and vessels may enter the chamber. Key elements of a glovebox include a front-mounted light, a color touch human-machine interface, a purge valve for catching waste gas evaporating from samples, adjustable shelving, teflon gloves for sample manipulation, a large and a small antechamber for insertion of items without exposing the main chamber to the air outside of the chamber, gas purification system, a vacuum pump for reducing pressure in the main chamber and the antechambers, leveling casters for ensuring proper balance.

3.3. Spectrophotometry

Spectrophotometry is the quantitative measurement of the interaction of electromagnetic radiation in the range of visible light (i.e., 390–700 nm). The method measures the absorption of a specific spectrum of radiation by the compound of interest.

The relation between the intensity of the incident and the transmitted beam is key for spectrophotometry. The dependence of concentration of the compound on transmitted beam is characterized by the Beer-Lambert law (Formula 7).

$$\log_{10}\frac{I_o}{I} = \varepsilon * c * l$$

Formula 7 Beer-Lambert law. Io is the intensity of the incident beam, I is the intensity of the transmitted beam, is the molar decadic extinction coefficient, c is the concentration of an absorbing species and l is the length of a path traveled by the light during measurement (typically the width of a test cuvette).

The results can be the measure of decomposition of compound sensitive to air oxygen (Ramešová et al., 2012). This information can then be used by pharmaceutical companies to determine the product's clinical usability, shelf life and commercial viability.

3.4. Electroanalytical methods

In vitro pre-clinical experiments have to demonstrate the interactions between the drug and endogenous molecules, particularly metabolizing enzymes, such as CYPs (*Brandon et al., 2003). Some of these interactions, however, make it difficult to detect the formation of reactive metabolites or intermediates with short half-lives, since these species bind covalently to DNA and proteins (Lohmann & Karst, 2007). CYP-induced metabolism can be mimicked using a purely instrumental electrochemical technique. This approach proves to require a lot less time and resources than commonly used in vivo and in vitro methods involving liver cells or isolated enzymes. Possible metabolites can be detected and identified efficiently by on-line EC/MS in the early stages of drug discovery process (Lohmann & Karst, 2006). This approach is very useful as it allows a clean and rapid generation and direct identification of both stable species and metabolites with short half-lives, omitting the use of organ extracts and animal models at this stage (*Álvarez-Lueje et al., 2012).

3.4.1. Cyclic voltammetry

Cyclic voltammetry is one of the most widely used techniques among all modern electroanalytical methods. It is a type of potentiodynamic electrochemical measurement. Electrochemical properties are measured using a three-electrode system consisting of a working electrode, a counter electrode and a reference electrode. The method observes the potential of the working electrode with respect to the reference electrode and measures the current between the working and the counter electrodes. The resulting graph then portrays the dependency of electrical current at the working electrode on the applied potential. An example of a typical electrochemical cell setup is included below (Figure 6).

The working electrode is usually constructed from inert materials, such as allotrope derivatives of carbon (graphite or glassy carbon) or metals (mercury, platinum or gold). The reference electrode is often made from saturated calomel electrode or Ag|AgCl|KCl(sat) and the auxiliary electrode is usually a platinum wire or a platinum net (*Álvarez-Lueje et al., 2012).

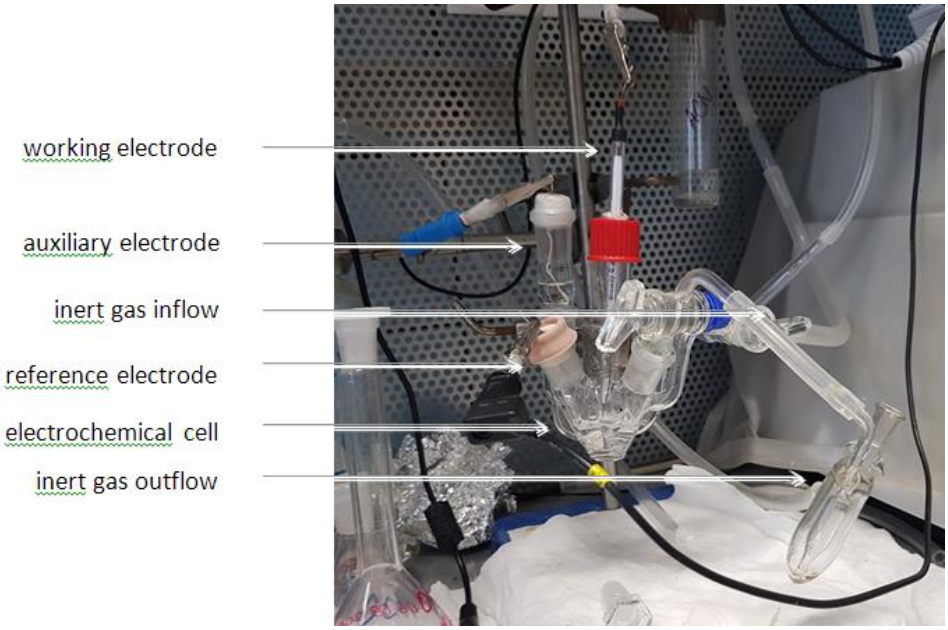

Figure 6 Electrochemical cell setup for cyclic voltammetry

The typical cyclic voltammogram (taken from (Bogdan et al., 2014)) is shown in Figure 7. On the x-axis are the independent values of an imposed parameter (in this case potential in volts). On the y-axis, the corresponding values of the dependent parameter are generated (in this case current in ampers). The forward portion of the graph depicts the signal obtained by oxidation, while the reverse portion depicts the signal obtained by re-reduction of the obtained product.

The Nernst equation (Formula 8) helps to predict the system's response to a change in concentration or the electrode potential.

$$E = E^0 + \frac{RT}{nF} \ln \frac{(Ox)}{(Red)} = E^0 + 2.3026 \frac{RT}{nF} \log_{10} \frac{(Ox)}{(Red)}$$

Formula 8 Nernst equation. E stands for the potential of an electrochemical cell, E^0 stands for the standard potential of a species. (Red) and (Ox) stand for the relative activities of the reduced and oxidized analyte in the system at equilibrium, F stands for Faraday's constant 96 485.3329 s A / mol, r is the universal gas constant and T is temperature in K.

If the potential is scanned in a negative direction, it is referred to as a cathodic scan (reduction). If the potential is scanned in a positive direction, it is referred to an anodic scan (oxidation) (Elgrishi et al., 2018).

The electrochemical processes that can occur at the electrode can be heterogeneous electron-transfer reactions or electron-transfer reactions coupled to homogenous reactions. In the case of a heterogeneous electron-transfer reaction, the electroactive species (O) is converted to the product (R). This reaction can be a simple one-electron transfer, such as an outer-sphere reaction in which no new bonds are formed in species O, nor any bonds are eliminated.

