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INFLUENCE OF DIABETES ON CYTOCHROME P450 ENZYME MEDIATED DRUG METABOLISM – CASE STUDIES ON DICLOFENAC AND K-48

Ph.D. Thesis

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ABBREVIATIONS

ADME	adsorption, distribution, metabolism, elimination
ADP	adenosine diphosphate
AGGTCA	adenine-guanine-guanine-thymine-cytosine-adenine
AhR	aryl hydrocarbon receptor
Ala	alanine (amino acid)
Amu	atomic mass unit
Arg	arginine (amino acid)
Arnt	aryl hydrocarbon receptor nuclear translocator
Asn	asparagine (amino acid)
ATP	adenosine triphosphate
AUC	area under the plasma concentration-time curve
BBB	blood brain barrier
BZY	benzydamine
BZYNO	benzidamine N-oxide
Ca^{2+}	calcium ion
cAMP	cyclic adenosine monophosphate
CAR	constitutive androstane receptor
cDNA	complementary deoxyribonucleic acid
CL	clearance
CL_{int}	intrinsic clearance
CL_{nr}	nonrenal clearance
CL_r	renal clearance
CSF	cerebrospinal fluide
Ct	threshold cycle
CYP	cytochrome P450
D55	55 mg/kg streptozotocin treated diabetic rats
D70	70 mg/kg streptozotocin treated diabetic rats
DAB	3,3'-diaminobenzidine
DNA	deoxyribonucleic acid
EDTA	ethylenediamine-tetraacetic acid
FAD	flavin adenine dinucleotide
FMO	flavin-containing monooxygenase
FXRα	farnesoid x receptor
GADPH	glyceraldehyde-3-phosphate dehydrogenase
Gly	glycine (amino acid)
GR	glucocorticoid receptor
GRE	glucocorticoid response element
HNF-1α	hepatic nuclear factor – 1α
HNF-4α	hepatic nuclear factor – 4α
HPLC	high performance liquid chromatography
HPLC-ECD	high performance liquid chromatography with electrochemical detector
HPLC-MS	high performance liquid chromatography with mass spectrometry
i.m.	intramuscular
i.p.	intraperitoneal
ID55	insulin treated 55 mg/kg streptozotocin induced diabetic rats

ID70	insulin treated 70 mg/kg streptozotocin induced diabetic rats
IDDM	insulin-dependent diabetes mellitus
K_M	Michaelis constant
LXRα	liver X receptor α
Lys	lysine (amino acid)
MGB	minor groove binder
mRNA	messenger ribonucleic acid
NADPH	nicotinamide adenine dinucleotide phosphate, reduced
NIDDM	non-insulin-dependent diabetes mellitus
NSAID	non-steroidal anti-inflammatory drug
OP	organophosphonates
p.o.	per os
P450	cytochrome P450
PACER	pyridinium aldoxime cholinesterase reactivator
PAH	polycyclic aromatic hydrocarbon
PCN	pregnenolon 16-carbonitrile
PKA	cAMP dependent protein kinase A
PMSF	phenylmethanesulphonyl fluoride
PPARα	peroxisome proliferator activated receptor α
PXR	pregnane X receptor
RAR	retinoic acid receptor
RNA	ribonucleic acid
RQ	relative quantitation
RT	retention time
RXRα	retinoid X receptor α
S.D.	standard deviation
SIM	single ion monitoring
STZ	streptozotocin
TBS	Tris buffer solution
TCA	trichloroacetic acid
TCDD	2,3,7,8-tetrachlorodibenzo –*p*-dioxin
TRIS	tris hydroxymethyl aminomethane
Tyr	tyrosine (amino acid)
VDR	vitamin D receptor
V_{max}	maximum velocity
Vss	volume of distribution at steady state
YY1	zinc finger protein

1. INTRODUCTION

In the process of drug candidate screening and development *in vitro* drug metabolism studies are aimed at the evaluation of the potential for metabolism based drug interactions. The bioavailability of orally administered drugs is determined mainly by the first pass effect of the liver; however, the barrier function of the small intestine is also essential. Any alteration in the metabolising capacity of the liver and intestine may increase the incidence of altered biotransformation of drugs. Sex, age, nutrition, endogenous compounds (e.g., hormones), genetic polymorphism and other xenobiotics have effects on the metabolism. Furthermore, the adverse effect of drugs can also be caused by altered hepatic or intestinal P450 mediated drug metabolism in pathological conditions.[1] Diabetes mellitus is a complex metabolic disorder which produces serious complications during the course of the disease and also changes in the drug metabolising enzyme expression and activity of the intestine and the liver.[2,3,4,5,6,7,8,9,10] The altered biotransformation capacity of diabetic intestine or liver due to inhibition, inactivation or induction of the enzymes can result in altered drug exposures. Therefore, studying both intestinal and hepatic drug metabolising systems in diabetes is of great importance.

The altered biotransformation and pharmacokinetic of drugs in diabetes have not been investigated in dept. Nowadays, when the drug therapy endeavours to adjust to the individual needs, addressing the genetic polymorphism and phenotypical differences, it is surprising that the potential effect of diabetes on drug bioavailability is somewhat neglected, especially given the fact that such individuals often receive multiple and lifelong therapies. This study concentrates on the metabolism of diclofenac which is a clinically used NSAID drug and on K-48 which is a promising antidote on the basis of preclinical studies in organophosphate intoxication.

1.1. DRUG METABOLISM

Xenobiotics entering into the living organism are usually lipophilic. This property facilitates their transport through the membranes of high lipid content. Lipophilicity, however, is not an advantage from the aspect of elimination; therefore,

the organism transforms foreign compounds by enzymatic reactions to increase their polarity, and water solubility. The pharmacological activity of the drugs is usually changed as a consequence of biotransformation, although this does not necessarily mean a reduced efficacy. Metabolism may potentially lead to either drug activation, or inactivation, and it may also be responsible for the development of toxic side effects. Irrespective of the pharmacological outcome, metabolism makes drugs usually more hydrophilic.[11,12,13,14]

Metabolic enzymes can be classified in two main groups: the enzymes catalysing phase I reactions generally participate in oxidative transformations, mostly hydroxylation and dealkylation, however, they may catalyse reduction and hydrolysis, too. The pharmacological activity of compounds usually decreases but it can also increase or remain unchanged in phase I reactions. Phase II enzymes catalyse conjugation reactions characterized by the coupling of an endogenous, polar small molecule (glucuronic acid, sulphate, glutathione, etc.) to the functional groups or moieties already present in the parent compound or created in phase I reactions. The majority of phase II reactions lead to reduced pharmacological activity.[12,14] It may also happen that the parent drug is eliminated, without being metabolised, via excretion in an unchanged form.

In addition to the classical phase I and II reactions, phase III processes can also be mentioned. These are transport processes with the involvement of carrier proteins transporting both endogenous and exogenous compounds and playing a decisive role in the ADME profile of drugs. Although in the sense of the classical definition transport processes do not qualify as metabolic reactions, yet on account of their importance comparable to those of the other two phases, they are often referred to as phase III processes in the literature.[13]

The drugs entering the human organism are primarily metabolised in the liver. The greatest variety and amount of metabolic enzymes are localised here. Nevertheless, metabolising enzymes can be found in other organs, as well. The organs to be mentioned in this respect are mainly those through which xenobiotics may enter the organism („gate organs”): the gastrointestinal tract, the lungs and the skin and mucous membranes. Certain other organs such as the kidneys, the brain, the pancreas, the spleen, the adrenals, the testis and the ovary are also known to contain drug

metabolising enzymes; even the placenta has the means needed for the biotransformations of xenobiotics.[15] It is common for orally administered drugs to undergo biotransformation in the gastrointestinal tract and in the liver before they can reach the systemic circulation. This phenomenon is termed first-pass effect, and it may play a decisive role with respect to the systemic bioavailability of orally applied medicinal products.[13] The most obvious evidence for the extrahepatic metabolism of a drug is the systemic clearance value exceeding the hepatic blood flow.[16]

1.2. THE CYTOCHROME P450 ENZYME SYSTEM

The first report on cytochrome P450 was published in 1958.[17] It was not referred to as such name, however, until 1962, when Omura and Sato made the observation that following reduction and binding of carbon-monoxide to the CYP pigment result in a complex displaying an absorption maximum at 450 nm.[18] At that time cytochrome P450 was regarded as a single enzyme, however, it has become clear by now that cytochrome P450 is a superfamily of enzymes with more than 200 family members.

Structure of cytochrome P450s

Cytochrome P450 enzymes are hemoproteins, more accurately, they belong to the family of hemothiolate enzymes. They can be found in every kingdom of the living world, in bacteria, in fungi, in plants and in animals. P450 occurs in a great variety of mammalian tissues and cells, specifically, in the subcellular membranes including the endoplasmic reticulum, the nuclear membrane and the inner membrane of mitochondria.

CYP enzymes are membrane-bound proteins. Solubilisation of these enzymes (e.g. by surfactants) destabilizes the protein and may lead to complete inactivation. High glycerol concentrations can protect cytochrome P450 enzymes against the effects of surfactants.[19]

Concerning their structure, CYP enzymes are built from a heme moiety and a globular protein. The binding of these enzymes to the membrane is ensured by a sequence from 20-25 amino acids at the N-terminal of the protein. Heme is located between two long α-helices in the inner part of the protein lined with hydrophobic amino acids. This highly hydrophobic inner pocket is appropriate to receive

hydrophobic substrates. The 5th ligand of the heme is a thiolate anion originating from cysteine and absorption maximum of the CO-reduced P450 complex at 450 nm is associated with this anion. Structural derangement in the P450 enzymes, e.g. the replacement of the thiolate by an imidazole group from histidine, or changes in heme vicinity leads to a shift in the absorption maximum to 420 nm.[20,21] These alterations are associated with the inactivation of P450 enzymes.

Classification, polymorphism and contribution

Cytochrome P450 enzymes are classified on the basis of the isoenzyme homology. Enzymes with higher than 40 % homology in their amino acid sequence belong to the same family (arabic numerals), in the case of higher than 55 % homology they belong to the same subfamily (capital letter). The isoenzymes of the same subfamily are again distinguished by arabic numeral (e.g. CYP1A1).[14,22]

The first three families of cytochrome P450 contribute to the biotransformation of xenobiotics (CYP1, CYP2, and CYP3). There are 18 isoenzymes that may play a role in the pharmacokinetics and interactions of drugs in humans. There are, however, just a few among them, which are of particular importance (CYP1A2, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1 and CYP3A4).[14,15,22] The enzymes of the other families are primarily involved in the transformation of endogenous substrates (steroid and bile acid biosynthesis, fatty acid and arachidonic acid metabolism). CYPs are generally sensitive to homeostatic changes (e.g. sexual maturation, age, pathological, pathophysiological changes) and adapt their function to the actual needs of the organism.[1,22]

Cytochrome P450 isoenzymes are characterized by inter-species, inter-individual and tissue specificity. The variations in the enzyme activity are often related to enzyme polymorphism. Certain enzymes with a minor overall contribution to drug metabolism may assume greater importance on account of enzyme polymorphism (e.g. the amount of CYP2D6 is less than 2.5 % in the human liver, but its contribution to drug metabolism is almost 20 %).[15] The contribution of various isoenzymes to drug metabolism is demonstrated in Table 1.

CYP	*Relative amount of cytochrome P450 in the liver (%)*	*Contribution to drug metabolism (%)*
1A1	<1	2.5
1A2	~13	8.2
1B1	<1	n.d.
2A6	~4	2.5
2B6	<1	3.4
2C8, 2C9	~18	15.8
2C18, 2C19	~1	8.3
2D6	≤2,5	18.8
2E1	≤7	4.1
2F1	≤1	~1.3
3A4, 3A5	≤28	34.1
4A11	~14	n.d.

Table 1.
Relative amount of the isoenzymes in human liver and their percentile contribution to the metabolism of drugs (approximate values); n.d. = no data.[15]

The main isoenzymes contributing to the drug metabolism in human liver are the members of CYP3A and CYP2C subfamilies. The role of these two enzyme subfamilies in drug biotransformation is more than 50 %. The extrahepatic expression level of CYPs is different -usually lower - than that of the liver, moreover certain isoenzymes are dominantly expressed in certain tissues but may not or only hardly detectable in others.[16] For instance, CYP2C is the dominant rat liver isoform while it is hardly detectable in rat small intestine. In human liver and intestine the CYP3A is mainly expressed and it is also presenting in rat intestine.[23]

Function of cytochrome P450

The cytochrome P450 enzyme system is responsible for the metabolism of a wide range of endogenous and exogenous substrates. The isoenzymes have overlapping substrate specificity. The members of certain CYP families are specific to chemical structures. Hence, the structurally similar drugs can metabolise on the same isoenzyme leading to drug-drug interactions in many cases.[14,15]

The most important function of the cytochrome P450 enzymes is monooxygenation that requires molecular oxygen and a reducing agent (NADPH, NADH). Eukaryotic cytochrome P450 enzymes (either mitochondrial or microsomal) use NADPH as an electron donor. In the microsomal metabolising system there are two standard components: the membrane-bound cytochrome P450 and the NADPH-cytochrome P450 reductase. The catalytic cycle of P450 is presented in Figure 1.

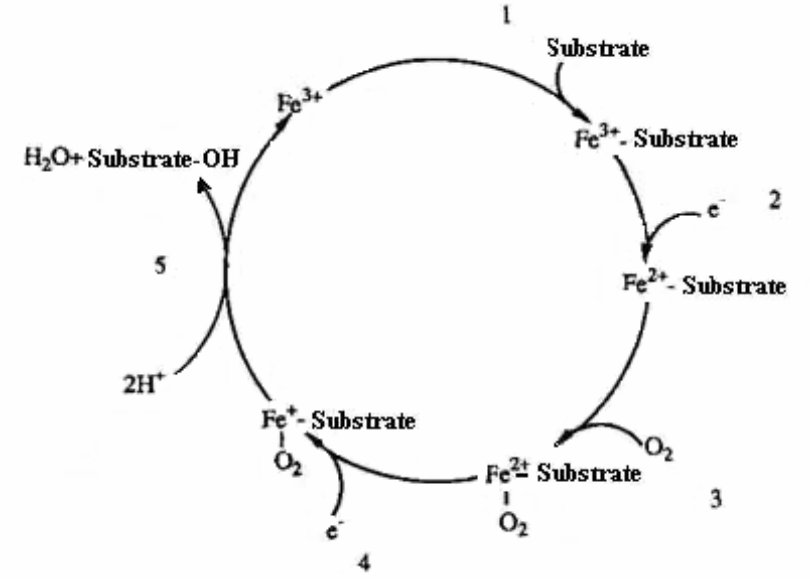

Figure 1.
Catalytic cycle of cytochrome P450.[15]

The first step of the catalytic cycle is the binding of the substrate. The Fe^{3+} form of the heme is then reduced to Fe^{2+} form by the electron received from NADPH (Step 2.). The electron transport is performed by NADPH-cytochrome P450 reductase. The reduced iron in the heme can bind molecular oxygen (Step 3.) that is transformed into an activated state through the uptake of another electron (Step 4.) and one proton. The activated oxygen can then oxidise the substrate. Only one oxygen atom reacts with the substrate, the other oxygen atom leaves the cycle as water following the uptake of another proton (Step 5.). The oxidized substrate will then dissociate from the enzyme, which returns to its initial state and becomes ready to bind another substrate molecule.[15,19]

In the course of the cycle, reactive oxygen radicals such as superoxide anion and hydrogen peroxide are formed. In a complete cycle these free radicals facilitate the

transformation of the substrate, however, if the cycle is interrupted at any stage, the reactive oxygen species may accumulate and may lead to cyto- and genotoxicity.[14,15,19]

More than half of the drugs used in clinical practice is metabolised by the cytochrome P450 enzyme system[24], therefore it is important to be aware of the factors that influence the functions of this enzyme system.

1.2.1. Regulation of the cytochrome P450 enzyme system

Many CYP isoforms are regulated by endogenous (e.g. gonadal hormones, pituitary hormones, transcription factors) and exogenous molecules (xenobiotics).[25] P450s can be regulated at transcriptional, posttranscriptional, translational or posttranslational levels. Transcriptional regulation by nuclear receptors, posttranscriptional regulation by mRNA stabilization and posttranslational regulation by enzyme stabilization or phosphorylation of the isoenzymes are common.[24,25] Regulation of cytochrome P450 at different stages is presented in Table 2. Due to the regulation, the amount and activity of enzymes can be increased by de novo enzyme synthesis or can be decreased by down-regulation, inhibition or destruction. Alteration in endogenous hormone and transcription factor levels induced by pathological conditions (diabetes, hypertonia) can influence the P450 enzymes.[1]

Mechanism of induction	*CYPs known to be regulated at specific stages*
Gene transcription	CYP1A1, CYP1A2, CYP1B1, CYP1B2, CYP2B2, CYP2C7, CYP2C11, CYP2C12, CYP2D9, CYP2E1, CYP3A1, CYP3A2, CYP3A6, CYP4A1
mRNA stabilization	CYP1A1, CYP2B1, CYP2B2, CYP2C12, CYP2E1, CYP3A1, CYP3A2, CYP3A6
Enzyme stabilization	CYP2E1, CYP3A1, CYP3A2, CYP3A6

Table 2.
Regulation of cytochrome P450 isoenzymes.[15]

Transcriptional regulation is carried out by nuclear receptors. The nuclear receptor superfamily constitutes a group of ligand-dependent DNA binding transcription factors.

They have at least four specific functions: ligand binding, nuclear translocation, DNA binding and activation or repression of gene transcription.[26] They all have a similar modular structure in which various domains are involved in the receptor function: the ligand-independent AF-1 transactivation domain is located in the N-terminal part of the protein, the DNA binding domain consists of two highly conserved zinc finger motifs, the dimerization domain and finally the AF-2 ligand-dependent transactivation domain.[26] The nuclear receptors regulating P450s are active either as homodimers (GR) or as heterodimers (CAR, PXR). The inducing agents can bind to the receptors directly (PXR, AhR) or indirectly, by signal transduction (CAR).[27,28] The nuclear receptors are originally found in cytoplasm, translocate to the nucleus following ligand activation and bind to the AGGTCA sequence in the promoter of the DNA inducing gene transcription.[28] The dominant receptors involved in the xenobiotics metabolising CYP regulation and their targets are summarized in Table 3.