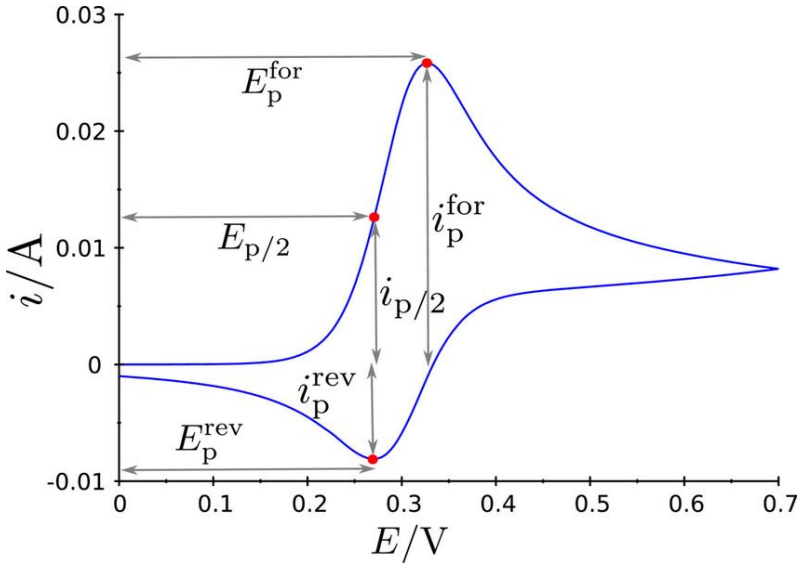

Figure 7 Typical cyclic voltammogram. Indicated in this graph are the forward peak, half peak, and reverse peak potentials (E_p^{for}, $E_{\frac{p}{2}}$, E_p^{rev}), and their corresponding currents (i_p^{for}, $i_{\frac{p}{2}}$, i_p^{rev}) according to (Bogdan et al., 2014)

The shape and the electrochemical parameter (which specifies the working conditions and the electrochemical instrumentation) of the cyclic voltammogram can identify charge-transfer as reversible (Nernstian), quasi-reversible, or irreversible (Bard & Faulkner, 2001). Diagnostic criteria for reversible charge-transfer have been summarized in Table I. Obtaining a different shape of cyclic voltammogram (showing a different ratio of peak currents for the forward and the reverse scan), the dependence of peak potential on scan rate allows us to detect an irreversible redox reaction or participation of coupled chemical reactions (Bard & Faulkner, 2001; Rossiter, 1986). This way, the presence of short living intermediates can be determined and the knowledge will lead to oxidation or reduction mechanism of bioactive compound. Another efficient tool for obtaining the information about intermediates is UV-Vis and IR spectroelectrochemistry (Sokolová et al., 2015).

Prior to measuring, a potential window must be chosen as the range of potentials in which neither the solvent nor the electrolyte is likely to undergo an electron-transfer reaction (Bard & Faulkner, 2001). Here, the importance of oxygen-free preparation of some solutions is evident, as the presence of oxygen might result in an obstruction in the shape of the voltammogram and can be recorded as peaks in absence of an analyte.

Diagnostic Criteria for Cyclic Voltammetry and Reversible Charge Transfer **Reversible Charge Transfer** **$O + ne^- \rightleftharpoons R$**
Properties of the potential response (at 25 °C) *Ep* is independent of *v* ΔE_p = *Ep (anodic) – Ep (cathodic)* = 59/n mV
Properties of the anodic-to-cathodic current ratio *Ip(anodic)/Ip(cathodic)* is independent of *v*
Other Wave shape is independent of *v*

Table 1 Diagnostic Criteria for Cyclic Voltammetry and Charge Transfer (reduction), where Ep is the peak potential, Ip is the current peak, the current function is Ip divided by the square root of the scan rate, v is the scan rate, n is the number of electrons (Rossiter, 1986).

3.4.2. Electrolysis (Coulometry)

Electrolysis is an efficient method used for generation of redox products of bioactive compound. Two main electrodes are essential in an electrochemical cell – a cathode and an anode. Cathodic and anodic compartments are separated by a porous frit. A cathode is an electrode at which reduction occurs, an anode is one at which oxidation occurs. Electrons transfer from the surface of the electrode to a species in the solution in a cathodic current. Simultaneously, electrons flow from a species in the solution to an electrode in an anodic current. Below, the setup of a typical electrochemical cell is depicted in Figure 8.

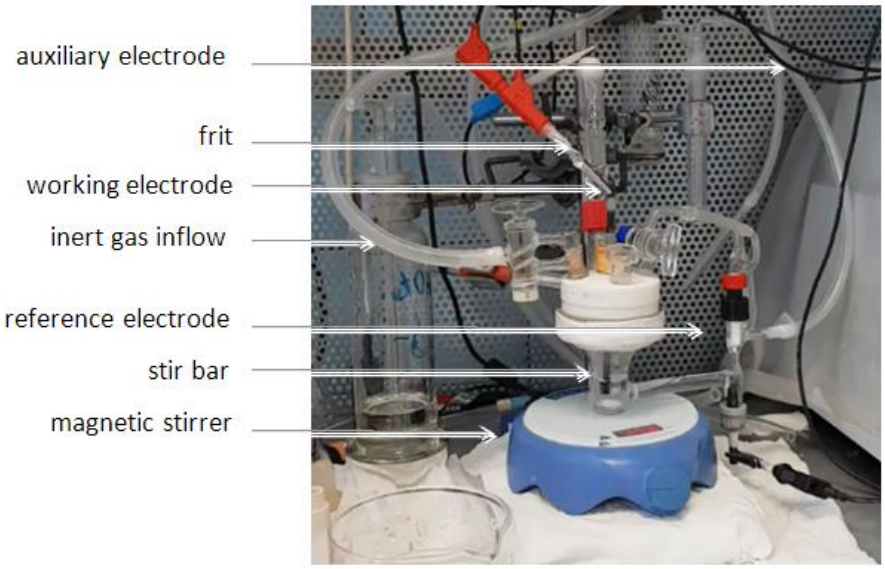

Figure 8 The electrolysis setup

The consumed charge Q during electrolysis is equal to the amount of oxidized compound according to Faraday's laws of electrolysis (Formula 9, Bard & Faulkner, 2001).

$$Q = \frac{m * n * F}{M}$$

Formula 9 Faraday's law of electrolysis, m stands for the mass of the compound, n stands for the number of electrons, F stands for the Faraday constant corresponding to the amount of electricity that is carried by 1 mol of electrons and equals to 96,485 C/mol, and M stands for the molar mass of the compound

3.5. Analytical separation methods

3.5.1. HPLC - DAD

High-performance liquid chromatography is a method that uses high-pressure pumping of a sample (analyte) dissolved in a solution (mobile phase) through a column of immobilized chromatographic packing material (stationary phase). An HPLC system consists of a pumping unit for pumping the eluent at a constant flow rate and pressure, a sample-injection unit for injecting the sample into the flow path, a separation unit selected to suit both the sample and the purpose of separation, a detection unit selected to suit the sample and data-processing unit for computing the concentration of each detected component from the height or area of each corresponding peak (Petrova & Sauer, 2017).

Diode array detector (DAD) is a type of detection unit for detecting data from the ultraviolet to visible light range (Worsfold & Zagatto, 2017).