Nuclear Receptors	*Functional structure*	*Regulated P450 enzymes*	*Inducers/ prototypes*
AhR	AhR-Arnt heterodimer	**CYP1A1**, CYP1A2, CYP1B1	PAH, TCDD
CAR	CAR-RXRα heterodimer	**CYP2B6**, CYP3A4, CYP2C9, CYP2C19	phenobarbital
RAR		CYP2C	retinoic acid
PXR	PXR-RXRα heterodimer	**CYP3A4, CYP3A7** CYP2B6, CYP2C9	PCN (rat), dexamethasone (rat), rifampicin (human),Hyperforin
HNF-4α	homodimer	**CYP2A, CYP2C, CYP2D**	phenobarbital (CYP2A), not known (CYP2D)
PPARα	PPARα-RXRα heterodimer	**CYP 4A,** peroxisomal acetyl-CoA oxidase	fatty acids, fibrates
LXRα	LXRα-RXRα heterodimer	**CYP 7A**	oxisterols (in except for cholesterol)
FXRα	FXRα-RXRα heterodimer	**CYP 7A**	bile acids, xenobiotics
GR	GR-GR homodimer	**CYP 2C,** CYP 3A, CYP 2B	glucocorticoids
VDR	VDR-RXRα heterodimer	CYP 3A, CYP 2B6, CYP 2C9	D-vitamin,bile acids

Table 3.
Nuclear receptors involved in the regulation of cytochrome P450 enzyme system and their main targets and inducers.[14,26,27,28,29]

Besides the long-term regulation of foreign compound metabolising enzymes by induction, a short-term regulation by posttranslational modifications of the proteins also exists.[24,25,30,31,32] The posttranslational modifications can occur via the processes of phosphorylation, acetylation, glycosylation or deamidation.[25] The regulation of cytochrome P450 by phosphorylation is supported by the fact that CYPs are substrates for protein kinases. In addition, the cytochrome P450 and cAMP dependent protein kinase A (PKA) are localized in the same subcellular fraction (microsomes) in intact cells.[33] Generally the PKA phosphorylates the adequate amino acid (serine, threonin or tyrosine), but the protein kinase C and the Ca^{2+}/Calmodulin dependent protein kinase II are also suitable for their modulation.[25,31] The PKA recognizes the Arg-Arg-X-Ser sequence where the serine is usually at 128 or at 129 positions.[34] The recognition sequence is conserved in CYP2 enzyme family members as in CYP2B1/2, CYP2A1, CYP2C11, CYP2D1/2, and CYP2E1 in rats; [25,35] and e.g. CYP2B6, CYP2E1 in humans[33] and CYP2B4 in rabbits.[34] The presence of this recognition sequence is also not always sufficient for phosphorylation to occur (e.g. CYP2C11) and isoenzymes involving threonin, in spite of serine (e.g. CYP2A2, CYP2C6, CYP2C7, CYP2C13 and CYP2C12 in rats) can not be phosphorylated by PKA.[25,32,35] However, it also has been published that the mutation of Ser^{129} in rat CYP2E1 to Ala or Gly brings about the PKA-mediated phosphorylation at another site of CYP2E1 (Lys-Lys-Ser^{209}-Tyr and Lys-Lys-Ser^{449}-Ala) or in another cooperating molecule and that leads to an opposite effect (increase in catalytic activity) as compared with its effect on wild-type CYP2E1.[33,36]

The activity of PKA depends on the level of cellular cAMP. This in turn depends on the concentration of hormones (e.g. glucagon, insulin, adrenalin, pituitary hormones and thyroid hormones), on diseases (e.g. diabetes, hypertonia) and on the presence and concentration of some drugs (e.g. adenylate cyclase stimulators, phosphodiesterase inhibitors).[25,31]

The CYP2E1 isoenzyme is mostly regulated at the posttranscriptional level. Inducing agents (ethanol, isoniazid, pyridine, pyrazole, imidazole etc.), starvation, chronic alcohol consumption or diabetes can increase the enzyme activity without enhanced gene transcription.[24] The CYP2E1 protein level increases but mRNA remains unchanged.[37] The enzyme stabilization is also based on a phosphorylational mechanism.

The phosphorylation of CYP2B and CYP2E1 results in a decrease of their catalytic activities.[30,32,35] The phosphorylation of CYP2E1 at the Ser^{129} position but not CYP2B1 is followed by heme loss and degradation by proteases.[24,38] However, Oesch-Bartolomowich and co-workers show that the cAMP-mediated phosphorylation of CYP2E1 leads to loss of activity without enzyme protein degradation.[39]

1.2.2. Intestinal cytochrome P450 enzyme system

The physiological conditions of the gastrointestinal tract (the presence or absence of food, biliary excretion etc.), the pathological alterations of the small intestine and the first-pass metabolism can all lead to the poor bioavailability of an orally administered drug.[40] Owing to its huge surface area and very good blood supply, the small intestine is the primary organ of drug absorption. Nevertheless, the small intestine may have an important role in the first pass metabolism of certain orally administered drugs,[41] including midazolam,[42] nifedipine,[43] tacrolimus,[44] sirolimus,[45] cyclosporine,[46] verapamil,[47] etc. and resulting in a reduced oral bioavailability. Furthermore, many prodrugs require the action of these metabolising enzymes pointing out to a substantial role of intestinal drug metabolism. Although the clinical importance of intestinal metabolism is a matter of debate nowadays, it is unquestionable that the small intestine can also be responsible for the inter- and intra-individual variation in the bioavailability of intensively metabolising drugs. It is worth mentioning that such variations may arise from the variations in the metabolic processes and from the variations in the function of the efflux transport proteins (e.g. p-glycoprotein) located in the intestinal wall, or from the combination of both. Owing to these two mechanisms, the small intestine forms not only the organ of absorption but it is also endowed with some barrier properties.[40]

The intestinal metabolism depends on the intracellular residence time of the drug molecule and on the catalytic activity of the enzymes present in the cell. Nevertheless, due to the anatomical structure of the capillary network of intestinal villi, it is also true, that the drugs entering into the systemic circulation directly and not through absorption, do not always reach the drug metabolising enzymes in the intestinal villi. The underlying mechanism is the free diffusion of drug molecules between the ascending

and descending capillaries, the so-called counter-current exchange that allows the free exchange of small molecules between the two branches of the capillaries.[48]

Distribution of intestinal P450s

The enzymes within the gut lumen come from two sources: mammalian (gastric, pancreatic and intestinal enzymes) and bacteria-associated enzymes, the latter of which are concentrated in the ileum and the colon.[16] Phase I and phase II drug metabolising enzymes and phase III transport proteins are expressed in the intestinal mucous membrane. The amount of drug metabolising enzymes is highest in the upper part of the small intestine i.e. in the duodenum and jejunum; however, cytochrome P450 activities do vary also along the longitudinal axis of intestinal villi. The cells in the apex of the villi are mature, that they are fully differentiated epithelial cells, while the crypt cells at the base are immature. The enzyme activity in the intestinal crypt cells is hardly measurable or completely absent.[49] On the other hand, maximum enzyme activities can be detected in the apex of the intestinal villi. The epithelial cells are densely covered by the intestinal microvilli. The turnover of the microvilli is 2 to 6 days in humans (2 days in rats); the cells detached from the surface leave the body by the means of the stools.[48,50] Presumably, the continuous renewal of cytochrome P450-containing cells plays an important role in protection against toxins.[50]

Nearly all hepatic drug metabolising enzymes can be found in the intestinal epithelium, too. As in the liver, the biotransformation of drugs is primarily catalysed by the cytochrome P450 enzymes in the small intestine. The expression level of cytochrome P450 enzymes is much lower in the extrahepatic organs than in the liver. For instance, the expression level of CYP3A isoenzymes which are the most abundant enzymes in the human small intestine is roughly 30 times lower than that in the human liver.[48] The isoenzymes of other CYP families are also present, though they are much less abundant.[16] The distribution of cytochrome P450 isoenzymes in human small intestine is shown in Figure 2.

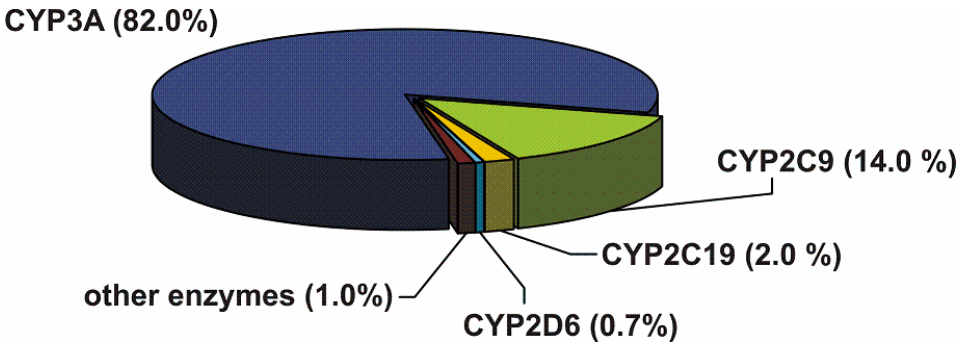

Figure 2.
Relative abundance of cytochrome P450 isoenzymes in human small intestine.[51]

Induction of intestinal P450s

The cytochrome P450 enzymes of the small intestine are also inducible; however, their induction differs from that of the liver enzymes.[48] The well-known inducers such as 3-methylcholantrane, ß-naphtoflavone, phenobarbital and dexamethasone also increase the activity and mRNA expression of the CYP1A1 (phenacetin- O-deethylation), CYP2B1/2 (7-ethoxycoumarin O-deethylation) and CYP3A1/2 (erythromycin N-demethylation) in rat intestine, respectively. [50,52,53,54] In the case of low oral doses the induction is significantly higher in the small intestine than in the liver.[55] The intestinal metabolism enhanced via induction relieves the liver to some extent from the burden of too many drugs as it prevents a portion of the drug molecules from reaching the liver. In the case of higher doses, the enzyme induction is much more pronounced in the liver than in the intestine. Furthermore, recent studies show that intestinal CYP3A cannot be regulated by PXR agonists; however, PXR is the main nuclear receptor regulating the CYP3A isoenzymes in the liver.[56] These facts can be taken as evidence for the independent regulation of the cytochrome P450 enzyme system in the liver and that in the small intestine.[48,56,57]

At low inducer doses the induction does not only depend on the dosage but also on the route of the administration. While high doses of intravenously administered inducer does not only induce the cytochrome P450 enzymes system in the liver but also in the small intestine. In other words, in the case of high enough doses, the induction of intestinal metabolising enzymes is independent of the route of administration.[48]

Although the consensus on the importance of intestinal metabolism is yet to come, the aforementioned processes clearly indicate that the epithelium of the small intestine can undoubtedly serve as the site of drug interactions. In addition to the classical induction- or inhibition-based drug interactions, gastrointestinal diseases, alterations of gastrointestinal motility and food supplements can all influence the efficacy of orally administered drugs.[49] Moreover, through compensating for the reduced hepatic metabolism, intestinal metabolism may assume a special role in diseases.[16,41]

1.3. FLAVIN-CONTAINING MONOOXYGENASE ENZYMES

Flavin-containing monooxygenase (FMO) family is a non-heme containing enzyme system involved in drug metabolism. FMO enzymes catalyse the oxygenation of numerous nucleophilic heteroatom-containing (i.e. nitrogen, sulphur, selenium, phosphorus) xenobiotics.[58] Flavin monooxygenase and cytochrome P450 have similar tissue, cellular and organelle expression.[59] They have overlapping substrate specificity and both FMO and CYP require NADPH and O_2 as cofactors to their function.[60,61] However, the mechanism of reactions catalysed by FMO is different from that of CYP-mediated metabolism. In contrast to CYP, FMO binds the substrate only when its FAD component has been already reduced by the two electrons received from NADPH and the reduced flavin reacted with molecular oxygen to form 4α-hydroxyperoxyflavin. The FMO oxygenates soft nucleophilic xenobiotics which shape, size and charge permit its access to the well-protected substrate binding channel. Oxygenation proceeds with the attack of nucleophilic heteroatom on the terminal hydroperoxy flavin oxygen atom to produce the product.[60,62]

The FMO class of monooxygenases has unique properties such as relative thermal lability,[60] pH dependency (the optimal pH for FMO enzyme function varies among species, but it is between pH 8-10),[63] detergent dependency (sodium cholate and fatty acids inhibit FMO)[64]and they generate relatively stabile hydroperoxy flavin intermediates.[60,65]

There are currently eleven FMO genes, but only the first five are functionally active the others are pseudogenes.[66,67] The FMO1 and FMO3 are currently thought to

play an important role in drug metabolism. Both have broad substrate specificity and high expression levels in most tissues. In one hand, the FMO takes part in the detoxication of xenobiotics such as synthetic therapeutic drugs and natural products in the diet like alkaloids. The other hand, FMO enzyme family has been implicated in the bioactivation of several xenobiotics resulted in metabolites with greater electrophilicity. FMOs play also important role in the biotransformation of numerous endogenous substrates. The nitrogen-containing biogen amines (e.g. phenethylamine, tyramine), the trimethylamine, the sulphur-containing cysteamine, disulfide lipoic acid, methionine, S-farnezylcysteine etc. can transform into their N- or S-oxides by FMO.[62,63,65]

FMO isoforms exhibit tissue-, species-, gender-, age- and substrate-specificity. Regarding to species differences FMO1 is the major hepatic isoenzyme in experimental animals while FMO3 is mainly expressed in human livers.[59] It is also shown that the expression of FMO3 is affected by the gender. In mice and rats a much greater FMO3 activity was observed with females than with males since testosterone repress and oestradiol increase the expression of FMO1 and FMO3 in mouse. Moreover, no gender effect was observed for humans, dogs and rabbits.[68,69]

FMO is regulated at transcriptional and posttranscriptional level. HNF-1α, HNF-4α and YY1 regulatory domains are found in the promoter of rabbit FMO1. HNF-1α and HNF-4α were demonstrated to enhance while YY1 suppressed the FMO1 promoter activity. These regulatory elements are also conserved in human FMO1. The posttranscriptional regulatory effect could be the N-glycolisation of FMO1 on Asn (asparagine) amino acid at the 120 position. It is also shown that NO appears to modify FMO3. The number of mechanisms to regulate CYP including enhancement of mRNA stability, enzyme phosphorylation and protein-protein interactions either do not occur for FMO or have not been reported.[70] Nevertheless FMO are under dietary-, developmental-, hormonal- (steroids, growth hormones) and genetic control and their expression and activity alter in pathophysiological status such as pregnancy, starvation, ascorbic acid deficiency, gonadectomy and diabetes.[71,72,73,74,75]

Our study primarily focused on the changes of cytochrome P450 enzyme system in streptozotocin induced diabetes. The expression and activity of FMO enzymes were determined to gain an overall picture about the metabolic changes in diabetes.

1.4. IN VITRO STUDIES TO ASSESS THE FUNCTION OF CYPs

The application of *in vitro* methods for the prediction of the pharmacokinetic and metabolic properties of drugs is a widely used approach in the pharmaceutical industry. The needs of industry concerning the development of the safest possible drugs at the lowest possible cost represented a great impetus to the development of such techniques. The use of *in vitro* methods is a cost-effective approach particularly in the early stages of research and development.

The use of biological models has a long established tradition in pharmacology; however, the models used for the elucidation of the ADME profile of drug candidates have a more recent history. Nevertheless, owing to the numerous advantages they offer over *in vivo* experimentation, *in vitro* methods became wide-spread rapidly.[14] *In vitro* techniques are much less demanding in terms of the amount of test substance and human resources than *in vivo* studies. The reduction of the utilization of experimental animals by *in vitro* methods is not only cost-saving but it is advantageous also from an ethical point of view. *In vitro* methods provide a possibility for the use of human cells and subcellular fractions in the early phases of research and development.[76,77] In view of the known inter-species differences in drug metabolism, the use of human tissues in *in vitro* experiments is of special importance. The *in vitro* models are also supplying pools of subcellular fractions from several animals or individuals which give a chance to circumvent the problem of inter-individual variations and to obtain a population mean directly.

There are a great number of *in vitro* techniques that can be used to elucidate the metabolism of a drug. In metabolism studies, the biological sample can be microsomes, S9 fraction, hepatocytes, precision-cut organ slices, recombinant enzyme systems, or perfused liver.[14] Microsomal fractions are frequently used systems. Microsomes are subcellular fraction consisting of spherical particles generated from disrupted, ultracentrifuged and resuspended endoplasmic reticulum.[78] Easy preparation and the possibility of long-term storage at –80°C without a significant loss of enzyme activity make microsomes especially advantageous. Owing to the role of endoplasmic reticulum in protein synthesis, the microsomal fraction contains a great variety of proteins, even metabolising enzymes. Both phase I and some phase II enzymes are present in this

subcellular fraction, nevertheless microsomes are most suitable for the investigation of cytochrome P450 enzyme system. Liver microsomal fraction characterized by containing the highest level of metabolising enzymes is the most frequently used biological sample in microsomal metabolism studies. As a prerequisite in the *in vitro* study of metabolism, optimal physiological conditions (ionic strength, pH, NADPH as an electron donor for CYPs, FMOs etc., oxygen of the air) have to be provided for the functioning of microsomal enzymes**.**

Microsomal fractions can be used to explore the metabolic pathways and profile of a drug candidate, to determine the first-pass metabolism and metabolic stability of new chemicals or to study the underlying mechanisms of drug-drug interactions through the elucidation of inhibition and induction processes. *In vitro* metabolism studies with probe substrates can successfully be used in the determination of the enzyme kinetic parameters (CL, V_{max}, K_M). Probe substrate reactions offer a means of investigating the overall picture of the metabolising properties of different tissues both under physiological and pathological conditions.

In *in vitro* metabolism studies, certain ADME parameters are usually neglected; therefore, one always has to keep the limitations of the model system in mind in order to avoid the over-interpretation of the results.[14]

1.5. CHANGES OF CYTOCHROME P450 FUNCTION IN DIABETES

1.5.1. Streptozotocin induced diabetes model

Hormonal changes associated with certain types of diseases such as diabetes or hypertension can have an effect on the amount and the activity of drug metabolising system.[79] There are a great number of publications concerning the effects of type I diabetes (insulin dependent diabetes mellitus, IDDM) on CYP enzymes,[2,3,4,5,6,7,8,9,10] nevertheless, and in spite of the similarity of reported results, little is known about the drug interactions that maybe induced by the pathological state.

In the experimental modelling of diabetes there are several chemicals known to provoke diabetic symptoms. Alloxan and streptozotocin (STZ) are the most frequently used and most suitable to elicit diabetes and to create an experimental model of the

disease.[80] Despite of the great number of results obtained with the alloxan model of diabetes and considering the low specificity of alloxan, STZ-induced diabetes as the model in our experiments was selected. The results obtained in the two models cannot be compared, since alloxan and STZ have different effects on the cytochrome P450 enzyme system, presumably due to the less specific effect of the former one.[6]

Streptozotocin (MW = 265 g/mol) is an N-nitroso derivative of D-glucoseamine. The chemical structure of streptozotocin can be seen in Figure 3.