3.5.2. HPLC - MS/MS

Mass spectrometry (MS) is a technique that is used to measure charged molecules in the gaseous phase based on their mass-to-charge ratio. Prior to mass analysis, the analyte must be ionized. The main parts of a mass spectrometer are the ion source, the mass analyzer and the detector (*Leurs et al., 2016).
Tandem mass spectrometry (MS/MS or MS^2) is a method for sequential mass analysis of the same population of ions. Prior to the analysis, a gas-phase fragmentation event often occurs. The sequential steps of tandem mass analysis can be achieved by their separation in space or in time. Space-separated MS/MS uses a tandem mass spectrometer, which is a single instrument using two (or more) mass analyzers. The individual elements of the mass spectrometer are connected by quadrupoles. The sample is filtered by its m/z ratio in the first quadrupole, only a certain range of m/z can continue to the collision cell (i.e., the second quadrupole). There, product ions are generated by the fragmentation of the precursor ion. The third quadrupole then analyzes those product ions (*Leurs et al., 2016).

Time-separated MS/MS uses separation or collision of ions that are trapped in the same physical space. The first among the steps separated in time is selection of the precursor ion with one specific m/z value and expulsion of others from the trap. Next, the precursor ion is fragmented in the trap. Lastly, the product ions are analyzed.

Since multiple rounds of selection, fragmentation and analysis can be performed on the product ions, the level of MS/MS analysis is indicated as the "n" in MS^n.
The benefit of MS/MS in comparison with other analytical methods is the decreased time-per-compound, since it allows to analyze molecular mass, structure and the purity in the same experiment (*Leurs et al., 2016).

4. Discussion

The study of mechanism of xenobiotics metabolism has a large impact in pharmacy, toxicology, food industry and monitoring of pollutants in environment. Enzymes listed in Chapter 1 and Chapter 2 play an important role during processes of detoxification or bioactivation of xenobiotics. The overall metabolism of a xenobiotic can be divided into 4 phases, Phases 0 (Chapter 2.1.1) and III (Chapter 2.1.4) involving the transport into and out of the cell, although Phase 0 might also include the effect of xenobiotic-ligand-activated

transcription factors. Phase I metabolism involves enzymes that mediate oxidation (Chapter 2.1.2.1, Chapter 2.1.2.2), reduction (Chapter 2.1.2.3), hydrolysis (Chapter 2.1.2.4). Their products can then be further processed in the reactions of Phase II, however reactions of either phase can also occur on their own. Although most Phase II reactions (described in chapters 2.1.3.1 to 2.1.3.4) result in a more soluble product, which would facilitate excretion, acetylation (described in Chapter 2.1.3.5) and methylation (described in Chapter 2.1.3.6) can also result in a less soluble product. The knowledge of the resulting metabolites is desired not only for example for detection of degradation products of bioactive compounds or medicaments in blood and urine, but also the identification of the structure of formed intermediates is important. For this reason, studies on efficacy, stability and pharmacokinetic properties are conducted (Chapter 2.2). As mentioned in Chapter 1, mechanism of reactions mediated by Phase I enzymes (Chapter 2.1.2) is initiated by a single-electron oxidation. The cleavage of the first electron from the molecule is followed by a series of coupled chemical reactions, such as aromatic hydroxylation, S-oxidation, P-oxidation, dehydrogenation, alcohol oxidation, and O- and N-dealkylation, as mentioned in Chapter 2.2.1. Reductive processes (Chapter 2.1.2.1, Chapter 2.2.2.), although less extensively studied, can also play a role in biotransformation and, as well as oxidation, can be detected and described using electrochemical methods. Reversibility or irreversibility of the reaction as well as participation of a coupled reaction can be predicted using cyclic voltammetry (Chapter 3.4.1). The electroanalytical methods can be helpful in determination of the reaction schemes as summarized the Chapter 3.4., in identification of redox products summarized in Chapter 3.4.2. Intermediates can be identified during reductive or oxidative electrolysis by HPLC and by applying of on-line spectrometrical methods (Chapter 3.5.1, Chapter 3.5.2). Reactions of Phase II enzymes (Chapter 2.1.3) can be observed through substrate depletion method (Chapter 3.1) involving incubation with hepatic microsomes (Chapter 3.1.1) for reactions mediated by enzymes localized at the endoplasmic reticulum, or cytosolic fractions (Chapter 3.1.2) for those mediated by cytosolic enzymes. The involvement of Phases 0 and III transporters can be of relevance but cannot be observed via incubation with microsomal or cytosolic fractions, as the experiment would require the use of whole cells.

As mentioned in Chapter 3.3, stability of the compound is tested using spectrophotometry. Experiments with compounds of high reactivity with atmospheric oxygen require that all manipulation happens in deoxygenated environment, which can be achieved with the use of a glovebox (Chapter 3.2)

5. Conclusion

Xenobiotics, such as pharmaceuticals, food additives, environmental pollutants, and dietary bioactive compounds are metabolized by various human enzymes, resulting in their bioactivation and detoxification. Understanding the underlying mechanisms as well as the consequences of this metabolism is an essential part of the drug discovery process and research of xenobiotic impact on the human organism.

Identification of resulting metabolites is important for their detection in bodily fluids and tissues for diagnostic and forensic purposes.

This thesis describes metabolic activity of enzymes most significant for the detoxification or biotransformation of foreign compounds. This biotransformation can be divided into 4 sections – Phase 0 during which the xenobiotic is uptaken into the cell (Ernst *Petzinger & Geyer, 2006), Phase I where oxidation, reduction and hydrolysis reactions take place, Phase II where conjugation of aminoacids, methyl-, acetyl-, sulfate, glucuronide or glutathione moiety takes place (Skálová, 2017), and Phase III of xenobiotic efflux out of the cell and into the bile (*Döring & Petzinger, 2014). There is a question posed by one author whether transcription factors that bind xenobiotics or molecules induced by xenobiotic stress (for example AhR, CAR, PXR) should be considered as a part of the Phase 0. The argument behind this claim is that these molecules are an initial line of defense and their activation leads to a response that allows individuals to acclimate to the chemical insult (*Baldwin, 2019).

Arguably the most important enzyme families for Phase I xenobiotic metabolism is CYP, accounting for approximately 75 % of the biotransformation reactions of the most commonly prescribed drugs. For Phase II, the main enzyme is UGT, accounting for about 13 % of biotransformation reactions (Williams et al., 2004).

In vitro biochemical methods, such as substrate depletion in microsomal and cytosolic fractions help identify the enzymes involved in the decomposition of a compound of interest (Jones & Houston, 2004).

Given that biochemical reactions are to a major extent composed of electron-transfer reactions (i.e., oxidation and reduction), the thesis includes a section dealing with the practical approaches to determination of the oxidative or reductive mechanism of bioactive compounds using electrochemistry. The described methods include spectrophotometry, cyclic

voltammetry, electrolysis and HPLC. Identification of reaction intermediates with the use of electrochemistry and biochemical methods may contribute to the characterization of the metabolism of compounds of interest.

The thesis is based on the author's personal experience with described methods, which were performed at Jaroslav Heyrovsky Institute of Physical Chemistry in Prague and Department of Biochemical Sciences at the Faculty of Pharmacy in Hradec Kralove.