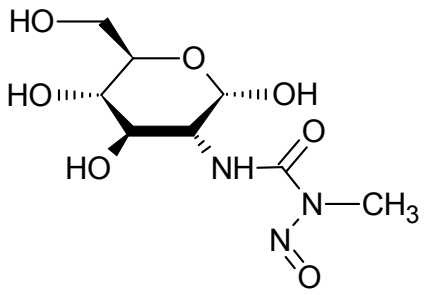

Figure 3.
Chemical structure of streptozotocin
(2-deoxi-2-(3-(methyl-3-nitrosoureido)-D-glucopyranose)) (Merck Index).

A single administration of streptozotocin damages the β-cells of the pancreatic Langerhans' islets specifically.[81] STZ enters the β-cells with the help of GLUT-2 transporter and it causes cell necrosis through DNA-alkylation. STZ is a nitrogen-monoxide donor, which represents another cytotoxic mechanism. Its cytotoxic effects equally prevail in rats, mice, rabbits, dogs, monkeys and humans.[82] STZ elicits hyperglycaemia due to insulin deficiency i.e. it provokes insulin dependent, type I diabetes mellitus (IDDM). It increases the activity of mitochondrial xanthine-oxidase, the enzyme catalysing the formation of superoxide anion, thus, those of hydrogen-peroxide and hydroxyl radicals, which in turn facilitate cellular necrosis. STZ is an inhibitor of the Krebs' cycle, whereby it reduces mitochondrial oxygen utilisation, thus, restricts ATP-production up to the complete depletion of ATP in the β-cells. As an additional mechanism, the DNA damage by STZ activates the poly-(ADP-ribosylase) enzyme that reduces the cellular NAD^+ content, which contributes to the depletion of

ATP.[81] Because of the effect of STZ, insulin production and secretion cease with a parallel increase of the hepatic glucose production, reduction of glucose utilisation, and decrease of the activity of enzymes involved in lipogenesis and gluconeogenesis.[5]

1.5.2. Effect of streptozotocin induced diabetes and insulin treatment on the hepatic monooxygenase enzymes

It is generally accepted that the alterations of the cytochrome P450 enzyme system in diabetes are due to the metabolic and hormonal alterations associated with the disease.[79]

The total cytochrome P450 content of rat liver is increased. The protein level of CYP1A2, CYP2A1, CYP2B1, CYP2C6, CYP2C7, CYP3A1, CYP3A2, CYP4A2, CYP4A3 and CYP2E1 isoenzymes are elevated in STZ-induced diabetes.[4,7,79] The protein expression of CYP4A1 enzyme is enhanced by some authors, and unchanged by others, whereas the expressions of CYP2C11, CYP2C13 and CYP2A2 are decreased.[4,7,79] In general, insulin substitution fully complements the diabetic alteration of gene expression, except in the case of CYP2C11, where insulin has only partial effect. The alterations described in the literature are summarized in Table 4.

Isoenzyme	*Alterations in diabetes*	*Effects of insulin substitution*	*References*
total cytochrome P450 content	induction	compensated induction	Shimojo et al. (1993) Reinke et al. (1978) Vega et al. (1993)
CYP1A2	induction	compensated induction	Sakuma et al. (2001)
CYP2A1	induction	compensated induction	Shimojo et al. (1993)
CYP2A2	inhibition	compensated inhibition	Sakuma et al. (2001)
CY 2B1	induction	compensated induction	Sakuma et al (2001)
CYP2C6	induction	compensated induction	Shimojo et al. (1993)
CYP2C7	induction	compensated induction	Favreau et al. (1988) Shimojo et al. (1993)
CYP2C11	inhibition	partially compensated inhibition	Favreau et al. (1988) Shimojo et al. (1993) Sakuma et al. (2001)
CYP2C13	inhibition	compensated inhibition	Favreau et al. (1988) Shimojo et al. (1993)
CYP2E1	induction	compensated induction	Favreau et al. (1988) Shimojo et al. (1993) Sakuma et al (2001)
CYP3A1	induction	compensated induction	Sakuma et al. (2001)
CYP3A2	induction	compensated induction	Shimojo et al. (1993) Sakuma et al. (2001)
CYP4A1	induction / no change	compensated induction / no change	Sakuma et al. (2001)/ Shimojo et al. (1993)
CYP4A2	induction	compensated induction	Shimojo et al. (1993) Sakuma et al. (2001)
CYP4A3	induction	compensated induction	Shimojo et al. (1993) Sakuma et al. (2001)

Table 4.
Alterations of cytochrome P450 protein expression in STZ-induced diabetes with and without insulin-replacement in rats.[4,5,6,7,8,79]

Diabetes also causes alterations in the catalytic activity of enzymes. In STZ-induced experimental diabetes the activity of aniline hydroxylase (CYP2E1), 7-ethoxycoumarin O-dealkylase (CYP2A6), testosterone 2ß-, 6ß-hydroxylase, ethylmorphine- and erythromycin N-demethylase (CYP3A2), testosterone 7α-hydroxylase (CYP2A1), ethoxyresorufin O-deethylase (CYP1A) and laurylic acid ω-, and (ω-1)-hydroxylase (CYP4A2, CYP4A3) all increase. Following insulin administration the enzyme activities return to control levels, except in the case of ethoxycoumarin O-dealkylase. Testosterone 2α- and 16α-hydroxylation (CYP2C11) and aminopyrine N-demethylation (CYP2B, CYP3A) decrease in experimental diabetes and

insulin substitution restores the enzyme activities.[4,5,8,9,80,83] No significant alteration with respect to p-nitrophenol hydroxylase activity (CYP2E1) was published by Barnett et al. (1994) and Ackermann and Leibman (1977).[9,80] Although in other studies the most marked increase in CYP2E1 catalytic activity was described by 3-8-fold.[4,5,8,9] The alterations in the catalytic activity of the enzymes are summarized in Table 5.

Isoenzyme	*Alteration of catalytic activity in diabetes*	*Effects of insulin substitution*	*Reference*
ethoxyresorufin O-deethylase (CYP1A)	increased	compensated	Yamazoe et al. (1989)
testosterone 7α- hydroxylase (CYP2A1)	increased	compensated	Shimojo et al. (1993)
7-ethoxycoumarin O-dealkylase (CYP2A6)	increased	no change	Shimojo et al. (1993)
aminopyrine N-demethylase (CYP2B1)	decreased	compensated	Reinke et al. (1978)
testosterone 2α-hydroxylase (CYP2C11)	decreased	compensated	Shimojo et al. (1993) Vega et al. (1993)
testosterone 16α-hydroxylase (CYP2C11)	decreased	compensated	Shimojo et al. (1993)
aniline 4-hydroxylase (CYP2E1)	increased	compensated	Reinke et al. (1978) Shimojo et al. (1993)
p-nitrophenol hydroxylase (CYP2E1)	increased/ no change	no change	Ackermann et al. (1977) Barnett et al. (1994) / Reinke et al. (1978) Shimojo et al. (1993)
testosterone 2ß-hydroxylase (CYP3A2)	increased	compensated	Shimojo et al. (1993)
testosterone 6ß-hydroxylase (CYP3A2)	increased	compensated	Yamazoe et al. (1989) Shimojo et al. (1993)
ethylmorphine N-demethylase (CYP3A2)	increased	no data	Shimojo et al. (1994)
erythromycin N-demethylase (CYP3A2)	increased	no data	Shimojo et al. (1994)
laurilic acid ω-hydroxylase (CYP4A2)	increased	compensated	Shimojo et al. (1993)
laurilic acid (ω-1)-hydroxylase (CYP4A3)	increased	compensated	Shimojo et al. (1993)

Table 5.
Alterations of the catalytic activity of cytochrome P450 isoenzymes in insulin-treated and untreated rats with STZ-induced diabetes.[4,5,6,8,9,80,83]

The alteration of xenobiotic metabolism in diabetes has long been recognised. The regulation mechanisms behind this phenomenon are, however, still the subject of investigations. The altered protein expression may be related to the decreased levels of pituitary growth hormones (e.g. at CYP1A2, CYP2B, CYP2E1, CYP2C11 isoenzymes), to decreased testosterone (e.g. at CYP2A1, CYP2C12 isoenzymes) or thyroid hormone levels (e.g. in the case of CYP2A1, CYP2B enzymes).[79] In addition, the growth hormone levels are decreased in diabetes, and the increase of CYP3A1 expression and the decrease in CYP2C11 enzyme are presumably related to these changes.[84,85]

It is also known that the marked elevation in the activity of CYP2E1 isoenzyme in diabetes can be attributed to the increase of the enzyme stability by phosphorylation rather than to transcriptional regulation.[37] Diabetes leads to an increased level of ketone bodies which may also involved in the regulation of CYP2E1, however, pancreatic insulin has been shown to be a direct regulator in the posttranslational modification of rat CYP2E1 and CYP2B expression.[7,86]

The alteration of FMO activities was also observed in modified physiological states of animals such as diabetes.[71] An increased N-oxidation of imipramine was shown in diabetic mouse liver and elevated hepatic FMO activity accompanied by enhanced FMO mRNA level was demonstrated in diabetic rats.[87,88] In our previous short-term diabetic study (Borbás et al., 2006) it was observed that FMO activity increased in diabetic rats in a streptozotocin dose-dependent manner. Insulin restored the FMO activity to the control level, while insulin had no effect on non-diabetic animals. Based on these results, it was indicated that insulin is involved in the regulation of hepatic FMOs.

1.5.3. Effect of streptozotocin induced diabetes and insulin treatment on the intestinal cytochrome P450s

The effects of diabetes on the intestinal metabolism are much less represented in the literature than that of hepatic metabolism. In spite of the overall body weight loss, the animals' intestinal epithelium becomes thicker in diabetes. The increase of mucous membrane mass is particularly substantial in the proximal regions. In spite of this increase, however, no increase could be demonstrated in the mass of epithelial cells.[89]

Al-Turk and associates observed an increase in intestinal 7-ethoxycoumarin O-deethylase (CYP2A) and aromatic hydrocarbon hydroxylase (CYP1A) activity in male and female diabetic rats.[2,3] It was shown by the same authors that both ovariectomy and castration lead to alterations in the catalytic activity of both hepatic and intestinal cytochrome P450 enzyme systems. Regarding the small intestine, 7-ethoxycoumarin O-deethylase activity did not differ in castrated diabetic animals from those of sham-operated, diabetic control animals, indicating that hormonal regulation had no effect on P450 activity in this case. Aromatic hydrocarbon hydroxylase activity was found induced in both sexes of gonadectomised diabetic rats. Based on the results above, it appears that the catalytic activity of aromatic hydrocarbon hydroxylase is regulated by both the sex and the pancreatic hormones.[3]

1.6. OVERVIEW OF THE SUBSTRATES STUDIED

1.6.1. Metabolism of NSAID drug, Diclofenac

Diclofenac sodium is the sodium salt of o-[(2,6-dichlorophenyl) amino] phenylacetic acid (structure see below in Figure 4.).[90] Diclofenac, the nonsteroidal anti-inflammatory drug (NSAID) is widely used for the treatment of rheumatoid arthritis, osteoarthritis, ankylosing spondylitis and acute muscular pain conditions.[91] It can rarely cause severe hepatic injury. Although the mechanism of hepatotoxicity is still not clear, extensive studies have focused on biotransformation of diclofenac into chemically reactive metabolites produced by cytochrome P450 mediated metabolism.[92] Diclofenac is metabolised mainly into two phenolic metabolites, 4'-hydroxy diclofenac and 5-hydroxy diclofenac. The latter one is a reactive metabolite.[93] The main metabolic pathway of diclofenac is shown in Figure 4.

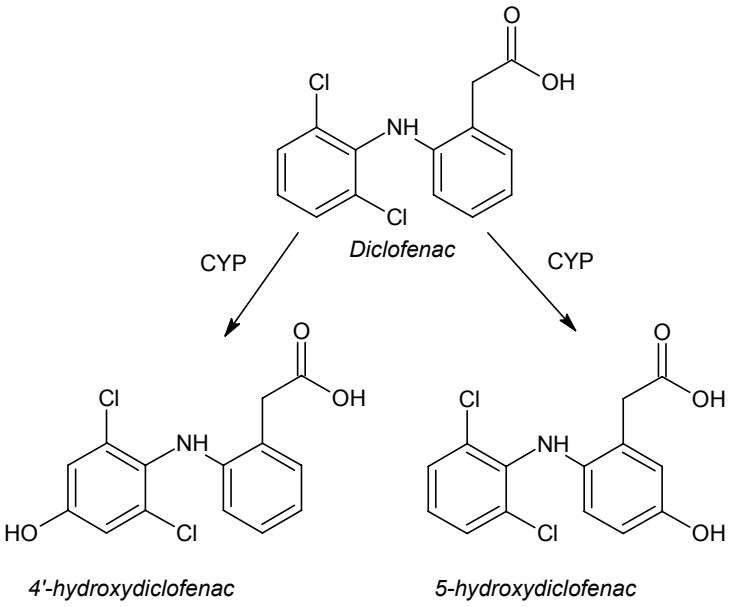

Figure 4.

Major pathway of the oxidative metabolism of diclofenac in rat liver microsomes.[93]

It has been demonstrated that the diclofenac metabolising enzyme, CYP2C11 is one of the microsomal target proteins of the covalent binding of the reactive metabolite and the enzyme is inactivated selectively in a mechanism-based manner in rats.[93,94] No evidence is available to show, that mechanism-based inactivation of human CYP2C9 occurs, which is the major human CYP isozyme responsible for diclofenac metabolism.[93,95]

The CYP2B, CYP2C, CYP3A enzymes are involved in the diclofenac metabolism in rats by 13 %, 66 % and 23 %, respectively. The CYP2C6 and male-specific CYP2C11 are the most dominant isoenzymes.[93,94,96,97,98] Conjugation reactions take place at the carboxyl group of diclofenac with glucuronic acid and taurin (in dogs) as ligands. The route of metabolic excretion is predominantly the renal route in humans and biliary route in rats.[90,97] Therefore, decrease in bile flow rate, altered bile compositions, gastrointestinal disorders and impaired kidney functions can change the ADME profile of diclofenac. These altered conditions may explain the pharmacokinetic changes of diclofenac in diabetes. After intravenous administration, the significantly greater AUC values due to significantly slower clearance values of diclofenac were

determined in the rat model of diabetes. However, after oral administration the significant change which was found between diabetic and control rats may be due to decreased absorption of diclofenac.[97] The smaller CL values after intravenous administration were due to the slower nonrenal clearance. Hence, the reduced catalytic activity of CYP2C11 may explain the altered pharmacokinetic parameters of diclofenac.[97]

1.5.2. The organophosphate antidote, K-48

Organophosphorus compounds (organophosphates and organophosphonates) are serine esterase and protease inhibitors. Organophosphates are widely used in agriculture as insecticides, in industry and technology as softening agents and lubricants.[99] Poisoning occurs as a result of accidental exposure or by the means of suicide or rarely homicide.[100,101] Organophosphonates (OP) are declared as chemical warfare agents used as "nerve gases" in terrorist attacks.[99]

The OPs are inhibitors of the butyrilcholine- and acetylcholine esterases, which mostly can be found in nerve tissues and erythrocytes. They react covalently with the active centre serine of cholinesterases by phosphorylation.[99,100,102] The inhibition of cholinesterase activity leads to accumulation of cholines in synapses, causing overstimulation of neurotransmission in both central and peripheral nervous systems.[100] Oximes are believed to be effective, and be especially useful in treating moderate or severe OP poisoning. The major pharmacological action of oximes is to reactivate the cholinesterase by removal of the phosphate group bound to the esteratic site.[101] Oximes are commonly used in combination with atropine.[99,103,104] Atropin relieves muscarinic signs and symptoms of the peripheral nervous system and the oximes are supposed to ameliorate the effect on CNS (e.g. respiratory muscle paralysis).[99] Pralidoxime salts have been used in many years for the treatment of OP poisoning; however, the clinical experiences are disappointing.[99,102] Over the years new potential reactivators have been developed. From the chemical point of view, the oximes can be separated into different groups: monopyridinium compounds (e.g. pralidoxime), bisquaternery symmetric (e.g. obidoxime, methoxime) and bisquaternery asymmetric compounds (e.g. HI-6, BI-6, K-48).[99] All the molecules are quaternary amino compounds with alkyl substituents on the

nitrogen(s) of the pyridinium ring(s). They have a strong hydrophilic character with a moderate hydrophobicity.[105] The chemical structure of oxime reactivators are presented in Fig. 5.