References

Review articles *

*Álvarez-Lueje, A., & Bollo Dragnic, S. (2010). Stability and Drug Metabolism Assessed by Electrochemical Methods. *Combinatorial Chemistry & High Throughput Screening*, *13*, 712–727. https://doi.org/10.2174/138620710791920329

*Álvarez-Lueje, A., Pérez, M., & Zapata, C. (2012). Electrochemical Methods for the In Vitro Assessment of Drug Metabolism. In D. J. Paxton (Ed.), *Topics on Drug Metabolism* (p. 294). InTech. https://doi.org/10.5772/28647

*Anzenbacher, P., & Anzenbacherová, E. (2001). Cytochromes P450 and metabolism of xenobiotics. *Cellular and Molecular Life Sciences*, *58*, 737–747. https://doi.org/10.1007/PL00000897

*Anzenbacher, Pavel, Dawson, J. H., & Kitagawa, T. (1989). Towards a unified concept of oxygen activation by heme enzymes: the role of the proximal ligand. *Journal of Molecular Structure*, *214*, 149–158. https://doi.org/10.1016/0022-2860(89)80011-0

*Axelrod, J. (1966). Methylation reactions in the formation and metabolism of catecholamines and other biogenic amines. *Pharmacological Reviews*, *18*, 95–113. http://www.ncbi.nlm.nih.gov/pubmed/5323771

*Baldwin, W. S. (2019). Phase 0 of the Xenobiotic Response: Nuclear Receptors and Other Transcription Factors as a First Step in Protection from Xenobiotics. *Nuclear Receptor Research*, *6*. https://doi.org/10.32527/2019/101447

*Barker, S. A., McIlhenny, E. H., & Strassman, R. (2012). A critical review of reports of endogenous psychedelic N, N-dimethyltryptamines in humans: 1955-2010. *Drug Testing and Analysis*, *4*, 617–635. https://doi.org/10.1002/dta.422

*Barski, O. A., Tipparaju, S. M., & Bhatnagar, A. (2008). The aldo-keto reductase superfamily and its role in drug metabolism and detoxification. In *Drug Metabolism Reviews* (Vol. 40, Issue 4, pp. 553–624). https://doi.org/10.1080/03602530802431439

*Beaumont, C., Young, G. C., Cavalier, T., & Young, M. A. (2014). Human absorption, distribution, metabolism and excretion properties of drug molecules: a plethora of approaches. *British Journal of Clinical Pharmacology*, *78*, 1185–1200. https://doi.org/10.1111/bcp.12468

*Brandon, E. F. A., Raap, C. D., Meijerman, I., Beijnen, J. H., & Schellens, J. H. M. (2003). An update on in vitro test methods in human hepatic drug biotransformation research: Pros and cons. *Toxicology and Applied Pharmacology*, *189*, 233–246. https://doi.org/10.1016/S0041-008X(03)00128-5

*Brockhausen, I., & Kuhns, W. (1997). Role and Metabolism of Glycoconjugate Sulfation. *Trends in Glycoscience and Glycotechnology*, *9*, 379–398. https://doi.org/10.4052/tigg.9.379

*Chen, X., Jia, W., Zhu, L., Mao, L., & Zhang, Y. (2020). Recent advances in heterocyclic aromatic amines: An update on food safety and hazardous control from food processing to dietary intake. *Comprehensive Reviews in Food Science and Food Safety*, *19*, 124–148. https://doi.org/10.1111/1541-4337.12511

*Döring, B., & Petzinger, E. (2014). Phase 0 and phase III transport in various organs: Combined concept of phases in xenobiotic transport and metabolism. *Drug Metabolism Reviews*, *46*(3), 261–282. https://doi.org/10.3109/03602532.2014.882353

*Habuchi, O. (2000). Diversity and functions of glycosaminoglycan sulfotransferases. *Biochimica et Biophysica Acta - General Subjects*, *1474*, 115–127. https://doi.org/10.1016/S0304-4165(00)00016-7

*Hanna, P. E., & Anders, M. W. (2019). The mercapturic acid pathway. *Critical Reviews in Toxicology*, *49*, 819–929. https://doi.org/10.1080/10408444.2019.1692191

*Knights, K. M., Sykes, M. J., & Miners, J. O. (2007). Amino acid conjugation: Contribution to the metabolism and toxicity of xenobiotic carboxylic acids. *Expert Opinion on Drug Metabolism and Toxicology*, *3*, 159–168. https://doi.org/10.1517/17425255.3.2.159

*Krueger, S. K., & Williams, D. E. (2005). Mammalian flavin-containing monooxygenases: Structure/function, genetic polymorphisms and role in drug metabolism. *Pharmacology and Therapeutics*, *106*, 357–387. https://doi.org/10.1016/j.pharmthera.2005.01.001

*Leurs, U., Mistarz, U. H., & Rand, K. D. (2016). *Applications of Mass Spectrometry in Drug Development Science* (pp. 253–289). https://doi.org/10.1007/978-1-4939-4029-5_7

*Ma, Z., Liu, H., & Wu, B. (2014). Structure-based drug design of catechol-O-methyltransferase inMa, Z., Liu, H., & Wu, B. (2014). Structure-based drug design of catechol-O-methyltransferase inhibitors for CNS disorders. British Journal of Clinical Pharmacology, 77, 410–420. https://doi.or. *British Journal of Clinical Pharmacology*, *77*, 410–420. https://doi.org/10.1111/bcp.12169

*McLeod, H. L., Krynetski, E. Y., Relling, M. V., & Evans, W. E. (2000). Genetic polymorphism of thiopurine methyltransferase and its clinical relevance for childhood acute lymphoblastic leukemia. *Leukemia*, *14*, 567–572. https://doi.org/10.1038/sj.leu.2401723

*Mueller, J. W., & Shafqat, N. (2013). Adenosine-5′-phosphosulfate - A multifaceted modulator of bifunctional 3′-phospho-adenosine-5′-phosphosulfate synthases and related enzymes. *FEBS Journal*, *280*, 3050–3057. https://doi.org/10.1111/febs.12252

*Petzinger, E, Blumrich, M., Brühl, B., Eckhardt, U., Föllmann, W., Honscha, W., Horz, J. A., Müller, N., Nickau, L., Ottallah-Kolac, M., Platte, H. D., Schenk, A., Schuh, K., Schulz, K., & Schulz, S. (1996). What we have learned about bumetanide and the concept of multispecific bile acid/drug transporters from the liver. *Journal of Hepatology*, *24*, 42–46. http://www.ncbi.nlm.nih.gov/pubmed/8926368

*Petzinger, Ernst, & Geyer, J. (2006). Drug transporters in pharmacokinetics. *Naunyn-Schmiedeberg's Archives of Pharmacology*, *372*, 465–475. https://doi.org/10.1007/s00210-006-0042-9

*Ramsay, E. E., & Dilda, P. J. (2014). Glutathione S-conjugates as prodrugs to target drug-resistant tumors. *Frontiers in Pharmacology*, *5*, 1–34. https://doi.org/10.3389/fphar.2014.00181

*Roth, M., Obaidat, A., & Hagenbuch, B. (2012). OATPs, OATs and OCTs: the organic anion and cation transporters of the SLCO and SLC22A gene superfamilies. *British Journal of Pharmacology*, *165*, 1260–1287. https://doi.org/10.1111/j.1476-5381.2011.01724.x

*Sherratt, H. S. A. (1986). Hypoglycin, the famous toxin of the unripe Jamaican ackee fruit. *Trends in Pharmacological Sciences*, *7*, 186–191. https://doi.org/10.1016/0165-6147(86)90310-X

*Sowjanya, G., Ganapaty, S., & Sharma, R. (2019). In vitro test methods for metabolite identification: A review. *Asian Journal of Pharmacy and Pharmacology*, *5*, 441–450. https://doi.org/10.31024/ajpp.2019.5.3.2

*Williams, J. A., Hyland, R., Jones, B. C., Smith, D. A., Hurst, S., Goosen, T. C., Peterkin, V., Koup, J. R., & Ball, S. E. (2004). Drug-drug interactions for UDP-

glucuronosyltransferase substrates: A pharmacokinetic explanation for typically observed low exposure (AUC 1/AUC) ratios. In *Drug Metabolism and Disposition* (Vol. 32, Issue 11, pp. 1201–1208). American Society for Pharmacology and Experimental Therapy. https://doi.org/10.1124/dmd.104.000794

*Yang, G., Ge, S., Singh, R., Basu, S., Shatzer, K., Zen, M., Liu, J., Tu, Y., Zhang, C., Wei, J., Shi, J., Zhu, L., Liu, Z., Wang, Y., Gao, S., & Hu, M. (2017). Glucuronidation: driving factors and their impact on glucuronide disposition. *Drug Metabolism Reviews*, *49*, 105–138. https://doi.org/10.1080/03602532.2017.1293682

*Zhou, X., Ma, Z., Dong, D., & Wu, B. (2013). Arylamine N-acetyltransferases: A structural perspective. *British Journal of Pharmacology*, *169*, 748–760. https://doi.org/10.1111/bph.12182

Aono, S., Keino, H., Yamada, Y., Adachi, Y., Nanno, T., Uyama, E., Koiwai, O., & Sato, H. (1995). Analysis of genes for bilirubin UDP-glucuronosyltransferase in Gilbert's syndrome. *The Lancet*, *345*, 958–959. https://doi.org/10.1016/S0140-6736(95)90702-5

Axelrod, J., & Daly, J. (1968). Phenol-O-methyltransferase. *BBA - Enzymology*, *159*, 472–478. https://doi.org/10.1016/0005-2744(68)90131-9

Balíková, M. (2017). Cíle toxikologických vyšetření. In L. Houdek (Ed.), *Forenzní a klinická toxikologie* (2nd ed., pp. 9–10). Galén.

Bard, A. J., & Faulkner, L. F. (2001). *Electrochemical Methods Fundmentals and Applications* (D. Harris, E. Swain, C. Robey, & E. Aiello (eds.); 2nd ed.). John Wiley & Sons, Inc.

Bock, K. W., & Köhle, C. (2005). Ah receptor- and TCDD-mediated liver tumor promotion: clonal selection and expansion of cells evading growth arrest and apoptosis. *Biochemical Pharmacology*, *69*(10), 1403–1408. https://doi.org/10.1016/j.bcp.2005.02.004

Bogdan, M., Brugger, D., Rosenstiel, W., & Speiser, B. (2014). Estimation of diffusion coefficients from voltammetric signals by support vector and gaussian process regression. *Journal of Cheminformatics*, *6*, 1–13. https://doi.org/10.1186/1758-2946-6-30

Bosma, P. J., Chowdhury, J. R., Bakker, C., Gantla, S., de Boer, A., Oostra, B. A., Lindhout, D., Tytgat, G. N. J., Jansen, P. L. M., Elferink, R. P. J. O., & Chowdhury, N. R. (1995). The Genetic Basis of the Reduced Expression of Bilirubin UDP-Glucuronosyltransferase 1 in Gilbert's Syndrome. *New England Journal of Medicine*, *333*, 1171–1175. https://doi.org/10.1056/NEJM199511023331802

Bosma, P. J., Chowdhury, N. R., Goldhoorn, B. G., Hofker, M. H., Elferink, R. P. J. O., Jansen, P. L. M., & Chowdhury, J. R. (1992). Sequence of exons and the flanking regions of human bilirubin-UDP-glucuronosyltransferase gene complex and identification of a genetic mutation in a patient with Crigler-Najjar syndrome, type I. *Hepatology*, *15*, 941–947. https://doi.org/10.1002/hep.1840150531

Chen, C.-H. (2020). Conjugation Reactions. In *Xenobiotic Metabolic Enzymes: Bioactivation and Antioxidant Defense* (pp. 39–41). Springer International Publishing. https://doi.org/10.1007/978-3-030-41679-9

Chen, C., Staudinger, J. L., & Klaassen, C. D. (2003). Nuclear receptor, pregnane X receptor, is required for induction of UDP-glucuronosyltransferases in mouse liver by 16aα-carbonitrile. *Drug Metabolism and Disposition*, *31*(7), 908–915. https://doi.org/10.1124/dmd.31.7.908

Chowdhury, J. R., Wolkoff, A. W., Chowdhury, N. R., Arias, I. M. (2001). Hereditary jaundice and disorders of bilirubin metabolism. In D. Scriver, C. R.; Beaudet, A. L.; Sly, W. S.; Valle (Ed.), *The Metabolic and Molecular Bases of Inherited Disease* (8th ed.,

Vol. 2, pp. 3063–3101). McGraw-Hill. https://doi.org/10.1036/ommbid.154

Dean, J. G. (2018). Indolethylamine-N-methyltransferase polymorphisms: Genetic and biochemical approaches for study of endogenous N, N,-dimethyltryptamine. *Frontiers in Neuroscience*, *12*, 1–16. https://doi.org/10.3389/fnins.2018.00232

Dean, J. G., Liu, T., Huff, S., Sheler, B., Barker, S. A., Strassman, R. J., Wang, M. M., & Borjigin, J. (2019). Biosynthesis and Extracellular Concentrations of N,N-dimethyltryptamine (DMT) in Mammalian Brain. *Scientific Reports*, *9*, 1–11. https://doi.org/10.1038/s41598-019-45812-w

Dorszewska, J., Prendecki, M., Lianeri, M., & Kozubski, W. (2014). Molecular Effects of L-dopa Therapy in Parkinson's Disease. *Current Genomics*, *15*, 11–17. https://doi.org/10.2174/1389202914666131210213042

Elgrishi, N., Rountree, K. J., McCarthy, B. D., Rountree, E. S., Eisenhart, T. T., & Dempsey, J. L. (2018). A Practical Beginner's Guide to Cyclic Voltammetry. *Journal of Chemical Education*, *95*, 197–206. https://doi.org/10.1021/acs.jchemed.7b00361

FAO and WHO. (2019). *Carryover in feed and transfer from feed to food of unavoidable and unintended residues of approved veterinary drugs* (No. 13). FAO Animal Production and Health. https://doi.org/10.4060/ca6296en