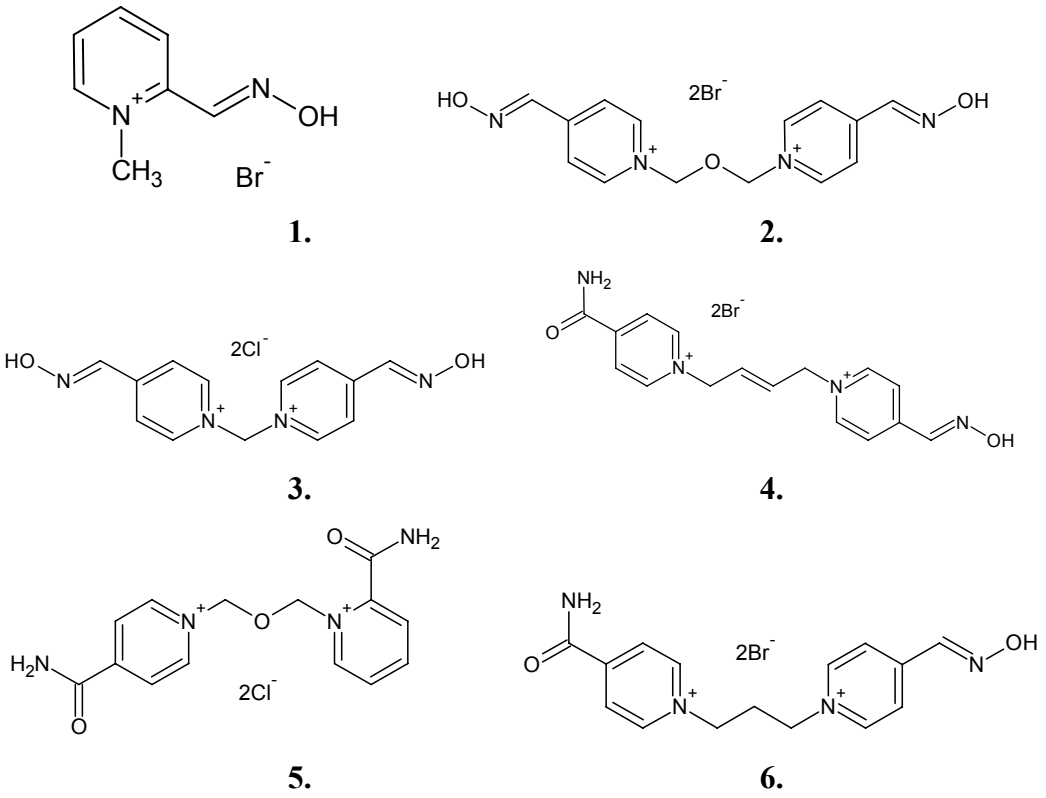

Figure 5.
The chemical structure of clinically used oximes
(**1.**, pralidoxime; **2.**, obidoxime; **3.**, methoxime; **4.**,BI-6; **5.**, HI-6; **6.**, K-48).[99,106]

There is no oxime which is able to sufficiently counteract acute toxic effects on all nerve agents.[104] Therefore, a clear demand is for a high efficacy, "broad spectrum" reactivator. K-48 compound is one of the newly synthesized oximes with a functional aldoxime group at position four of the pyridinium rings.[107] K-48 provided protection to rats exposed to paraoxon,[99] methyl-paraoxon,[107] tabun, but is not suitable for the treatment of cyclosarin poisonings.[104]

The metabolism and disposition of K-48 are barely known. The brain is the primary target organ of organophosporous agents; therefore, it is one of the crucial

points of the newly synthesized oximes to be able to cross the blood-brain barrier and reactivate the brain cholinesterase enzymes.[104] Poor penetration of K-48 molecule through the BBB is observed which is related to its hydrophilic properties. Its entry is not similar to or even far less than that of pralidoxime and obidoxime.[108] K-48 shows relatively high dose-normalized plasma AUC value compared to pralidoxime which might be beneficial as an antidote. Following i.m. and i.p. administration of K-48, a rapid rise in its blood concentration was found. The $t_{1/2}$ after i.m. administration is 83 minutes, the elimination of parent compound followed zero-order kinetics. The pharmacokinetics of the K-48 compound seems to be favourable in comparison to pralidoxime.[109] An important contribution to understand the mechanism of action of K-48 and other oximes is to investigate their disposition, metabolism and elimination.

2. RESEARCH OBJECTIVES

Determination of the effect of streptozotocin induced diabetes and insulin treatment on the rat intestinal metabolism. It is little known whether experimental diabetes influences the metabolising capacity of the small intestine. The study focused on the changes in total CYP content and CYP3A - a predominant isoenzyme in human and also highly expressed in rat intestine - catalytic activity in insulin treated and untreated diabetes in comparison to control. The questions were as follows:

- How does the diabetic state influence the intestinal P450 mediated metabolism?
- Is there any correlation between intestinal CYP3A catalytic activity and blood glucose concentration (an inverse indicator of insulin level)?

Study of the predominant hepatic isoenzymes in insulin treated and untreated long-term diabetes mellitus. The aim of this study was to observe the changes in mRNA expression of the CYP1A2, CYP2B1/2, CYP2C11, CYP2C13, CYP2C22, CYP2C23, CYP2D2, CYP2E1, CYP3A1, CYP3A2 and FMOs. The activity of phenacetin, aminopyrine, mephenytoin, tolbutamide, bufuralol, chlorzoxazone, testosterone and benzydamine metabolising enzymes were determined. Our inquiries were as follows:

- How does long-term diabetes affect the cytochrome P450 mRNA expression and catalytic activity?
- Is the altered cytochrome P450 mediated metabolism sensitive for a nine day period insulin treatment?
- Is the mRNA expression of CYP2C11 also reflected in the protein expression and catalytic activity in treated and untreated diabetes?
- Have diabetes and insulin treatment any effect on other CYP2C isoenzymes?

Metabolism of diclofenac in streptozotocin induced diabetes. Our experiments were focused on the determination of enzyme kinetic parameters of diclofenac 4'-hydroxylase with control, diabetic and insulin treated rat liver microsomes. The questions were as follows:

- Does long-term diabetes modify the enzyme kinetic parameter of diclofenac 4'-hydroxylase?
- Does the metabolism of diclofenac change in diabetic state according to *in vitro* prediction?

***In vitro* and *in vivo* ADME parameters of the organophosphate antidote, K-48.** The distribution, metabolism and elimination of PACERs are essential to understand their mechanism. *In silico*, *in vitro* and *in vivo* experiments were used for the assessment of K-48 metabolism, its distribution in serum, CSF and in the brain and its elimination by means of urine was investigated. The questions were as follows:

- Has diabetes an effect on the metabolism of K-48?
- Do the *in silico* predicted metabolites also appear in microsomal metabolism studies?
- Can K-48 or its metabolite be found in rat serum, CSF and urine following i.m. injection?
- Is it possible for K-48 or its metabolite to enter the brain?

3. MATERIALS AND METHODS

3.1. MATERIALS

All biochemicals used were of the highest purity available from commercial sources. Streptozotocin, diclofenac sodium, sodium dithionite, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, EDTA, dithiotreitol, dimethyl sulfoxide, sodium citrate, 6ß-hydroxy testosterone, aminopyrine, acetaminophen, benzydamine, bufuralol, chlorzoxazone, 6-hydroxy chlorzoxazone, tolbutamide, 1-octane sulfonic acid sodium salt, phosphoric acid and trifluoroacetic acid were purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA). 1'-hydroxy bufuralol, S-mephenytoin, 4'-hydroxy S-mephenytoin, 4'-hydroxy tolbutamide were obtained from Ultrafine (Manchester, UK). Testosterone, phenacetin, PMSF and 70 % perchloric acid were supplied by Fluka (Buchs, Switzerland). Potassium chloride, Tris, sodium pyrophosphate, NADPH, magnesium chloride and glycerin were acquired from Reanal (Budapest, Hungary). Heparin (Heparibene®) was obtained from Ratiopharma GmbH (Ulm, Germany). 4'-hydroxy diclofenac was purchased from BD Bioscience (Woburn, MA, USA). K-48 was synthesized in the Laboratory of Kamil Kuca (Department of Toxicology, Faculty of Military Health Sciences, Hradec Kralove, Czech Republic) by a method published earlier.[106] Benzydamine N-oxide metabolite was synthesized in the Chemistry Faculty of Gedeon Richter Plc. (Budapest, Hungary). Methanol gradient grade, acetonitril gradient grade, ammonium acetate, disodium hydrogen phosphate dehydrate and citric acid monohydrate from Merck (Darmstadt, Germany) were used for HPLC analysis.

For insulin treatment two different protocols were used. Insulin (Ultratard) purchased from Novo Nordisk (Novo Allé, Denmark) was used in intestinal metabolism studies. The protocol for the long-term diabetes prescribed Humulin N insulin obtained from Eli Lilly and Company (Indianapolis, IN, USA) and Insulatard acquired from Novo Nordisk (Novo Allé, Denmark). The blood glucose level was measured by an AccuCheck® Active Glucotrend kit supplied by Roche Diagnostic Ltd. (Mannheim, Germany).

3.2. ANIMALS AND INDUCTION OF DIABETES

3.2.1. Model for intestinal metabolism studies

The study was carried out in accordance with the Declaration of Helsinki. Male Sprague-Dawley rats (160-170 g) were purchased from Harlan (Holland). Rats were maintained at 20-25 °C on a 12-h light/12-h dark cycle, with access to water and food *ad libitum*. Diabetes was induced by a single intraperitoneal injection of buffered solution (0.1 mol/L citrate, pH 6.0) of streptozotocin at doses of 70 mg/kg. The animals were considered diabetic if their blood glucose concentrations rose above 350 mg/dL on the 4th day following streptozotocin treatment. The animals were separated in 3 groups: control (n=8); diabetic rats treated with streptozotocin at 70 mg/kg dose (n=8), D70; diabetic rats treated with streptozotocin at 70 mg/kg dose and from the 5th day insulin treatment was initiated with gradually increased doses (on day 1: 20, day 6: 40, day 7: 50 and from the day 8: 2x75 IU/kg/day) (n=8), ID70. During insulin treatment the blood glucose level was checked twice daily by Glucotrend kit (Mannheim, Germany). Animals were sacrificed by cervical dislocation 2 weeks after the streptozotocin treatment in a short interval so as to avoid circadian variation. Approximately the first 30 centimetres of duodenum and jejunum were removed and intestinal microsomes were prepared.

3.2.2. Model for hepatic metabolism studies

Male Sprague-Dawley rats (200-250 g) were purchased from ToxiCoop (Budapest, Hungary). Rats were maintained at 20-25 °C on a 12-h light/ 12-h dark cycle, with access to water and food *ad libitum.* Diabetes was induced by a single injection of a buffered solution (0.1 mol/L citrate, pH 6.0) of streptozotocin at dosage of 55 mg/kg intravenously (on day 0). The control animals (n=5) were treated by a single intravenous injection of 0.1 mol/L citrate buffer solution (pH 6.0) without streptozotocin at the same time. The animals were considered diabetic if their blood glucose concentrations increased above 350 mg/dL on the 20th day following

streptozotocin treatment. At that time the streptozotocin treated rats were further separated into two groups: diabetic rats (D55; n=12) and insulin treated diabetic rats (ID55; n=14). Insulin was administered subcutan twice daily from the twentieth day at a dosage of 20 IU/kg at 8.30 a.m. (Humulin N) and again 30 IU/kg at 4.30 p.m. (Insulatard) for duration of a nine day period. All animals were starved for two hours before measuring their blood glucose levels and carrying out of insulin treatment. During insulin administration, the blood glucose concentrations were checked daily on diabetic rats and twice a day on insulin treated diabetic rats. Animals were sacrificed 28 days after streptozotocin treatment, the liver was isolated from the animals and hepatic microsomes were prepared.

3.3. PREPARATION OF INTESTINAL AND HEPATIC MICROSOMES

The livers were homogenized in 1.15 % KCl containing Tris-HCl buffer (0.1 M, pH 7.4) with the volume/mass ratio of 2:1 buffer (mL) and liver (g) or intestine (g).[110] The buffer also contained 0.2 mM EDTA, 0.1 mM dithiotreitol, 0.1 M Tris, 1.15 % KCl and 10 % glycerin. The pH was adjusted with HCl to 7.4.

The intestinal microsomes were prepared according to the method of Stohs and co-workers.[110] The proximal part of the intestine was removed, perfused by ice-cold, isotonic, buffered saline, gently scraped with a glass microscope slide and suspended in the same buffer as described above but it contained PMSF and heparin additionally.

Individual rat liver and intestinal microsomes were prepared by differential centrifugation (the homogenate was centrifuged for 15 min at 9000 x g, the supernatant was ultracentrifuged for 60 min at 105 000 x g). The microsomal pellets obtained were resuspended in the same buffer.

The microsomal protein content was measured with alkaline Folin phenol reagent by the method described by Lowry et al. (1951) with bovine serum albumin as standard.[111] 2 mL mixture of three reagents (A: 10 mL 0.2 N NaOH + 4 % Na_2CO_3, B: 0.2 mL 2 % K-Na-tartrate, C: 0.2 mL 1 % $CuSO_4$) were added to equal volume of microsomal suspension (0.25 %). After 10 minutes, 0.2 mL of Folin-Ciocalteu phenol reagent was added to it. Following 30 minutes of exposure the absorbance of violet Cu-

complex in the samples was determined at 750 nm by Beckman DU-30 spectrophotometer (Beckman Instruments Inc., Fullerton, CA, USA).

Total cytochrome P450 content was essentially determined with a Hitachi U-3300 spectrophotometer (Nissei Sangyo Co. Ltd., Tokyo, Japan) by the method established by Greim.[112] 1.2 mL of microsomal suspension was bubbled by carbon monoxide for 15-20 seconds (20-30 bubbles) and divided into two aliquots (i.e. sample and reference cell). The aliquot in the sample cell was reduced by a few crystals of solid dithiotreitol and the differential spectrum was recorded. P450 concentrations (nmol/mg protein) were calculated from the difference of absorbancies measured at 450 and 490 nm using ε=91 $mM^{-1}cm^{-1}$.

3.4. ENZYMATIC ASSAYS

3.4.1. CYP1A index reaction: Phenacetin O-dealkylation

CYP1A2 specific activity was measured by the deethylation of phenacetin. Incubations were carried out in incubation mixtures containing 6.25 mM sodium pyrophosphate, 5 mM $MgCl_2$, 5 mM glucose 6-phosphate, 1 U/mL glucose 6-phosphate dehydrogenase in a final volume of 0.5 mL, 0.1 mM Tris-HCl buffer, pH 7.4. The hepatic microsomal protein content was 0.25 mg/mL, the substrate concentration was 400 μM. The reactions were initiated by adding 0.5 mM NADPH. The incubation was carried out for 20 minutes in a shaking water bath at 37 °C. After the indicated time the reaction was stopped by 500 μL of ice-cold methanol. Samples were placed at -20 °C for ten minutes. Then the samples were centrifuged for 10 minutes at 10000 x g, at 4 °C. The supernatant was injected onto HPLC. The analytical measurement was performed on a Merck-Hitachi LaChrom HPLC system equipped with a UV detector. Purospher STAR RP-18e 125 x 2 mm (5 μm) column (Merck, Darmstadt, Germany) operated at 0.2 mL/min flow rate, maintained at 40 °C. Acetaminophen (RT: 5.98 minutes) was determined by using mobile phases of the mixture of 35 mL methanol and 265 mL 25 mM ammonium-acetate (A) and methanol (B) with a gradient (A of 100 % (0 min), 60 % (15 min), and 100 % (20-35 min)). UV detection was at 244 nm.

3.4.2. CYP2B/3A index reaction: Aminopyrine N-demethylation

Aminopyrine N-demethylation was determined by measuring formaldehyde formation by the method of Nash.[113] The substrate concentration was 4 mM; the microsomal protein concentration used was 1 mg/mL in a final volume of 2 mL. The reaction was initiated by the addition of NADPH. The incubation was carried out for 15 minutes in a shaking water bath at 37 °C. After the indicated time the reaction was stopped by an equivalent amount of 20 % TCA. Then the samples were centrifuged for 20 minutes at 3000 x g, at 4 °C. In order to develop the colour reaction 2 mL of Nash reagent was added to 1.5 mL of supernatant and left in a water bath at 37 °C, without shaking. After 30 minutes, the samples were measured at 410 nm by spectrophotometer (Beckman DU-30, Beckman Instruments Inc., Fullerton, CA, USA). The produced formaldehyde concentration was determined.

3.4.3. CYP2C index reactions: Tolbutamide and mephenytoin 4'-hydroxylation

CYP2C specific activity was measured by the hydroxylation of tolbutamide and mephenytoin. Incubations were carried out in incubation mixtures containing 6.25 mM sodium pyrophosphate, 5 mM $MgCl_2$, 5 mM glucose 6-phosphate, 1 U/mL glucose 6-phosphate dehydrogenase in a final volume of 0.5 mL, 0.1 mM Tris-HCl buffer, pH 7.4. The hepatic microsomal protein content was 0.25 mg/mL and the substrate concentration was 1.5 mM in the reaction of tolbutamide hydroxylation. 1 mg/mL hepatic microsomal protein concentration and 600 μM substrate concentration were used for mephenytoin hydroxylation. The reactions were initiated by adding 0.5 mM NADPH. The incubation was carried out for 10 minutes in the case of tolbutamide and 30 minutes in the case of mephenytoin, in a shaking water bath at 37 °C. After the indicated time the reaction was stopped by 500 μL of ice-cold methanol. Samples were placed at -20 °C for ten minutes. Then the samples were centrifuged for 10 minutes at 10000 x g, at 4 °C. The supernatant was injected onto HPLC. The analytical measurement was performed on a Merck-Hitachi LaChrom HPLC system equipped with a UV detector.

4’-hydroxy tolbutamide (RT: 6.93 minutes) was determined by using Purospher STAR RP18e 125 x 4 mm (5 μm) column (Merck, Darmstadt, Germany) operated at 0.8 mL/min flow rate, maintained at 40 °C. Elution was applied to the mixture of 23 % methanol and 77 % 0.1 M ammonium-acetate (A) and methanol (B) with a gradient (A of 100 % (7.5 min), 60 % (11-13 min), and 100 % (13.5-22 min)). UV detection was at 230 nm.

4’-hydroxy S-mephenytoin (RT: 9.16 minutes) was determined by using Purospher STAR RP-18e 250 x 3 mm (5 μm) column (Merck, Darmstadt, Germany) operated at 0.4 mL/min flow rate, maintained at 35 °C. Elution was applied to a mixture of 165 mL methanol, 300 mL 0.1 % ammonium-acetate and 2 mL 10 % Ammonium hydroxide. UV detection was at 228 nm.

3.4.4. CYP2D index reaction: Bufuralol 1’-hydroxylation

CYP2D catalytic activity was determined by the production of 1’-hydroxy bufuralol. Incubations were carried out in incubation mixtures containing 6.25 mM sodium pyrophosphate, 5 mM $MgCl_2$, 5 mM glucose 6-phosphate, 1 U/mL glucose 6-phosphate dehydrogenase in a final volume of 0.5 mL, 0.1 mM Tris-HCl buffer, pH 7.4. The hepatic microsomal protein content was 0.25 mg/mL, the substrate concentration was 500 μM. The reactions were initiated by adding 0.5 mM NADPH. The incubation was carried out for 10 minutes in a shaking water bath at 37 °C. After the indicated time the reaction was stopped by 500 μL of ice-cold methanol. Samples were placed at -20 °C for ten minutes. Then the samples were centrifuged for 10 minutes at 10000 x g, at 4 °C. The supernatant was injected onto HPLC. The analytical measurement was performed on a Merck-Hitachi LaChrom HPLC system equipped with an UV detector. Purospher STAR RP-18e 125 x 4 mm (5 μm) column (Merck, Darmstadt, Germany) operated at 0.8 mL/min flow rate, maintained at 40 °C. 1’-hydroxy bufuralol (RT: 9.55 min) was determined by using mobile phases of 30 % methanol and 70 % 0.1 M ammonium-acetate (A) and methanol (B) with a gradient (A of 100 % (9 min), 40 % (15-18 min), and 100 % (20-35 min)). UV detection was at 250 nm.