Fujihara, J., Soejima, M., Yasuda, T., Koda, Y., Agusa, T., Kunito, T., Tongu, M., Yamada, T., & Takeshita, H. (2010). Global analysis of genetic variation in human arsenic (+ 3 oxidation state) methyltransferase (AS3MT). *Toxicology and Applied Pharmacology*, *243*, 292–299. https://doi.org/10.1016/j.taap.2009.11.020

Gibson, G. G., & Skett, P. (1986). Enzymology and molecular mechanisms of drug metabolism reactions. In *Introduction to Drug Metabolism* (1st ed., pp. 35–76). Chapman & Hall. https://doi.org/10.1007/978-1-4899-3188-7

Hagenbuch, B., & Stieger, B. (2013). The SLCO (former SLC21) superfamily of transporters. *Molecular Aspects of Medicine*, *34*, 396–412. https://doi.org/10.1016/j.mam.2012.10.009

Hickman, D., Pope, J., Patil, S. D., Fakis, G., Smelt, V., Stanley, L. A., Payton, M., Unadkat, J. D., & Sim, E. (1998). Expression of arylamine N-acetyltransferase in human intestine. *Gut*, *42*, 402–409. https://doi.org/10.1136/gut.42.3.402

Hindorf, U., Lindqvist, M., Hildebrand, H., Fagerberg, U., & Almer, S. (2006). Adverse events leading to modification of therapy in a large cohort of patients with inflammatory bowel disease. *Alimentary Pharmacology and Therapeutics*, *24*, 331–342. https://doi.org/10.1111/j.1365-2036.2006.02977.x

Hines, R. (2007). Phase I biotransformation reactions-flavin monooxygenase. In S. J. Enna & D. B. Bylund (Eds.), *xPharm: The Comprehensive Pharmacology Reference* (pp. 1–11). Elsevier Inc. https://doi.org/10.1016/B978-008055232-3.60280-6

Hines, R. N., Hopp, K. A., Franco, J., Saeian, K., & Begun, F. P. (2002). Alternative processing of the human FMO6 gene renders transcripts incapable of encoding a functional flavin-containing monooxygenase. *Molecular Pharmacology*, *62*, 320–325. https://doi.org/10.1124/mol.62.2.320

Hosoyamada, M., Sekine, T., Kanai, Y., & Endou, H. (1999). Molecular cloning and functional expression of a multispecific organic anion transporter from human kidney. *American Journal of Physiology - Renal Physiology*, *276*, 122–128. https://doi.org/10.1152/ajprenal.1999.276.1.f122

IARC. (1993). *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans. Some Naturally Occurring Substances: Food Items and Constituents, Heterocyclic Aromatic Amines and Mycotoxins* (IARC (ed.); Vol. 56). World Health Organization. https://publications.iarc.fr/Book-And-Report-Series/Iarc-Monographs-On-The-

Identification-Of-Carcinogenic-Hazards-To-Humans/Some-Naturally-Occurring-Substances-Food-Items-And-Constituents-Heterocyclic-Aromatic-Amines-And-Mycotoxins-1993

Ishikawa, T. (1992). The ATP-dependent glutathione S-conjugate export pump. *Trends in Biochemical Sciences*, *17*, 463–468. https://doi.org/10.1016/0968-0004(92)90489-V

Jančová, P., & Šiller, M. (2012, February 22). *Phase II Drug Metabolism* (J. Paxton (ed.)). Topics on Drug Metabolism; InTech. https://doi.org/10.5772/29996

Jones, H. M., & Houston, J. B. (2004). Substrate depletion approach for determining in vitro metabolic clearance: Time dependencies in hepatocyte and microsomal incubations. *Drug Metabolism and Disposition*, *32*(9), 973–982. https://doi.org/10.1124/dmd.104.000125

Kaivosaari, S., Finel, M., & Koskinen, M. (2011). N-glucuronidation of drugs and other xenobiotics by human and animal UDP-glucuronosyltransferases. *Xenobiotica*, *41*, 652–669. https://doi.org/10.3109/00498254.2011.563327

Kannt, A., Rajagopal, S., Kadnur, S. V., Suresh, J., Bhamidipati, R. K., Swaminathan, S., Hallur, M. S., Kristam, R., Elvert, R., Czech, J., Pfenninger, A., Rudolph, C., Schreuder, H., Chandrasekar, D. V., Mane, V. S., Birudukota, S., Shaik, S., Zope, B. R., Burri, R. R., … Dhakshinamoorthy, S. (2018). A small molecule inhibitor of Nicotinamide N-methyltransferase for the treatment of metabolic disorders. *Scientific Reports*, *8*, 1–15. https://doi.org/10.1038/s41598-018-22081-7

Kitamura, S., Tatsumi, K., Hirata, Y., & Yoshimura, H. (1981). Further studies on sulfoxide-reducing enzyme system. *Journal of Pharmacobio-Dynamics*, *4*, 528–533. https://doi.org/10.1248/bpb1978.4.528

Knights, K. M., Stresser, D. M., Miners, J. O., & Crespi, C. L. (2016). In vitro drug metabolism using liver microsomes. *Current Protocols in Pharmacology*, *74*, 1–24. https://doi.org/10.1002/cpph.9

Kurian, J. R., Bajad, S. U., Miller, J. L., Chin, N. A., & Trepanier, L. A. (2004). NADH cytochrome b5 reductase and cytochrome b5 catalyze the microsomal reduction of xenobiotic hydroxylamines and amidoximes in humans. *Journal of Pharmacology and Experimental Therapeutics*, *311*, 1171–1178. https://doi.org/10.1124/jpet.104.072389

Lautala, P., Ulmanen, I., & Taskinen, J. (2001). Molecular mechanisms controlling the rate and specificity of catechol O-methylation by human soluble catechol O-methyltransferase. *Molecular Pharmacology*, *59*, 393–402. https://doi.org/10.1124/mol.59.2.393

Lehmann, J. M., McKee, D. D., Watson, M. A., Willson, T. M., Moore, J. T., & Kliewer, S. A. (1998). The human orphan nuclear receptor PXR is activated by compounds that regulate CYP3A4 gene expression and cause drug interactions. *Journal of Clinical Investigation*, *102*, 1016–1023. https://doi.org/10.1172/JCI3703

Lohmann, W., & Karst, U. (2006). Simulation of the detoxification of paracetamol using on-line electrochemistry/liquid chromatography/mass spectrometry. *Analytical and Bioanalytical Chemistry*, *386*, 1701–1708. https://doi.org/10.1007/s00216-006-0801-y

Lohmann, W., & Karst, U. (2007). Generation and identification of reactive metabolites by electrochemistry and immobilized enzymes coupled on-line to liquid chromatography/mass spectrometry. *Analytical Chemistry*, *79*, 6831–6839. https://doi.org/10.1021/ac071100r

Lotta, T., Vidgren, J., Tilgmann, C., Ulmanen, I., Melén, K., Julkunen, I., & Taskinen, J. (1995). Kinetics of Human Soluble and Membrane-Bound Catechol O-Methyltransferase: A Revised Mechanism and Description of the Thermolabile Variant

of the Enzyme. *Biochemistry*, *34*, 4202–4210. https://doi.org/10.1021/bi00013a008