3.4.5. CYP2E1 index reaction: Chlorzoxazone 6-hydroxylation

Chlorzoxazone biotransformation into 6-hydroxy chlorzoxazone was used for the determination of CYP2E1 activity. Incubations were carried out in incubation mixtures containing 6.25 mM sodium pyrophosphate, 5 mM $MgCl_2$, 5 mM glucose 6-phosphate, 1 U/mL glucose 6-phosphate dehydrogenase in a final volume of 0.5 mL, 0.1 mM Tris-HCl buffer, pH 7.4. The hepatic microsomal protein content was 0.2 mg/mL, the substrate concentration was 80 μM. The reactions were initiated by adding 0.5 mM NADPH. The incubation was carried out for 15 minutes in a shaking water bath at 37 °C. After the indicated time the reaction was stopped by 500 μL of ice-cold methanol. Samples were placed at -20 °C for ten minutes. Then the samples were centrifuged for 10 minutes at 10000 x g, at 4 °C. The supernatant was injected onto HPLC. The analytical measurement was performed on a Merck-Hitachi LaChrom HPLC system equipped with an UV detector. Purospher STAR RP-18e 125 x 4 mm (5 μm) column (Merck, Darmstadt, Germany) operated at 0.5 mL/min flow rate, maintained at 40 °C. 6-hydroxy chlorzoxazone (RT: 6.05 min) was determined by using mobile phases of the mixture of 500 mL methanol, 1250 mL 20 mM ammonium-acetate and 10 mL 10% TFA. UV detection was at 285 nm.

3.4.6. CYP3A index reaction: Testosterone 6ß-hydroxylation

CYP3A specific activity was measured by the production of 6ß-hydroxy testosterone. Incubations were carried out in incubation mixtures containing 6.25 mM sodium pyrophosphate, 5 mM $MgCl_2$, 5 mM glucose 6-phosphate, 1 U/mL glucose 6-phosphate dehydrogenase in a final volume of 0.5 mL, 0.1 mM Tris-HCl buffer, pH 7.4. The hepatic and intestinal microsomal protein content was 0.25 and 1.75 mg/mL, respectively, and the substrate concentration was 500 μM in both cases. The reactions were initiated by adding 0.5 mM NADPH. The incubation was carried out for 5 or 15 minutes in a shaking water bath at 37 °C. After the indicated time the reaction was stopped by 500 μL of ice-cold methanol. Samples were placed at -20 °C for ten minutes. Then the samples were centrifuged for 10 minutes at 10000 x g, at 4 °C. The

supernatant was injected onto HPLC. The analytical measurement was performed on a Merck-Hitachi LaChrom HPLC system equipped with a UV detector according to the method of Dalmadi et al.[114] Purospher STAR RP-18e 125 x 4 mm (5μm) column (Merck, Darmstadt, Germany) operated at 0.8 mL/min flow rate, maintained at 40 °C. 6β-hydroxy testosterone (RT: 8.3 min) was determined by using mobile phases of methanol/ 0.1 M ammonium-acetate, 45:55 (A) and methanol (B) with a gradient (A of 100 % (7 min), 40 % (15-18 min), and 100 % (21-26 min)). UV detection was at 254 nm.

3.4.7. FMO index reaction: Benzydamine N-oxygenation

Benzydamine/ benzydamine N-oxide (BZYNO) transformation was used as an FMO specific index reaction. The incubation medium contained 0.2 mg/mL rat liver microsomal protein, 0.4 mM NADPH, 0.4 mM glucose 6-phosphate, 0.8 IU/mL glucose 6-phosphate dehydrogenase and 0.2 mM DETAPAC in a final volume of 0.5 mL of 50 mM phosphate buffer (pH 8.5). The final concentration of BZY was 500 μM. Incubation time and microsomal protein concentration was within the linear range of metabolite formation. After 30 minutes the reaction was stopped by 500 μL of ice-cold methanol. Samples were placed at -20 °C for ten minutes. Then the samples were centrifuged for 10 minutes at 10000 x g, at 4 °C. The supernatant was injected onto HPLC. The analytical measurement was performed on a Merck-Hitachi LaChrom HPLC system equipped with a UV detector. Purospher STAR RP-18e 125 x 4 mm (5μm) column (Merck, Darmstadt, Germany) operated at 0.8 mL/min flow rate, maintained at 30 °C. BZYNO was monitored at 306 nm. Mobile phase consisted of 58 % methanol in 0.1 M Ammonium acetate with isocratic elution. Under these chromatographic conditions BZYNO eluted at 6.8 minutes.

3.5. DETERMINATION OF mRNA EXPRESSION

Total RNA isolation was carried out using the RNeasy Mini Kit according to the instructions of the manufacturer (Qiagen Sciences, Germantown, MD, USA). Hence,

30-30 mg of liver samples of each examined animal was placed into 0.5 mL RNA stabilization reagent. Following homogenization in lysing buffer containing guanidine isothiocyanate, centrifugation and addition of 0.5 mL of 70 % ethanol to the supernatant, the sample was applied to an RNeasy mini column to discard from proteins and DNA. The mRNA was eluted by 50 µL of RNase-free water. The concentration and integrity of purified RNA were checked by Agilent 2100 bioanalyser using the RNA 6000 Nano Assay kit (Agilent Technologies, Santa Clara, CA, USA). Full-length first strand cDNA was synthesized using "Superscript III First-Strand Synthesis SuperMix for qRT PCR" (Invitrogen Corporation, Carlsbad, CA, USA) with a random primer and the addition of 0.5 µg total RNA. The reaction was carried out at 25 °C and at 50 °C over a period of 10 and 30 minutes, respectively. The quantitative real-time PCR was performed using Taqman Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA) with standard conditions on the 7900HT Real-Time PCR system (Applied Biosystem, Foster City, CA, USA). Specific amplifications of cDNA were performed using TaqMan® Gene Expression Assays (Applied Biosystem, Foster City, CA, USA) consisting of PCR primers and FAM™ dye-labelled TaqMan® MGB probe. Taqman assays for rCYP1A2 (Rn00561082_m1), rCYP2B1/2 (Rn01457879_m1), rCYP2C11 (Rn01502201_m1), rCYP2C13 (Rn00593388_g1), rCYP2C22 (Rn00593377_m1), rCYP2C23 (Rn00582954_m1), rCYP2D2 (Rn00562419_m1), rCYP2E1 (Rn00580624_m1), rCYP3A1 (Rn01412959_g1), rCYP3A2 (Rn00756461_m1), rFMO1 (Rn00562945_m1), rFMO3 (Rn00584825_m1) and endogenous control rGAPDH (Rn99999916_s1) were used. The threshold cycle (Ct) was determined and relative quantitation (RQ) was calculated using the comparative Ct method with RealTime Statminer (Integromics, Granada, Spain) as follows:

Ct_{GAPDH} = average for GAPDH of the sample in question

$Ct_{CYP/\ FMO}$ = individual value of the sample in question

$\Delta Ct = Ct_{CYP/\ FMO} - Ct_{GAPDH}$

$\Delta\Delta Ct_{CYP/\ FMO}$ treated = $\Delta Ct_{CYP/\ FMO}$ treated-average ($\Delta Ct_{CYP/\ FMO}$ control)

Fold-induction CYP or FMO treated = $2^{-\Delta\Delta CtCYP/\ FMO\ treated}$

3.6. WESTERN BLOT ANALYSIS OF CYP2C11 PROTEIN LEVEL

For Western blot analysis a 12 % (w/v) Tris-Glycine PAGEr GOLD Precast Gel (Lonza, Rockland, ME, USA) was used. Microsomes were solubilised in Laemmli Sample buffer solution (BioRad Laboratories, Hercules, CA, USA) containing 5 % (v/v) 2-mercaptoethanol and heated at 95 °C for 5 minutes. Equal amounts of proteins (protein concentration was determined by the method of Lowry et al, 1951) were analyzed. Proteins were transferred onto Immobilion-P PVDF membrane (10 V, 60 min) after the electrophoresis. 30 mM TBS (Tris buffer solution, pH 10.4) anode buffer and 40 mM TBS containing 40 mM glycine cathode buffer were applied for the protein transfer. All the solutions included 10 % methanol. Blocking was done with SuperBlock® Dry Blend (TBS) Blocking Buffer solution (Pierce, Rockford, IL, USA). Primary labelling was carried out with rabbit polyclonal anti-Cytochrome P450 2C11 (1:1500) purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and goat Actin C-11 (1:200) antibody obtained from Abcam Plc. (Cambridge, UK). Biotynilated anti-rabbit IgG (1:2000) and biotynilated anti-goat IgG (1:2000) acquired from Vector Laboratories (Burlingame, CA, USA) were used as secondary antibodies. Antibodies were diluted in the 10-fold diluted blocking solution. Washing was done between steps with Tris Buffer Solution (TBS). After incubating with extravidine peroxidase (Sigma-Aldrich Corp., St. Louis, MO, USA) in 1:5000 dilutions, DAB Substrate kit (Pierce, Rockford, IL, USA) was used for development.

3.7. INCUBATION CONDITIONS FOR THE DETERMINATION OF DICLOFENAC 4'-HYDROXYLASE ENZYME KINETIC PARAMETERS

Enzyme kinetic parameters K_M and V_{max} for the formation of 4'-hydroxy diclofenac from diclofenac were determined. Incubations were carried out in incubation mixtures containing 6.25 mM sodium pyrophosphate, 5 mM $MgCl_2$, 5 mM glucose 6-phosphate, 1 U/mL glucose 6-phosphate dehydrogenase and 0.25 mg/mL microsomal protein in a final volume of 0.5 mL, 0.1 mM Tris-HCl buffer, pH 7.4. The reactions were initiated

by adding 0.5 mM NADPH. The incubation medium contained 1.5625, 3.125, 6.25, 12.5, 25, 50, 100 μM diclofenac (dissolved in water) and allowed to incubate 20 minutes in a water bath shaker maintained at 37 °C. The reactions were terminated by adding an equal volume of ice-cold methanol. All incubations were run in triplicate.

Samples were analyzed by a Merck-Hitachi LaChrom Elite HPLC (Darmstadt, Germany) device following centrifugation (2500 x g, 0 °C, 30 min). The supernatant was injected onto a Purospher STAR RP18e 125 x 3 mm (5 μm) column (Merck, Darmstadt, Germany). Isocratic elution was applied by 0.1 M ammonium acetate containing 45 % methanol and the mobile phase was delivered at 0.45 mL/min flow rate. 4'-hydoxy diclofenac was detected at 267 nm with the retention time of 7.32 minutes.

The kinetic constants (K_M, V_{max}) were calculated using Lineweaver-Burk plot by linear regression. The intrinsic clearance (CL_{int}) for the disappearance of diclofenac was calculated by dividing the respective V_{max} by the respective K_M.

3.8. IN SILICO, IN VITRO AND IN VIVO STUDIES OF K-48

3.8.1. *In silico* prediction of K-48 metabolism

Prediction of metabolic pattern and calculation of lipophilicity (logP) were done using the Pallas Program of CompuDrug Ltd. (Budapest, Hungary).

3.8.2. Incubation conditions of *in vitro* microsomal metabolism study

K-48 (50 μM) was incubated with control rat liver or diabetic rat liver microsomes. Incubation medium contained 6.25 mM sodium pyrophosphate, 5 mM $MgCl_2$, 5 mM glucose 6-phosphate, 1 U/mL glucose 6-phosphate dehydrogenase in a final volume of 2 mL, 0.1 mM Tris-HCl buffer, pH 7.4 (37 °C). The microsomal protein content was 0.5 mg/mL. The reactions were initiated by adding 0.5 mM NADPH. Control incubation was carried out with microsomal protein and NADPH without K-48

for the duration of 30 minutes. The incubation was run in a shaking water bath at 37 °C, sampling was done at 0 and 30 minutes. After the indicated time the reaction was stopped by an equal volume of ice-cold methanol or by 70 % ice-cold perchloric acid (in the case of HPLC-ECD analysis). Samples were placed at -20 °C for ten minutes. Then the samples were centrifuged for 10 minutes at 10000 x g, at 4 °C. The supernatant was injected onto HPLC. Tentative metabolites and unchanged compounds were measured.

3.8.3. *In vivo* animal studies

Male Wistar rats (Toxicoop, Budapest, Hungary) were kept in an animal house regulated for temperatures (22–24 °C), humidity (55±6 %), and using a 12-h light–dark cycle. Five days after arrival, 22.31 mg (50 μM) of K-48 freshly dissolved in distillated water was injected intramuscularly into the animals (weighing 213.3 ± 1.9 g) in a volume of 0.2 mL. After treatment, the rats were placed in special individual cages, where urine was collected. The rats were sacrificed after 60 min of observation. Blood and cerebrospinal fluid (CSF) were taken. The brain was dissected immediately on an ice-cold aluminium surface. In parallel experiments urine was collected for four hours. All samples were kept frozen at −80 °C until analysis. Serum, CSF and urine samples were mixed with an equal volume of 20 % trichloroacetic acid (TCA) to precipitate the protein, and centrifuged in an Eppendorf centrifuge (A. Hettich, Tuttlingen, Germany) at 10,000 × g for 10 min at 4 °C. Whole-brain samples were homogenized in an equal volume of 20 % trichloroacetic acid (TCA) by an Ultra Turrax T25 Janke & Kunkel homogenizer (IKA Labortechnik, Staufen, Germany) at 20,000 rpm/min for 10 sec at room temperature, then 1.0 mL of the homogenate was diluted with an equal volume of 10 % TCA. The supernatant obtained after repeated centrifugation in the Eppendorf centrifuge at 10000 x g (10 min at 4 °C) was used for HPLC analysis.

3.8.4. HPLC analysis

Samples of *in vitro* metabolism studies of K-48 molecule in STZ diabetes were analyzed by reversed-phase high-performance liquid chromatography with electrochemical detection (HPLC-ECD). A Jasco pump (PU1580, Tokyo, Japan) was used; the samples were injected using a 50 μL loop and the column temperature was 35 °C. The precolumn was Zorbax RX-C18 12.5 x 4.6 mm while the analytical column was Zorbax RX-18 250 x 4.6 mm (5 μm); both columns were purchased from Agilent Technologies Inc. (Santa Clara, CA, US). The chromatogram was detected by an Intro amperometric detector (Antec; Leyden B.V., Zoeterwoude, the Netherlands) at E_{ox} = 0.65 V and a sensitivity of 10 nA, with a 0.1 sec time filter. The mobile phase contained 56.2 mM Na_2HPO_4, 47.9 mM citric acid, 0.027 mM Na_2EDTA, 0.925 mM octane sulfonic acid, and acetonitrile-phosphate buffer 80:950, v/v; its flow rate was 1mL/min. The pH was adjusted to 3.7 with 85 % H_3PO_4. The chromatograms were electronically stored and evaluated using Borwin 1.21 chromatographic software (JMBS, Le Fontanil, France).

Samples from *in vivo* and *in vitro* metabolism studies with control microsomes were also analyzed by HPLC-MS. A 1100 HPLC/MSD SL system from Agilent Technologies (Waldbronn, Germany) which consisted of a binary pump, degasser, automatic injector, diode array detector, thermostat, and mass-selective detector. An electrospray was used in positive ionization mode in the range of 100–1000 atomic mass units (amu). The step size was 0.2 min, while the drying gas flow and the temperature were 13 L/min and 350 °C, respectively. The nebulizer gas pressure was 35 psig, and a capillary voltage of 3000 V was applied. A 4.6 ×150 mm column containing 5 μm Zorbax Eclipse XDB-C8 (Agilent Technologies Inc., Santa Clara, CA, US) was used for separation. The mobile phase was methanol–0.1 M ammonium acetate (8:92) in isocratic mode. Ultraviolet absorbance was detected at 286 nm.

3.9. DATA ANALYSIS

Statistical analysis was performed by a GraphPad Prism 4.0 (GraphPad Software Inc., La Jolla, CA, US). Normality test (Shapiro-Wilk's test) and homogeneity test (Bartlett's test) were run for all datasets in order to determine whether they have Gaussian distribution and homogeneity in their variances. In the case of parametric distribution one-way analysis of variance was used which was followed by Tukey multicomparison test if any significant change was shown, the same was done for all enzyme activity and mRNA values. In the case of non-parametric distribution (despite transformations); a Kruskal-Wallis multicomparison test was used. Mean, S.D. and correlation coefficient were calculated by Confidence interval of 95%.

4. RESULTS

4.1. EFFECT OF DIABETES AND INSULIN TREATMENT ON INTESTINAL P450s

4.1.1. Physical and biochemical characteristics

Streptozotocin treated rats became diabetic with symptoms of polydipsia, polyuria, hyperglycaemia and decreased rate of body weight gain appeared. The changes in the physiological parameters among control, D70 and ID70 groups are summarized in Table 6. Control rats consistently had blood glucose levels of approximately 100 mg/dL, while rats receiving 70 mg/kg doses of STZ displayed about a five-fold increase in blood glucose levels: 536.1 mg/dL. Diabetic rats had a significant reduction in body weight and wet liver weight compared to the control, at the time of sacrifice. Insulin treatment of diabetic animals only partially compensated for the rise of blood glucose level (391.4 mg/dL), but fully for the changes in body weight and wet liver weight, which resulted in an enhanced relative wet liver weight.

Parameters	*Control animals*	*Diabetic animals*	
		70 mg/kg STZ i.m. *D70*	*70 mg/kg STZ i.m. + Insulin ID70*
Body wt. at sacrifice *(g)*	294 ± 13	175 ± 12***	240 ± 21†
Blood glucose *(mg/dL)*	101.2 ± 4.5	536.1 ± 18.7***	391.4 ± 51.2
Wet liver weight *(g)*	12.8 ± 1.2	8.1 ± 0.7***	14.8 ± 2.5†††
Relative wet liver weight *(mg liver/ g body wt.)*	43.5 ± 3.1	46.2 ± 2.4	61.3 ± 7.3**

Table 6.
Effect of streptozotocin induced diabetes and insulin treatment on various physical and biochemical parameters. *Mean ± S.D. were calculated (n=5-9).* $^{**}p \leq 0.01$ *vs. control values,* $^{***}p \leq 0.001$ *vs. control values,* $^{\dagger}p \leq 0.05$ *values of D70 vs. ID70,* $^{\dagger\dagger\dagger}p \leq 0.001$ *values of D70 vs. ID70*

4.1.2. Intestinal total cytochrome P450 content and CYP3A catalytic activity

The intestinal P450 content tended to increase in D70 rats in comparison to control; however the change was statistically not significant. The extent of elevation was reduced by insulin treatment in ID70 animals (Table 7.). The testosterone 6ß-hydroxylase activity significantly decreased to control level in diabetic rats. The insulin treatment caused an increase in CYP3A activity compared to D70 animals (40 %), although it was not restored to the control value. The turnover number of CYP3A (calculated with total CYP content) mediated activity was reduced by 74 % and 54 % in D70 and ID70 rats, respectively.

The regression analysis between blood glucose level and intestinal CYP3A isoenzyme activities resulted in inverse correlation (r= 0.6787; p= 0.0054; n=14).

Parameters	*Control animals*	*Diabetic animals*	
		70 mg/kg STZ i.m. *D70*	*70 mg/kg STZ i.m. + Insulin ID70*
Total cytochrome P450 content *(nmol/mg microsomal protein)*	0.031 ± 0.018	0.049 ± 0.017	0.039 ± 0.014
Testosterone 6ß-hydroxylase activity *(pmol mg^{-1} min^{-1})*	2.29 ± 0.57	1.13 ± 0.19***	1.58 ± 0.21**,†
Testosterone 6ß-hydroxylase turnover number *(pmol/nmol total P450)*	98.4 ± 56.9	25.4 ± 9.4***	45.3 ± 16.2*

Table 7.
Intestinal CYP content and CYP3A enzyme activity in streptozotocin induced diabetic rats with or without insulin treatment. *Mean ± S.D. were calculated (n=5-9).* $^{*}p \leq 0.05$ *vs. control values,* $^{**}p \leq 0.01$ *vs. control values,* $^{***}p \leq 0.001$ *vs. control values,* $^{\dagger}p \leq 0.05$ *values of D70 vs. ID70.*

4.2. EFFECT OF DIABETES AND INSULIN TREATMENT ON HEPATIC CYTOCHROME P450s

4.2.1. Physical and biochemical characteristics

Streptozotocin treated rats became diabetic with symptoms of polydipsia, polyuria, hyperglycaemia and decreased rate of body weight gain appeared. The changes in the physiological parameters among the control, diabetic and insulin treated diabetic groups are summarized in Table 8. The blood glucose concentration significantly increased in D55 animals (546.6 mg/dL) in comparison to control (99.8 mg/dL). There was a substantial decrease in the rate of body gain and only a tendentious decrease in wet liver weight (25 %) in the group of D55 compared to control. Insulin treatment of diabetic animals significantly but only partially compensated for the rise of blood glucose level (276.1 mg/dL); and changes in body weight but fully for the changes in wet liver weight, which resulted in an enhanced relative wet liver weight. There were no differences in the microsomal protein content among the three groups investigated (data not shown).

Parameters	*Control animals*	*Diabetic animals*	
		55 mg/kg STZ i.v. *D55*	*55 mg/kg STZ i.v. + Insulin ID55*
Body wt. at sacrifice *(g)*	358 ± 4	207 ± 33***	246 ± 27*†
Blood glucose *(mg/dL)*	99.8 ± 1.6	546.6 ± 28***	276.1 ± 85†††
Wet liver weight *(g)*	13.6 ± 0.3	10.2 ± 1.3	16.6 ± 3.7†††
Relative wet liver weight *(mg liver/g body wt.)*	37.8 ± 0.7	50.2 ± 9.9	68.4 ± 15.5***†

Table 8.
Effects of streptozotocin-induced diabetes and insulin treatment on various physiological parameters. *Mean ± SD were calculated (n=5-14)* $^{*}p \leq 0.05$ *vs. control;* $^{***}p \leq 0.001$ *values vs. control;* $^{\dagger}p \leq 0.05$ *values D55 vs. ID55;* $^{\dagger\dagger\dagger}p \leq 0.001$ *values D55 vs. ID55.*

4.2.2. Results of mRNA expression studies

The total RNA was isolated by RNeasy Mini Kit. The RNAs prepared from the samples analyzed were of high quality.

Gene expression of CYP1A2 isoenzyme

The mRNA level in rats received 55 mg/kg dose of STZ tended to increase by 24 %. Insulin treatment decreased significantly the gene expression of CYP1A2 which is resulted a non-significant but lower expression (28 %) in comparison to the control group (Figure 6.).

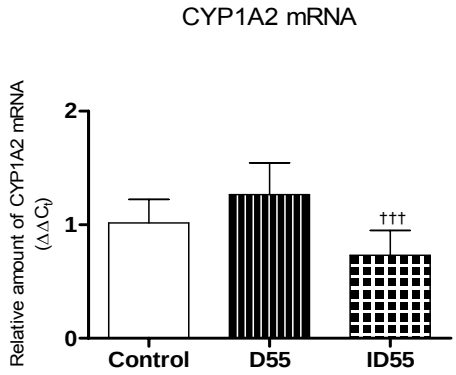

Figure 6.
Hepatic CYP1A2 mRNA level in diabetic rats with or without insulin treatment.
Mean ± S.D. were calculated (n=5-14). $^{†††}p \leq 0.001$ D55 vs. ID55.

Gene expression of CYP2B1/2

The mRNA level of CYP2B1/2 did not change statistically either in STZ induced diabetic or in insulin treated diabetic rats (Fig.7.).

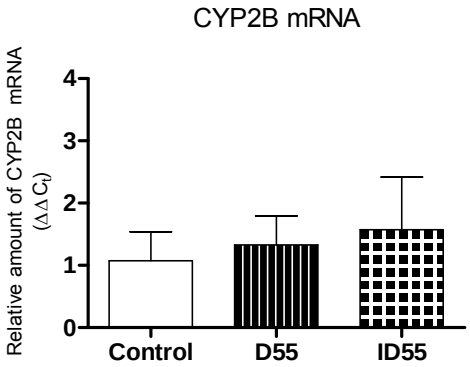

Figure 7.
Hepatic CYP2B mRNA level in diabetic rats with or without insulin treatment.
Mean ± S.D. were calculated (n=5-14).

Gene expression of CYP2D2

The gene expression of CYP2D2 significantly decreased in experimental diabetic rats by 55 %. The mean mRNA level tended to elevate following insulin treatment. However, the mRNA expression level in ID55 rats statistically not differed from the expression level determined in D55 and control animals. (Fig. 8.).

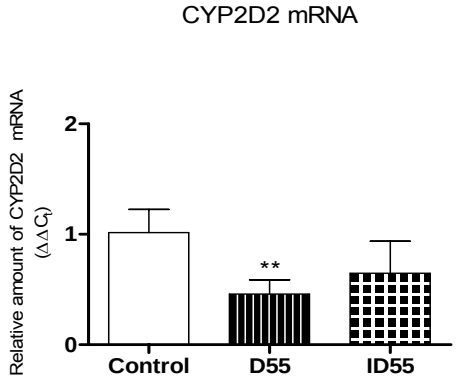

Figure 8.
Hepatic CYP2D2 mRNA level in STZ diabetic rats with or without insulin treatment.
Mean ± S.D. were calculated (n=5-14). $^{**}p \leq 0.01$ *values vs. control.*

Gene expression of CYP2E1

The gene expression of CYP2E1 elevated in rats which received 55 mg/kg dose of STZ by 2.2-fold. The insulin treatment restored the gene expression of CYP2E1 to control level. The decrease was significant when compared to D55 rats (Fig. 9.)

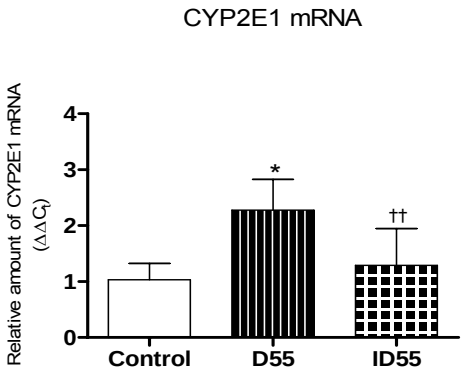

Figure 9.
Hepatic CYP2E1 mRNA level in insulin treated and not treated streptozotocin induced diabetes. *Mean ± S.D. were calculated (n=5-14).* $^{*}p \leq 0.05$ values *vs. control,* $^{\dagger\dagger}p \leq 0.01$ *D55 vs. ID55*

Gene expression of CYP3A1 and CYP3A2

The mRNA level of CYP3A1 tended to increase by 2.4 –fold in experimental diabetes; however, a very high S.D. occurred. There was a significant decrease following insulin treatment in gene expression level of CYP3A1 which resulted in the expression level of the control. The gene expression of CYP3A2 changed only tendentiously in untreated and insulin treated diabetic animals (Fig. 10.).

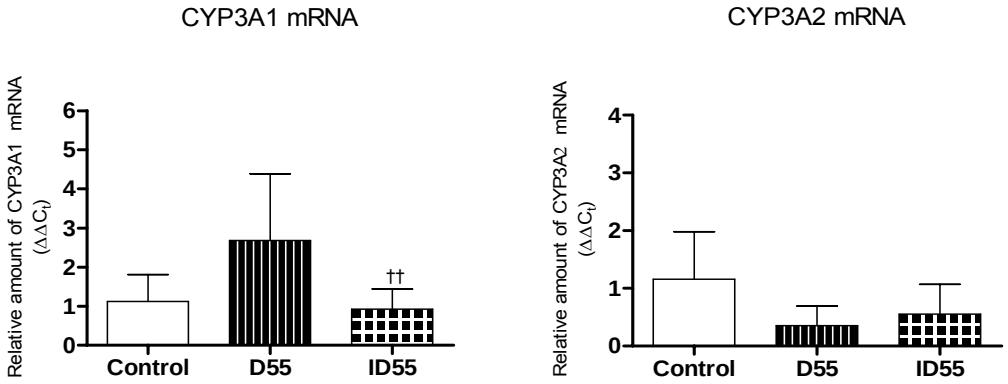

Figure 10.
Hepatic CYP3A1 and CYP3A2 mRNA level in STZ diabetic rats with or without insulin treatment. *Mean ± S.D. were calculated (n=5-14).* $^{\dagger\dagger}p \leq 0.01$ *D55 vs. ID55*

Gene expression of FMO1 and FMO3

In 55 mg/kg STZ treated diabetic rats only the FMO3 mRNA level increased. The increase of FMO3 mRNA level was 3-fold. The gene expression was tended to restore to the control level as a result of insulin treatment. The decreased gene expression level in ID55 animals did not differed significantly either D55 or control groups. FMO1 did not show any substantial changes (Fig.11.)

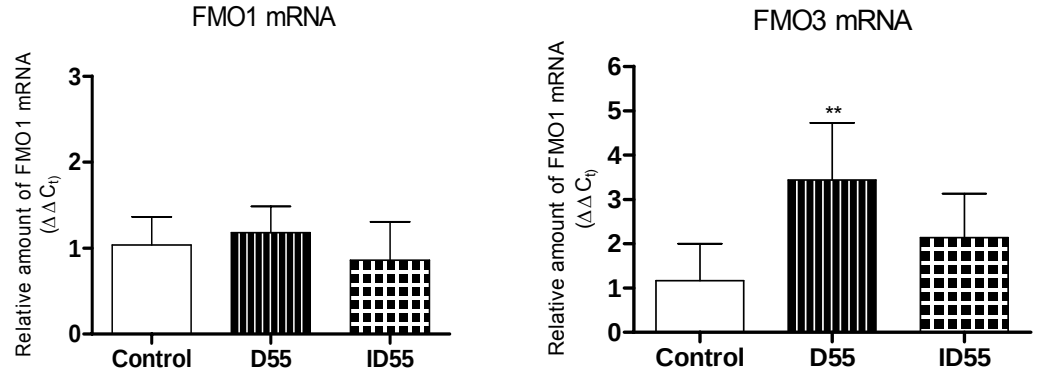

Figure 11.
Gene expression levels of hepatic FMO isoenzymes in experimental diabetic animals and following insulin treatment. *Mean ± S.D. were calculated (n=5-14).* $^{**}p \leq 0.01$ *values vs. control.*

Focusing on the changes in CYP2C isoenzymes

The mRNA expression of CYP2C11, CYP2C13 and CYP2C22 presented in a significantly lower level in streptozotocin-induced diabetic rats in comparison to the related control (Fig. 10.). The most evident decrease was seen at the gene expression of CYP2C11 (95 %). No substantial loss was noticed at the mRNA expression of CYP2C23. Insulin treatment only tended to compensate for the decrease of CYP2C11 and CYP2C13 mRNA expression. However, no significant differences could be shown between insulin treated diabetic and control samples. There was only a slight, if any, effect of insulin treatment on the gene expression level of CYP2C23 and CYP2C22. The results show that diabetes does not influence the mRNA expression level of CYP2C23 isoform (Fig. 12.).

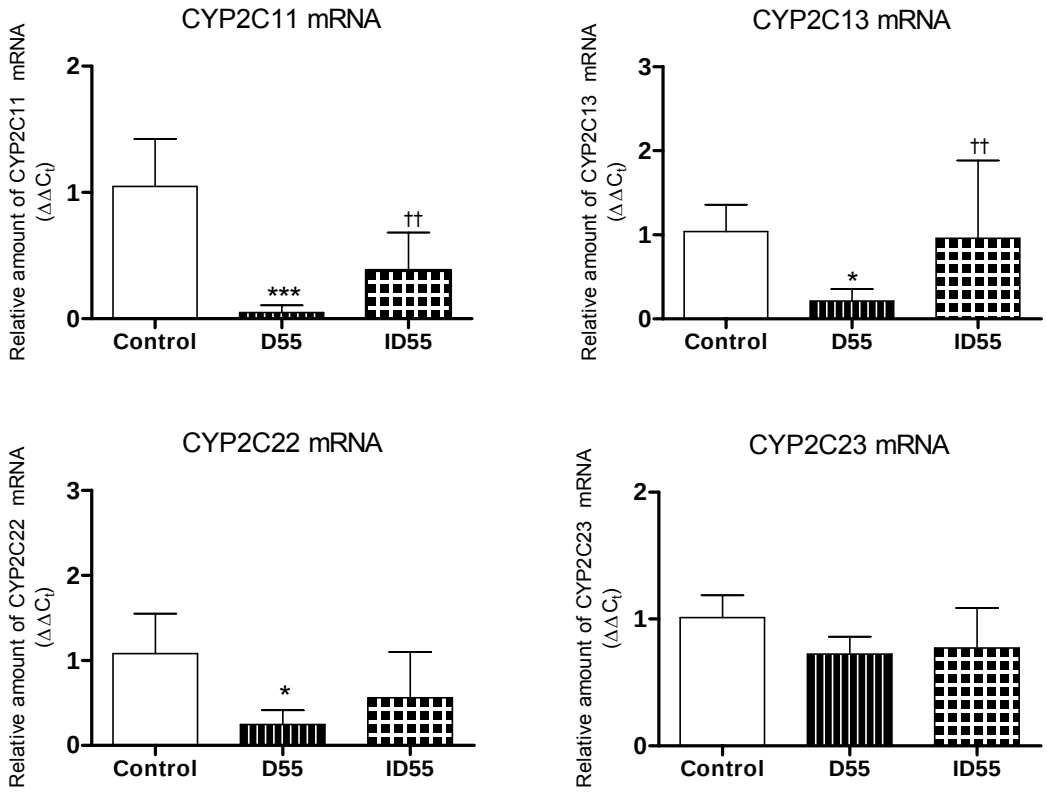

Figure 12.

Hepatic CYP2C11, CYP2C13, CYP2C22 and CYP2C23 mRNA levels in STZ diabetic rats and insulin treated diabetic rats. *Mean± S.D. were calculated (n=5-14).* $^{*}p \leq 0.05$ *values vs. control;* $^{***}p \leq 0.001$ *values vs. control;* $^{\dagger\dagger}p \leq 0.01$ *D55 vs. ID55*

4.2.3. Results of Western blot analysis

The protein level was determined by Western blot analysis. The most abundant rat liver CYP2C isoform, CYP2C11 was analyzed in control, STZ induced diabetic and insulin treated diabetic rat liver microsomes. CYP2C11 protein was highly expressed in the control animals, but no protein was detected in the D55 group (Figure 13.). Repeated experiments confirmed that the protein level in D55 animals was non-detectable. This result is in accordance with the decreased CYP2C11 mRNA expression level. The analysis showed a slight increase in CYP2C11 protein level following insulin treatment. The protein level of insulin treated samples was considerable lower in comparison to the control group. Actin was used as a standard. No differences were seen at the protein level of Actin among the groups in any experiment.

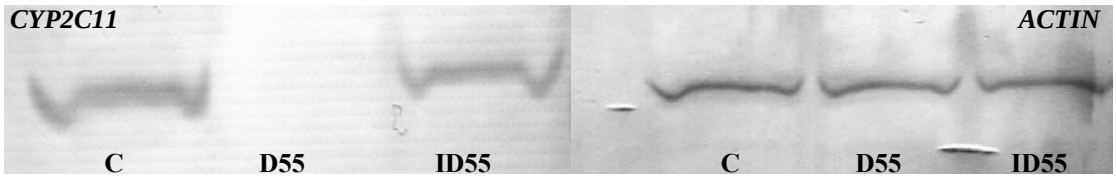

Figure 13.
Result of Western blot analysis of CYP2C11 (left) in control (C), streptozotocin-induced diabetic (D55) and insulin treated diabetic (ID55) rat liver microsomes. Actin was used as a protein amount standard in all samples (right).

4.2.4. Total P450 content and catalytic activities of hepatic CYP and FMO isoenzymes

The hepatic total P450 content and all of the catalytic activities measured are summarized in Table 9. The total P450 content significantly increased in diabetic animals and it decreased following insulin treatment which resulted in a substantially lower CYP level in insulin treated animals in comparison to control.

The phenacetin O-deethylase activity (CYP1A marker reaction) increased in diabetic rats by 61 % and decreased with insulin treatment, but none of the changes proved to be significant. The aminopyrine N-demethylase activity, a nonspecific index

reaction of CYP2B and CYP3A isoforms, did not change either in D55 or ID55 animals in our experiment. The tolbutamide 4'-hydroxlase activity, an index reaction of CYP2C, also did not change either in ID55 or D55 rats. However, the mephenytoin 4'-hydroxylase, an alternative probe substrate for CYP2C, tended to increase (33 %) in diabetes. The insulin treatment resulted in a non-significantly lower mephenytoin hydroxylase activity in comparison to control. The change in mRNA expression was not reflected in the CYP2C mediated activity. This observation led us to further studies with this subfamily. The CYP2D-mediated bufuralol 1'-hydroxylation decreased in D55 animals and increased following insulin treatment; however, these changes were not significant. The chlorzoxazone 6-hydroxylase considerable enhanced in diabetes by 3.3–fold. The insulin treatment fully compensated for the rise in CYP2E1 activity. There was no change in CYP3A-mediated testosterone 6ß-hydroxylase catalytic activity in either diabetic or insulin treated diabetic animals. FMO activity substantially increased in 55 mg/kg streptozotocin treated diabetic animals by 65 %. In insulin treated diabetic animals, the FMO completely restored to control level, moreover, FMO activity resulted in a significantly lower level in comparison to control.