Madsen, K. G., Skonberg, C., Jurva, U., Cornett, C., Hansen, S. H., Johansen, T. N., & Olsen, J. (2008). Bioactivation of diclofenac in vitro and in vivo: Correlation to electrochemical studies. *Chemical Research in Toxicology*, *21*, 1107–1119. https://doi.org/10.1021/tx700419d

Miley, M. J., Zielinska, A. K., Keenan, J. E., Bratton, S. M., Radominska-Pandya, A., & Redinbo, M. R. (2007). Crystal Structure of the Cofactor-Binding Domain of the Human Phase II Drug-Metabolism Enzyme UDP-Glucuronosyltransferase 2B7. *Journal of Molecular Biology*, *369*(2), 498–511. https://doi.org/10.1016/j.jmb.2007.03.066

Miyake, N., Elcioglu, N. H., Iida, A., Isguven, P., Dai, J., Murakami, N., Takamura, K., Cho, T. J., Kim, O. H., Hasegawa, T., Nagai, T., Ohashi, H., Nishimura, G., Matsumoto, N., & Ikegawa, S. (2012). PAPSS2 mutations cause autosomal recessive brachyolmia. *Journal of Medical Genetics*, *49*, 533–538. https://doi.org/10.1136/jmedgenet-2012-101039

Muller, P. (1994). Glossary of terms used in physical organic chemistry: (IUPAC Recommendations 1994). *Pure and Applied Chemistry*, *66*, 1077–1184. https://doi.org/10.1351/pac199466051077

National Toxicology Program. (2011). NTP 12th Report on Carcinogens. *Report on Carcinogens : Carcinogen Profiles / U.S. Dept. of Health and Human Services, Public Health Service, National Toxicology Program*, *12*.

Nicoll, C. R., Bailleul, G., Fiorentini, F., Mascotti, M. L., Fraaije, M. W., & Mattevi, A. (2020). Ancestral-sequence reconstruction unveils the structural basis of function in mammalian FMOs. *Nature Structural & Molecular Biology*, *27*(1), 14–24. https://doi.org/10.1038/s41594-019-0347-2

Olsen, J., Li, C., Bjørnsdottir, I., Sidenius, U., Hansen, S. H., & Benet, L. Z. (2005). In vitro and in vivo studies on acyl-coenzyme A-dependent bioactivation of zomepirac in rats. *Chemical Research in Toxicology*, *18*, 1729–1736. https://doi.org/10.1021/tx0501785

Palade, G. E., & Siekevitz, P. (1956). Liver microsomes; an integrated morphological and biochemical study. *The Journal of Biophysical and Biochemical Cytology*, *2*, 171–200. https://doi.org/10.1083/jcb.2.2.171

Petrova, O. E., & Sauer, K. (2017). High-performance liquid chromatography (HPLC)-based detection and quantitation of cellular c-di-GMP. In K. Sauer (Ed.), *Methods in Molecular Biology* (Vol. 1657, pp. 33–43). Humana Press Inc. https://doi.org/10.1007/978-1-4939-7240-1_4

Phillips, I. R., & Shephard, E. A. (2019). Flavin-containing monooxygenases: new structures from old proteins. *Nature Structural and Molecular Biology*, *27*, 3–4. https://doi.org/10.1038/s41594-019-0356-1

Ramešová, Š., Sokolová, R., Degano, I., Bulíčková, J., Žabka, J., & Gál, M. (2012). On the stability of the bioactive flavonoids quercetin and luteolin under oxygen-free conditions. *Analytical and Bioanalytical Chemistry*, *402*, 975–982. https://doi.org/10.1007/s00216-011-5504-3

Rini, J., Szumlanski, C., Guerciolini, R., & Weinshilboum, R. M. (1990). Human liver nicotinamide N-methyltransferase: Ion-pairing radiochemical assay, biochemical properties and individual variation. *Clinica Chimica Acta*, *186*, 359–374. https://doi.org/10.1016/0009-8981(90)90322-J

Riordan, J. R., Deuchars, K., Kartner, N., Alon, N., Trent, J., & Ling, V. (1985). Amplification of P-glycoprotein genes in multidrug-resistant mammalian cell lines. *Nature*, *316*, 817–819. https://doi.org/10.1038/316817a0

Robbins, W. P., & Lipmann, F. (1957). Isolation and identification of active sulfate. *J Biol*

Chem., *229*, 837–851.

Roninson, I. B., Chin, J. E., Choi, K., Gros, P., Housman, D. E., Fojo, A., Shen, D. W., Gottesman, M. M., & Pastan, I. (1986). Isolation of human mdr DNA sequences amplified in multidrug-resistant KB carcinoma cells. *Proceedings of the National Academy of Sciences of the United States of America*, *83*, 4538–4542. https://doi.org/10.1073/pnas.83.12.4538

Rossiter, B. W. (1986). Voltammetry with stationary and rotate electrodes. In J. F. Hamilton (Ed.), *Physical Methods of Chemistry* (Second, Vol. 2). John Wiley and Sons Inc.

Ruff, L. J., & Brass, E. P. (1991). Metabolic effects of pivalate in isolated rat hepatocytes. *Toxicology and Applied Pharmacology*, *110*, 295–302. https://doi.org/10.1016/S0041-008X(05)80012-2

Ruzza, P., & Calderan, A. (2013). Glutathione transferase (GST)-activated prodrugs. *Pharmaceutics*, *5*, 220–231. https://doi.org/10.3390/pharmaceutics5020220

Ruzza, P., Rosato, A., Rossi, C., Floreani, M., & Quintieri, L. (2009). Glutathione Transferases as Targets for Cancer Therapy. *Anti-Cancer Agents in Medicinal Chemistry*, *9*, 763–777. https://doi.org/10.2174/187152009789056895

Schrödinger LLC. (2020). *The PyMOL Molecular Graphics System* (2.3.4).

Sim, E., Fakis, G., Laurieri, N., & Boukouvala, S. (2012). Arylamine N-Acetyltransferases - from Drug Metabolism and Pharmacogenetics to Identification of Novel Targets for Pharmacological Intervention. In *Advances in Pharmacology* (Vol. 63, pp. 169–205). Academic Press Inc. https://doi.org/10.1016/B978-0-12-398339-8.00005-7

Skálová, L. (2017). *Metabolismus léčiv a jiných xenobiotik* (2nd ed.). Karolinum.