Index reactions	*Control animals*	*Diabetic animals*	
		55 mg/kg STZ	*55 mg/kg STZ i.v. + Insulin*
		D55	*ID55*
Cytochrome P450 protein content *(nmol/mg microsomal protein)*	0.51 ±0.09	0.64 ±0.07*	0.28 ±0.09***†††
Phenacetin O-deethylase (CYP1A) *(pmol mg^{-1} min^{-1})*	234.3 ± 99.9	377.1 ± 138.3	267.9 ± 64.7
Aminopyrine N-demethylase (CYP2B/3A) *(nmol mg^{-1} min^{-1})*	3.41 ± 0.88	2.93 ± 0.67	2.72 ± 0.52
Tolbutamide 4'-hydroxylase (CYP2C) *(pmol mg^{-1} min^{-1})*	333.8 ± 269	372.2 ± 211	318.9 ± 162
Mephenytoin 4'-hydroxylase (CYP2C) *(pmol mg^{-1} min^{-1})*	72.2 ± 26	95.9 ± 18	55.9 ± 15†††
Bufuralol 1'-hydroxylase (CYP2D2) *(pmol mg^{-1} min^{-1})*	1468 ± 663	998.9 ± 42	1098 ± 456
Chlorzoxazone 6-hydroxylase (CYP2E1) *(pmol mg^{-1} min^{-1})*	1458 ± 794	4787 ± 1680**	1316 ± 514†††
Testosterone 6ß-hydroxylase (CYP3A) *(pmol mg^{-1} min^{-1})*	3201 ± 1239	2874 ± 1402	2208 ± 1221
Benzydamine N-oxygenase (FMO) *(pmol mg^{-1} min^{-1})*	3345 ± 1197	5532 ± 1668**	1714 ± 541*†††

Table 9.

Hepatic CYP and FMO isozyme activities in STZ-induced diabetic rats with or without insulin treatment. *Mean ± S.D. were calculated (n=5-9). *p ≤ 0.05 values vs. control, **p ≤ 0.01 values vs. control, ***p ≤ 0.001 values vs. control, ††† p ≤ 0.001 ID55 vs. D55.*

4.3. METABOLISM OF DICLOFENAC IN EXPERIMENTAL DIABETES

Diclofenac metabolic oxidation into its hydroxyl metabolites is predominantly catalyzed by the CYP2C enzyme family. The background of the assessment of diclofenac metabolism in diabetes and insulin treated diabetes is the reduced CYP2C mRNA expression and protein level (results are described in Section 4.2.2. and 4.2.3). The K_M and V_{max} values were determined at the formation of 4'-hydroxy diclofenac with rat liver microsomes prepared from control, D55 and ID55 animals. The kinetic parameters determined are summarized in Table 10. The K_M and V_{max} values increased in streptozotocin induced diabetes in comparison to control and not fully restored following insulin treatment, although they did not significantly differ from control. The CL_{int} for the disappearance of diclofenac was calculated by dividing the respective V_{max} by the respective K_M. The changes did not appear in pharmacokinetic parameters as the CL_{int} calculated did not show any differences in the three investigated groups.

	Kinetic parameters for the formation of 4'-hydroxy diclofenac		
	V_{max} *pmol x min^{-1} x mg-^{1} protein*	K_M *μM*	CL_{int} *ml x min^{-1} x mg^{-1} protein*
Control animals	178.53 ± 32.6	12.84 ± 4.0	0.0146 ± 0.003
D55 animals	313.03 ± 62.3*	21.95 ± 4.3**	0.0148 ± 0.004
ID55 animals	208.23 ± 101.0	18.05 ± 2.4	0.0113 ± 0.005

Table 10.
Kinetic parameters for the formation of 4'-hydroxy diclofenac in control, streptozotocin-induced diabetic and insulin treated diabetic rats. *Mean ± S.D. were calculated (n=6);* $^{*}p \leq 0.05$ *vs. control;* $^{**}p \leq 0.01$ *vs. control.*

4.4. RESULTS OF K-48 METABOLISM STUDY

4.4.1. *In silico* prediction

Computer simulation of the possible metabolism of K-48 molecule indicated oxidative deamination of C-NH_2, N-demethylation (alkyl bridge splitting), and N-glucuronidation on the amino group of C-NH_2.[115] Lipophilicity was also calculated by Pallas Program. The values of lipophilicity (logP) for the parent compound (K-48), the hydroxylated K-48, the epoxyde substituted K-48, and the two N-dealkylated fragments were −2.61, −3.26, −3.10, −0.57 and +0.54, respectively. Both hydroxylation and epoxidation of K-48 decreases the lipophilicity, so the elimination of K-48 may be facilitated.

4.4.2. *In vitro* microsomal metabolism assessment

The biotransformation of K-48 was determined by HPLC-MS and HPLC-ECD following incubation with control or streptozotocin induced diabetic microsomes. The metabolic rate of K-48 after 30 minutes microsomal incubation was found to be very slight, only approximately 15 % decrease and a small fragment with a molecular peak of 122 amu resulted by HPLC-MS analysis. This compound represents either one of the substituted pyridinium rings present in the parent molecule. Another molecule showed a sharp signal at 315 amu, suggesting the hydroxylation of K-48. The parent compound was presented at 299 amu. The suggested *in vitro* metabolic patterns are shown in Figure 14.

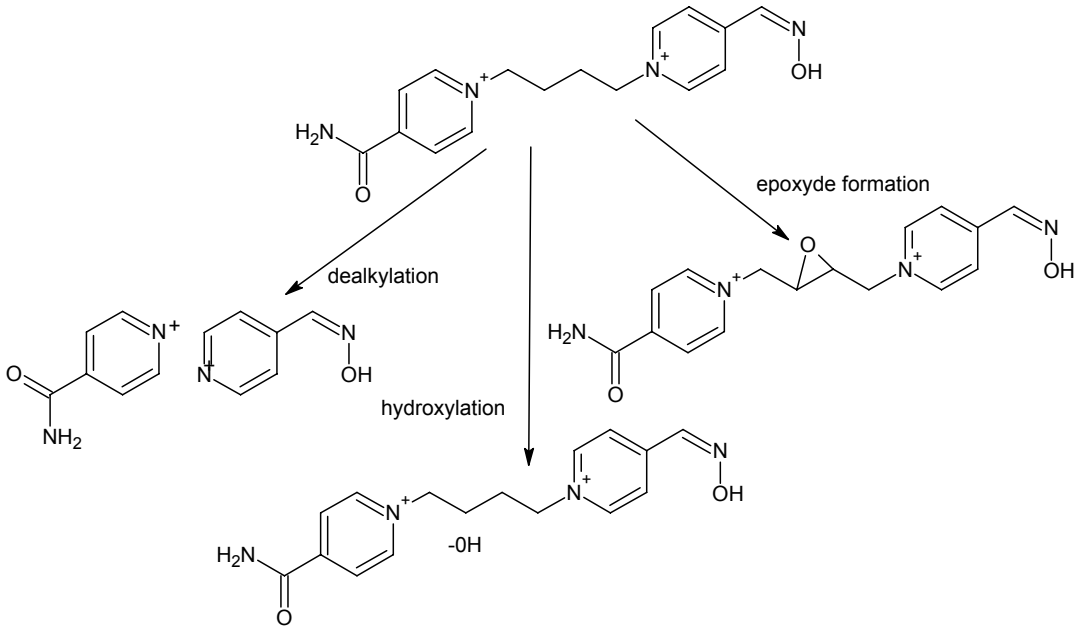

Figure 14.
Our suggested *in vitro* biotransformation of K-48

The analysis by HPLC-ECD was suitable for monitoring changes in K-48 decrease in *in vitro* conditions. The decrease in K-48 concentration was approximately 20 % following 30 minutes incubation. There were no significant changes in diabetic rat liver microsomes in comparison to control (Figure 15).

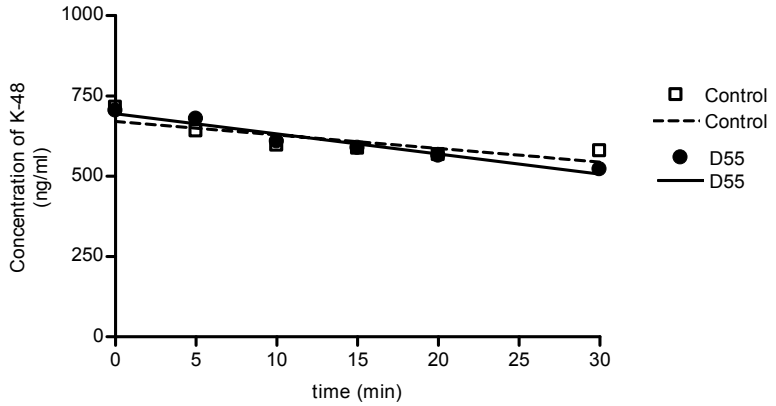

Figure 15.
Microsomal metabolism of K-48 with control and STZ diabetic rat liver microsomes.
□ *control rat liver microsomes, ---Linear regression of control,* ● *liver microsomes of 55mg/kg streptozotocin treated diabetic rats,* ¯ *Linear regression of D55.*

4.4.3. Results of *in vivo* study

Various body compartments (serum, CSF, urine and brain) of rats were subjected to HPLC-MS analysis after i.m. administration of K-48. The parent compound was found in the serum, but no metabolite was detected. Similarly, only K-48 was found in the rat CSF and in the homogenate of brain (Figure 16). In these physiological compartments (serum, CSF and brain) neither epoxidation nor fragmentation and hydroxylation were detectable. The predominant signal was from the unchanged K-48.

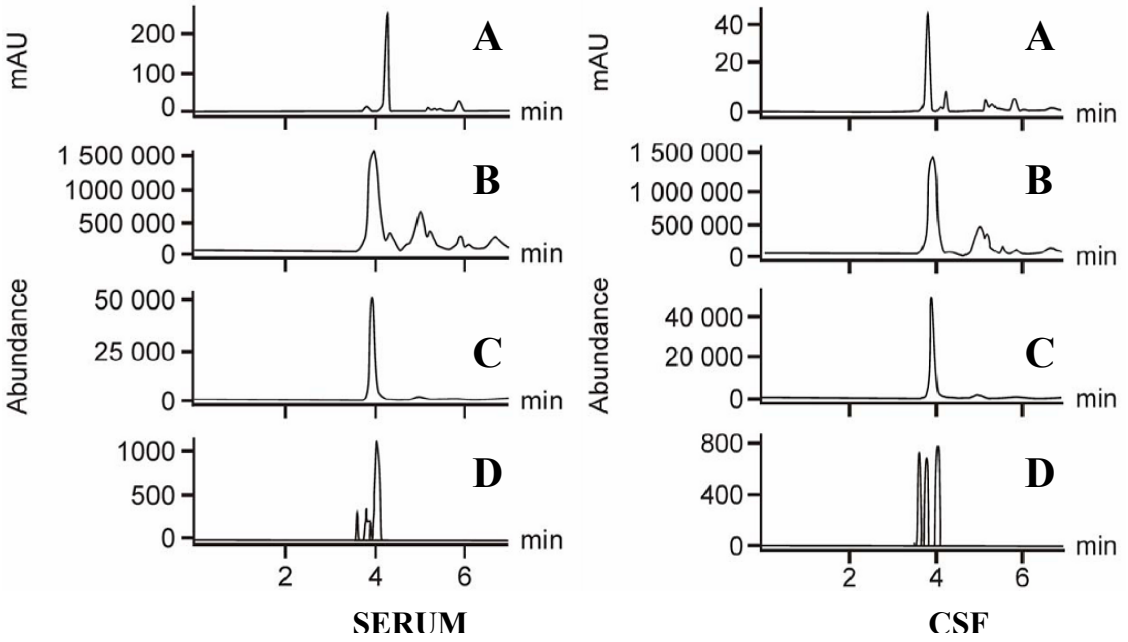

Figure 16.
HPLC-MS analysis of the serum (left) and cerebrospinal fluid (right) in rats treated with K-48 intramuscularly. *Detection was done with a diode array detector (**A**), total ion current: 100-1000 amu (**B**), 299 amu (**C**) and 313 amu(**D**)*

Urine was collected in a four hour period and was screened for K-48 and its metabolites. Urinary elimination of K-48 showed a single intensive peak that eluted in the range in which K-48 and its potential metabolites elute, between two and six minutes (Figure 17). It was monitored at 313 amu to search for tentative epoxide of K-48. The abundance of this peak was comparable to the abundance of 299 amu in the

serum. At the same time, no unchanged K-48 was found in the urine at a comparable amount to that of the epoxide (313 amu).

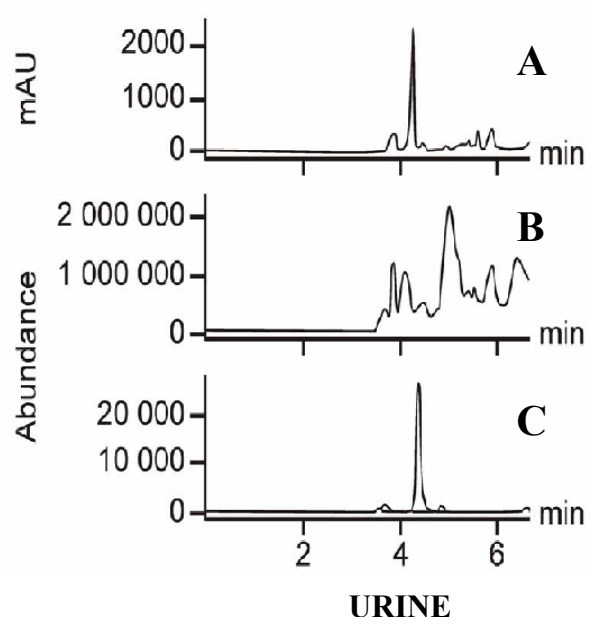

Figure 17.
HPLC-MS analysis of the urine of rat i.m. treated with K-48. *Detection was done with a diode array detector (**A**); total ion current: 100-1000 amu (**B**) and 313 amu (**C**)*

5. DISCUSSION

5.1. EXPERIMENTAL DIABETIC MODEL

Diabetes occurs when the nutritional system is unable to complete the process of breaking down and absorbing carbohydrates. Diabetic patients are unable to absorb glucose because of insulopenia or insulin resistance, therefore, sugar begins to accumulate in their blood. Diabetes mellitus influences the whole energy use; affects on the carbohydrate, lipid, protein and drug metabolism. The effect of diabetes mellitus on various drug metabolising enzyme systems depends on the type of disease. There are two classes of diabetes mellitus, type I and type II, with type II compromising over 80 % of clinical cases. Type I diabetes (also called juvenile or insulin-dependent diabetes mellitus – IDDM) is the most severe form which the disease takes and without treatment it is invariably fatal. It generally develops when patients are in adolescence and is characterized by the destruction of the β-cells in the islet of Langerhans which are responsible for the production of insulin. Bacterial infection triggers an immunological reaction in susceptible persons, which can produce this form. Type II diabetes (noninsulin dependent diabetes mellitus – NIDDM) generally develops later in life and is caused by insulin resistance. Obesity and family history are prime risk factors for the development of this form. Type I diabetes is treated with insulin supplementation, while type II can often be controlled with diet, exercise and oral hypoglycaemic agents.[116,117,118]

STZ diabetes is widely used for modelling type I diabetes mellitus. In our studies the alterations in physiological and biochemical parameters such as elevated blood glucose level and relative wet liver weight, the loss in body weight and the other symptoms (polidypsia, polyuria) indicated the development of the pathological state following both intraperitoneal and intravenous injection of streptozotocin. Administration of insulin did not completely restore the blood glucose level with any protocol we used; however, by varying the injection of Humulin N (8.30 am) and Insulatard insulin (4.30 pm) the blood glucose level decreased significantly in comparison to the Ultratard insulin administered twice a day. The insulin we used was human recombinant isophane insulin which did not seem to have sufficient effect on

rats. Due to the incomplete restoration to the physiological conditions of controls; the incomplete recovery in the parameters we measured may not be surprising. The different responsiveness of animals to insulin can explain the high S.D. values we observed. The repeated results excluded the technical failures and confirmed our hypothesis. The inter-individual variations could be responsible for the differences; however, we also starved the animals before insulin treatment to minimize the variations due to different food-uptake.

5.2. CHANGED INTESTINAL TOTAL CYP CONTENT AND CYP3A ACTIVITY IN DIABETES

Although the liver is well-known playing the major role in drug metabolism, the metabolic capacity of the intestine is increasingly recognized. *In vivo* studies eventually pointed out that significant first-pass metabolism by the intestinal wall have implications for the bioavailability of several compounds. In addition, it can occur that in severe disease states the extrahepatic pathways such as the small intestinal route might compensate for the impaired hepatic function. Therefore, it is necessary to clarify whether the intestinal metabolism altered as a result of a pathological state such as diabetes.

Decreased intestinal CYP3A activity

The CYP3A constitutes only approximately 30 % of total human hepatic cytochrome P450 content; it accounts for more than 80 % of the CYP content in human small intestine.[48,51] CYP3A subfamily is also highly expressed in rat intestine and it is widely studied.[119,120,121] Therefore; our investigations firstly focused on the changes in intestinal CYP3A functions in insulin treated and untreated experimental diabetic rats. Even though the total cytochrome P450 content was statistically unchanged, the metabolic capacity of CYP3A enzyme showed a marked decrease possibly due to inactivation in the diabetic state. The recovery of the enzymatic changes following insulin administration was also indicated. The reason for the decreased activity in spite of the unchanged total P450 content could be the outcome of a kind of covalent down-regulation (e.g. phosphorylation), which is supported by the known posttranslational

modification - via phosphorylation catalyzed by PKA - which have an outstanding role in CYP regulation. Phosphorylation controls the CYP function like a switch by which the enzymes get into their fully inactivated form, while reduction can not be observed in enzyme content.[31] CYP3A has the property to be regulated by phosphorylation, whereas it is not common.[15]

Another reason for the moderate increase in CYP amount in spite of decreased CYP3A activity could also be a remarkable induction of another isoform such as CYP2E1 known to be highly induced in diabetic liver.[4] This explanation is supported by the publication of Al-Turk and co-workers (1980) who reported an elevation in 7-ethoxycoumarin O-deethylase and aromatic hydrocarbon hydroxylase activity (CYP2E1 and CYP2A6, respectively) in the intestine of male and female rats in diabetes.[2,3]

Parallel to intestinal metabolism study, the hepatic characterization of dominant CYP isoforms were also determined (data not shown). The CYP3A activity does not change in the liver in treated and untreated streptozotocin induced diabetes. It suggests and confirms the literature data, that intestinal and hepatic monooxygenases are regulated independently.[16,119]

Difficulties in investigating intestinal metabolism

The microsomal monooxygenase content of the small intestine is much lower than that of the liver.[40] Moreover, the cytochrome P450 of a rat's small intestine suffers spontaneous degradation into the inactive cytochrome P420 form during the preparation of microsomes.[110,121] These facts render difficulties for the preparation and investigation of intestinal metabolism. The rapid preparation and additives (trypsin inhibitor, glycerin, and heparin) can increase the yield of functionally active intestinal P450s.[110]

Due to technical reasons (hardly detectable activities, aforementioned degradation, low expressions of the other isoenzymes abundant in the gut), the scrutinization of the mechanism of the changes was impossible. Anti-CYP3A2 antibody was used to determine the protein level of intestinal CYP3A by Western blot. It is known that CYP3A2 is not expressed in rat small intestine;[120] however, the antibody against rat hepatic CYP3A2 is published to cross react with CYP3A9 expressed in male and female rat small bowel.[119] The antibody did react with the CYP3A very slightly or not at all in our experiment (data not shown).