Sokolová, R., Nycz, J. E., Ramešová, Š., Fiedler, J., Degano, I., Szala, M., Kolivoška, V., & Gál, M. (2015). Electrochemistry and spectroelectrochemistry of bioactive hydroxyquinolines: A mechanistic study. *Journal of Physical Chemistry B*, *119*, 6074–6080. https://doi.org/10.1021/acs.jpcb.5b00098

Sonoda, J., Xie, W., Rosenfeld, J. M., Barwick, J. L., Guzelian, P. S., & Evans, R. M. (2002). Regulation of a xenobiotic sulfonation cascade by nuclear pregnane X receptor (PXR). *Proceedings of the National Academy of Sciences*, *99*(21), 13801–13806. https://doi.org/10.1073/pnas.212494599

Sugimura, T., & Sato, S. (1983). Mutagens-carcinogens in foods. *Cancer Research*, *43*, 2415–2421. http://www.ncbi.nlm.nih.gov/pubmed/6682010

Talaska, G., Al-Juburi, A. Z. S. S., & Kadlubar, F. F. (1991). Smoking related carcinogen-DNA adducts in biopsy samples of human urinary bladder: Identification of N-(deoxyguanosin-8-yl)-4-aminobiphenyl as a major adduct. *Proceedings of the National Academy of Sciences of the United States of America*, *88*, 5350–5354. https://doi.org/10.1073/pnas.88.12.5350

Teng, W. C., Oh, J. W., New, L. S., Wahlin, M. D., Nelson, S. D., Ho, H. K., & Chan, E. C. Y. (2010). Mechanism-Based Inactivation of Cytochrome P450 3A4 by Lapatinib. *Molecular Pharmacology*, *78*(4), 693–703. https://doi.org/10.1124/mol.110.065839

Tenhunen, J., Salminen, M., Lundström, K., Kiviluoto, T., Savolainen, R., & Ulmanen, I. (1994). Genomic organization of the human catechol O-methyltransferase gene and its expression from two distinct promoters. *European Journal of Biochemistry*, *223*, 1049–1059. https://doi.org/10.1111/j.1432-1033.1994.tb19083.x

Thompson, M. A., Moon, E., Kim, U. J., Xu, J., Siciliano, M. J., & Weinshilboum, R. M. (1999). Human indolethylamine N-methyltransferase: cDNA cloning and expression, gene cloning, and chromosomal localization. *Genomics*, *61*, 285–297. https://doi.org/10.1006/geno.1999.5960

Towles, J. K., Clark, R. N., Wahlin, M. D., Uttamsingh, V., Rettie, A. E., & Jackson, K. D. (2016). Cytochrome P450 3A4 and CYP3A5-catalyzed bioactivation of lapatinib. *Drug Metabolism and Disposition*, *44*(10), 1584–1597. https://doi.org/10.1124/dmd.116.070839

Van Haren, M. J., Sastre Toraño, J., Sartini, D., Emanuelli, M., Parsons, R. B., & Martin, N. I. (2016). A Rapid and Efficient Assay for the Characterization of Substrates and Inhibitors of Nicotinamide N-Methyltransferase. *Biochemistry*, *55*, 5307–5315. https://doi.org/10.1021/acs.biochem.6b00733

Vessey, D. A., Kelley, M., & Warren, R. S. (1999). Characterization of the CoA ligases of human liver mitochondria catalyzing the activation of short- and medium-chain fatty acids and xenobiotic carboxylic acids. *Biochimica et Biophysica Acta*, *1428*, 455–462. https://doi.org/10.1016/s0304-4165(99)00088-4

Wang, H., Iwasaki, M., Haiman, C. A., Kono, S., Wilkens, L. R., Keku, T. O., Berndt, S. I., Tsugane, S., & Marchand, L. Le. (2015). Interaction between Red Meat Intake and NAT2 genotype in increasing the risk of colorectal cancer in Japanese and African Americans. *PLoS ONE*, *10*, 1–11. https://doi.org/10.1371/journal.pone.0144955

Weinshilboum, R. (1989). Methyltransferase pharmacogenetics. *Pharmacology & Therapeutics*, *43*, 77–90. https://doi.org/10.1016/0163-7258(89)90048-x

Weitzel, K. W., Smith, D. M., Elsey, A. R., Duong, B. Q., Burkley, B., Clare-Salzler, M., Gong, Y., Higgins, T. A., Kong, B., Langaee, T., McDonough, C. W., Staley, B. J., Vo, T. T., Wake, D. T., Cavallari, L. H., & Johnson, J. A. (2018). Implementation of Standardized Clinical Processes for TPMT Testing in a Diverse Multidisciplinary Population: Challenges and Lessons Learned. *Clinical and Translational Science*, *11*, 175–181. https://doi.org/10.1111/cts.12533

Williams, R. (1959). Detoxication mechanisms: the metabolism and detoxication of drugs, toxic substances, and other organic compounds. *British Journal of Industrial Medicine*, *17*, 244.

Wood, T. C., Salavagionne, O. E., Mukherjee, B., Wang, L., Klumpp, A. F., Thomae, B. A., Eckloff, B. W., Schaid, D. J., Wieben, E. D., & Weinshilboum, R. M. (2006). Human arsenic methyltransferase (AS3MT) pharmacogenetics: Gene resequencing and functional genomics studies. *Journal of Biological Chemistry*, *281*, 7364–7373. https://doi.org/10.1074/jbc.M512227200

Worsfold, P. J., & Zagatto, E. A. (2017). Spectrophotometry | overview. In P. Worsfold, A. Townshend, C. Poole, & M. Miró (Eds.), *Encyclopedia of Analytical Science* (3rd ed., pp. 318–321). Elsevier. https://doi.org/10.1016/B978-0-12-409547-2.14265-9

Yamashita, M., Wakabayashi, K., Nagao, M., Sato, S., Yamaizumi, Z., Takahashi, M., Kinae, N., Tomita, I., & Sugimura, T. (1986). Detection of 2-amino-3-methylimidazo[4,5-f]quinoline in cigarette smoke condensate. *Japanese Journal of Cancer Research : Gann*, *77*, 419–422. http://www.ncbi.nlm.nih.gov/pubmed/3089973

Yano, J.K., Wester, M.R., Schoch, G.A., Griffin, K.J., Stout, C.D., Johnson, E. F. (2004). The Structure of Human Microsomal Cytochrome P450 3A4 Determined by X-ray Crystallography to 2.05-A Resolution. *J Biol Chem.*, *279*, 38091–38094. https://doi.org/10.1074/jbc.C400293200

Zhang, D., Fourie-O'Donohue, A., Dragovich, P. S., Pillow, T. H., Sadowsky, J. D., Kozak, K. R., Cass, R. T., Liu, L., Deng, Y., Liu, Y., Hop, C. E. C. A., & Cyrus Khojasteh, S. (2019). Catalytic cleavage of disulfide bonds in small molecules and linkers of antibody-drug conjugates. *Drug Metabolism and Disposition*, *47*, 1156–1163. https://doi.org/10.1124/dmd.118.086132

Zhou, S., Chan, E., Li, X., & Huang, M. (2005). Clinical outcomes and management of

mechanism-based inhibition of cytochrome P450 3A4. *Therapeutics and Clinical Risk Management*, *1*(1), 3–13. https://doi.org/10.2147/tcrm.1.1.3.53600

Zhu, B. (2005). Catechol-O-Methyltransferase (COMT)-Mediated Methylation Metabolism of Endogenous Bioactive Catechols and Modulation by Endobiotics and Xenobiotics: Importance in Pathophysiology and Pathogenesis. *Current Drug Metabolism*, *3*, 321–349. https://doi.org/10.2174/1389200023337586

Zimmerli, B., Valantinas, J., & Meier, P. J. (1989). Multispecificity of Na+-dependent taurocholate uptake in basolateral (sinusoidal) rat liver plasma membrane vesicles. *Journal of Pharmacology and Experimental Therapeutics*, *250*(1), 301–308.