Correlation between blood glucose concentration and CYP3A activity

In our study the insulin level was altered by insulin administration in diabetic rats (ID70). Nevertheless, the blood glucose level, an inverse parameter of insulin concentration, was regularly checked. The determination of glucose concentration is simple and characteristic of the severity of diabetes mellitus. The regression analyses showed a significant inverse correlation between CYP3A activity and average blood glucose concentration in diabetic rats.

The changed CYP3A activity in small bowel and gastrointestinal complications in diabetes seems to have importance especially when the decreased barrier function of intestine is considered. It is also suggested that the decrease of functionally active intestinal CYP3A enzyme in the diabetic state might lead to increased bioavailability of drugs which are substrates of intestinal CYP3A following p.o. administration.

5.3. CHANGES IN HEPATIC CYTOCHROME P450s IN DIABETES

A long-term diabetic state was investigated in our 28 days study. A group of diabetic animals received insulin for 9 days length of which was insufficient to restore the changes developed during the long-term diabetic state. However, regarding our results the changed cytochrome P450 enzyme content, mRNA expression and catalytic activity in the liver were mostly in accordance with those described in the literature.

The hypoinsulinaemia and hyperketonuria in diabetes

In the diabetic state total cytochrome P450 content, the gene expression of CYP1A2, CYP2E1 and CYP3A1 was increased as it was observed by Shimojo et al. (1993), Sakuma et al. (2001) and Favreau et al. (1988).[4,7,79] Following insulin treatment the hepatic microsomal CYP content was decreased to the control value as published by Vega et al. (1993).[6] The catalytic activity of CYP1A2 (phenacetin O-deethylation) and CYP2E1 (chlorzoxazone 6-hydroxylation) increased as was expected by the results of the mRNA expression study. The role of insulin and ketone bodies in the regulation of P450s (CYP1A2, CYP2B1, CYP2E1) has been proven.[122,123] The physiological functions of CYP2E1 involve lipid metabolism and ketone utilization in starvation,

obesity, and diabetes.[124] In our study, the induction of the mRNA expression level and catalytic activity of CYP2E1 due to insulin deficiency, whether or not accompanied by hyperketonuria, was 2.2-fold and 3.3-fold in diabetes, respectively. The CYP2E1 is regarded to be regulated mostly by mRNA and protein stabilization following phosphorylation.[24,37,38] Recent data also show that impaired secretion of growth hormone is also responsible for the induction of CYP2E1 in diabetic rats and starvation enhances the gene transcription of CYP2E1.[125,126] We showed the elevation of the mRNA expression in diabetes; however, the degree of the increase in catalytic activity was not fully in accordance with the induction in gene expression. This fact suggests both the transcriptional and posttranscriptional regulation of CYP2E1. The catalytic activity and also the gene expression of CYP2E1 restored following insulin treatment, indicating the suppressive effect of insulin on the CYP2E1 isoenzyme.[127]

The mRNA expression of CYP2B and the related aminopyrine N-demethylase activity does not change in insulin treated and untreated animals, although Sakuma and co-workers (2001) and Reinke et al. (1978) observed a decrease in both.[8,79]

The regulation of hepatic CYP3A isoforms

The aminopyrine N-demethylation is also catalyzed by the CYP3A isoenzymes. The gene expression of the CYP3A1 increased, although the changes proved not to be significant. The CYP3A2 was unaltered in both insulin treated and untreated diabetes which may be reflected in the unchanged aminopyrine N-demethylation and testosterone 6ß-hydroxylation. The CYP3A1 isoform decreased significantly following insulin treatment. Insulin has numerous and varied cellular effects, including increased glucose transport, promotion of DNA and protein synthesis, cell division and regulation of gene expression etc. The regulation of insulin is carried out by the insulin signalling pathway involving several hormones, mediators, enzymes, receptors etc.; therefore, it can elicit dramatic changes in the absence of any other hormonal alterations.[83,127] In diabetes, the hypoinsulinaemia is accompanied by impaired growth hormone secretion. Growth hormones are involved in the regulation of CYP3A and also of CYP2E1 isoenzyme; therefore, the increase of the protein content may be the consequence of the reduced growth hormone secretion.[4,122,128,129] The result was confirmed by the publication of Ackerman and co-workers (1977), that the lack of insulin in the insulin

signalling pathway may be responsible for the changes and not hyperglycemia.[80] This hypothesis is also supported by the fact that infusion of glucose and diabetogens has no effect upon the metabolising activities *in vitro* as published by the aforementioned authors.[80]

Altered CYP2D2 activity and mRNA expression

The CYP2D2 is known to be regulated only at transcriptional level by the nuclear receptor HNF-4α. It was reflected in the reduced hydroxylation of bufuralol following decreased CYP2D2 gene expression. The insulin treatment does not restore either the mRNA expression or the enzyme activity of CYP2D2. It seems insulin may not regulate the gene expression of CYP2D directly. The decrease of this enzyme activity in diabetes may result from another regulator.

Regulation of FMO isoenzymes by insulin

In contrast to CYPs, the regulation of FMO enzymes is not well known. The alteration of FMO activities were observed in modified physiological states of animals: pregnancy, starvation, ascorbic acid deficiency, gonadectomy and diabetes.[71,72,73,74,75] Our previous study indicated that short-term diabetic state elevates the catalytic activity and the gene expression of FMO1 and FMO3 isoenzymes and both, the activity and the expression were restored on insulin treatment. The results in long-term diabetic state also suggested that insulin has a role in the regulation of FMOs and the FMO3 is more sensitive on insulin-deficiency than FMO1.

5.4. CYP2C SUBFAMILY AND DICLOFENAC METABOLISM IN DIABETES

Regulation of CYP2C isoenzymes

Drug metabolising enzymes can play important roles in serious drug interactions. Pathophysiological conditions such as diabetes mellitus are known to influence microsomal metabolism of xenobiotics enhancing the incidence of drug interactions.[1] Our results indicate that type I diabetes mellitus model have a remarkable effect on the expression of CYP2C enzymes. The physiological role of the CYP2C subfamily is the

steroid hydroxylation in 2α and 16α position, whereby it contributes to the oxidative metabolism of testosterone and androstenedione in adult male rats.[130] The promoter region of CYP2C constitutes the GRE and CAR recognition sequences which mediate the transcriptional regulation of GR/GR, CAR/RXR and PXR/RXR nuclear receptor dimers.[26,27,28] The HNF-4α and PPARα also regulates the expression of CYP2C isoenzymes, but they seem not to be the major determinant for the liver specific expression of their genes.[131]

Our study, which is in agreement with the literature data, resulted in the decrease of protein expression of the major male-specific isoform, CYP2C11 in diabetes. The mRNA expressions of the CYP2C11, CYP2C13 and CYP2C22 isoenzymes were also suppressed in the streptozotocin treated experimental diabetic model used. The CYP2C11 and CYP2C13 are regulated by the status of pituitary, gonadal and thyroidal hormone secretion.[83,84] The serum glucose and ketone bodies increase and the serum level of insulin, pituitary growth hormones, androgen and thyroid hormones decrease in diabetes.[83] The reduction of the circulating pituitary hormones may explain the down-regulation of CYP2C protein level and mRNA expressions in diabetes. Insulin treatment can reverse many factors such as serum glucose, ketone bodies, and metabolic capacity of P450s.[84] In our study, the reversal of the protein level and mRNA expressions following insulin treatment was tendentious, but only the pituitary hormone regulated CYP2C11 and CYP2C13 increased significantly in comparison to the diabetic state.

The CYP2C22 is also involved in the metabolism of steroid hormones and shows similar sex-specific expression like CYP2C11 and CYP2C13 in rats; however the regulation of this isoform has not been investigated so far.[132] Its mRNA expression also substantially decreased in diabetes, but the insulin treatment resulted in an insignificant elevation.

The CYP2C23 was the single isoenzyme which did not alter in both STZ induced and insulin treated diabetic animals. The CYP2C23 is expressed mainly in kidney and liver and it is responsible for the metabolism of arachidonic acid into epoxyeicosatrienoic acids and hydroxyeicosatetraenoic acids.[133] It is reported that the streptozotocin induced diabetes increases the expression of CYP2C23 in rat kidney,[134] while Pass and co-workers published that sex and pathophysiological status have no effect on the expression of CYP2C23 in mice liver.[132] Our study confirms the results of

the aforementioned author; since no significant change was observed in the gene expression of rat hepatic CYP2C23 in diabetes. It is also published, that the generally used chemical inducers of human CYP2C isoenzymes and peroxisome proliferators (e.g. fibrates) which activate the PPARα that mostly express in rodents, have a contrary, suppressor effect on rat CYP2C11 and CYP2C23.[135,136] In spite of the repressive effect of exogenous chemicals, the hepatic CYP2C23 remain fairly stable in physiological conditions and seems not to be regulated by gonadal and pituitary hormones.[137] All these observations support the lack of alteration of CYP2C23 mRNA expression as we indicated in diabetic state.

Diclofenac metabolism in diabetes

To find evidence for substantially changed metabolism – consequently exposition - caused by suppressed CYP2C metabolising capacity in diabetes, we used a dominant CYP2C11 substrate, the anti-inflammatory drug, diclofenac. It is metabolised mainly by CYP2C11 and by CYP2C9 in rat and human liver, respectively.[93,138] Based on the production of 4'-hydroxy diclofenac we observed a significant change in the enzyme kinetic parameters of diclofenac in diabetes. It is reported that the disease brings about a smaller V_{ss}, and a greater AUC values of intravenously administered diclofenac due to slower CL_{nr} and faster CL_r values. It is also published that diabetes has no effect on these pharmacokinetic parameters after oral administration.[97] The changes in clearance following intravenous administration are explained by the reduced CYP2C11 activity in streptozotocin induced diabetic rats.[97] Our results do not correspond with these results, since we measured a significant increase in K_M, V_{max} values in diabetic rat liver microsomes in comparison to control. The insulin ameliorated the changes in the enzyme kinetic parameters and did not differ from control. In spite of the enhanced K_M and V_{max}, indicating faster and less affinitive enzymes in diabetes, the CL_{int} and metabolic bioavailability calculated do not show significant differences among the three groups. Based on these results, the possibility of interactions due to the decreased metabolising capacity is small. The apparent discrepancy between reduced CYP2C11 enzyme expression and altered enzyme kinetic parameters of diclofenac metabolism may be explained by a redistribution of the enzyme metabolising capacity or the differences in the investigated dose in the *in vivo* and *in vitro* experiment.

The study provided evidence for the alteration in CYP2C isoforms at mRNA and protein expression levels, however, the changes of enzyme kinetic parameters is not reflected in altered intrinsic clearance; therefore, the possibility of changed drug biotransformation in the case of diclofenac is *in vitro* not confirmed.

5.5. METABOLISM, DISPOSITION AND ELIMINATION OF K-48

Compound K-48 is a promising antidote against organophosphate intoxication. The pralidoxim and obidoxim used mostly in US and Europe, respectively, have not sufficient effect on the reactivation of acetylcholine esterase enzymes. Because of the threat of terrorist attacks it appears urgent to evaluate medical interventions that may be effective in mass exposures. The antidotes should have a broad spectrum of action against various OPs along with minimal adverse effects. The CNS efficacy and its administration by autoinjector intramuscularly would also be essential.[139]

Structurally K-48 is a bisquaternery asymmetric compound with an intact –C=N-OH and a -CO=NH_2 group. The former mentioned group is essential for the organophosphate removal; the latterly mentioned group may be suitable for hepatic first-pass metabolism to occur. Pallas Program was applied for the *in silico* prediction of the possible metabolites and logP values. Deamination of C-NH_2, N-demethylation (alkyl bridge splitting) and glucuronyl conjugation on the amino group was noted.[115] Liver microsomes from control and diabetic rats were used for *in vitro* metabolism study of K-48. Only a moderate, 15-20 % reduction in the concentration of parent compound could be measured after 30 minutes. The metabolism of K-48 was also tested by streptozotocin induced diabetic microsomes. No significant differences were found *in vitro* between the metabolism with streptozotocin treated and untreated (control) animals. The NADPH dependent metabolism of K-48 (no reduction of K-48 was seen in NADPH-free control incubation) refers to the CYP and FMO mediated metabolism. In spite of the changes in mRNA expression and catalytic activity of both monooxygenases in diabetic state, the metabolism of K-48 *in vitro* did not alter which may suggest the involvement of an unchanged P450 isoform in K-48 metabolism.

HPLC-ECD is suitable for monitoring changes in K-48 concentration in *in vitro* conditions. To identify the produced metabolite(s) use of advanced technique such as

HPLC with on-line MS detection was indispensable. A small fragment with a molecular peak of 122 amu resulted following 30 minutes incubation with rat liver microsomes. This compound is possibly one of the substituted pyridinium rings presented in the parent molecule. The other molecule was displayed at 315 amu, implying the hydroxylation of K-48. No other metabolites predicted *in silico* (N-dealkylation or epoxide formation) could be seen.

Oximes like pralidoxime are commonly used in combination with atropine. The most important requirement to oximes would be to cross the BBB and ameliorate the CNS effects of organophosphate poisoning. Therefore, the brain is the primary target organ for the newly synthesized oximes like K-48. Several compartments: serum, CSF and brain were analyzed for K-48 and its metabolites. Poor penetration of K-48 (3-8 %) through BBB was indicated by Kassa and co-workers (2005).[104] In concert with the above referred study, only K-48 was found in all three compartments in our experiments. Metabolites were absent in the serum, CSF and brain. It is known that K-48 has a better protective action than other PACERs.[99,104,107] However, this advantage may be caused by the higher efficacy of K-48 in the peripheral nervous system and not a better penetration to the CSF and brain.[108] The logP for K-48 and hydroxylated and epoxide substituted metabolite is -2.61, -3.26 and -3.10, respectively. The relatively high hydrophilic character and only moderate lipophilicity[104,105] predict only a moderate penetration through BBB and a relatively rapid elimination by means of urine. Both hydroxylation and epoxidation decrease the lipophilicity facilitating the elimination of compounds. Being aware of this fact, it is not surprising that any of the metabolites could not be found in the brain, and the elimination through urine is preferred. Single ion monitoring was used to detect any unchanged K-48 from samples derived from rat urine. A relatively sharp peak was found in the rat urine when HPLC was monitored at 313 amu which suggests an epoxide metabolite. Epoxide contamination in the treated substance was excluded; the epoxide metabolite of K-48 is probably due to its biotransformation.

To summarize our results and compared to the recently used oxime (pralidoxime), K-48 proved to metabolise to a lower extent which was not altered in experimental diabetes. Pralidoxime was reported to have an extensive metabolism *in vitro*.[140] The elimination of pralidoxime due to their polarity is rapid which brings about the

unchanged excretion of pralidoxime *in vivo*.[141] The elimination of K-48 by urine was also observed, but only epoxide metabolite and no unchanged K-48 could be detected by HPLC-MS. The efficacy of K-48 in the case of some OP poisoning seems to be more sufficient. Our study confirmed only a low penetration into brain and CSF, which shows that the better reactivating effect is not the outcome of a higher CNS efficacy.

6. CONCLUSION

- It was revealed that diabetic state has an effect on the intestinal CYP mediated metabolism. Reduced CYP3A mediated metabolism in spite of the statistically unaltered total CYP content was resulted in STZ induced diabetes, which can be explained either by posttranslational regulation of the enzyme via covalent down-regulation (e.g. phosphorylation) or by a change in the intestinal CYP enzyme composition. The intestinal CYP3A activity was sensitive to insulin administration.

- Inverse correlation was found between blood glucose concentrations (regarded as a marker for insulin level) and CYP3A function suggesting the involvement of insulin in the intestinal CYP3A regulation.

- We demonstrated that long term 28 days diabetes induced the most remarkable increase in hepatic total CYP content, and in hepatic CYP2E1 and FMO3 gene expression and function and a significant decrease in CYP2C11 and CYP2D2 mRNA expression. The CYP1A2, CYP2B, CYP3A1, CYP3A2 and FMO1 did not show statistical alterations either in expression or in function in diabetes.

- It was recognized that the hepatic total CYP content and CYP1A2, CYP2E1, CYP3A1 were significantly decreased while CYP2C11 gene expression increased following a nine day period insulin administration. The catalytic activities of FMO and CYP2E1 were the most sensitive to insulin and their function resulted in a lower activity in comparison to control after insulin treatment.

- Furthermore, it was shown that the CYP2C11 gene expression is not in concert with the tolbutamide and mephenytoin 4'-hydroxylase activity, although the decreased gene expression in untreated diabetes was also reflected in reduced CYP2C11 protein level. The insulin also restored the CYP2C11 mRNA and protein level.

- It was further revealed that insulin treated and untreated diabetic state had an effect on the gene expression of CYP2C13 and CYP2C22, however, the CYP2C23 isoenzyme did not show any alteration. We suggested that the different attitude of CYP2C23 gene may be due to its different physiological function (arachidonic acid metabolism) and regulation.

- We concluded that in spite of altered (increased) K_M and V_{max} values of diclofenac 4'-hydroxylase in diabetic state the intrinsic clearance calculated remained unchanged. The altered *in vivo* pharmacokinetics of diclofenac in diabetes published might be explained by the differences between the *in vivo* and *in vitro* effective dose.

- It was shown that the changes in CYP2C enzymes in diabetes do not bring about alteration in the biotransformation of diclofenac which may suggest the redistribution of metabolic pathways.

- K-48 showed only a moderate biotransformation (15-20 %) with both diabetic and control rat liver microsomes which suggests that diabetes does not affect the metabolism of K-48 molecule.

- In *in vitro* microsomal metabolism studies only a hydroxyl metabolite of the pyridinium aldoxime cholinesterase reactivator, K-48 was identified. No other metabolites predicted *in silico* were seen *in vitro*.

- It was demonstrated that only the parent compound, K-48 was found in serum, CSF and brain. Metabolites were absent in all three compartments. We did not find any unchanged K-48 in rat urine; presumably an epoxide metabolite could be identified by HPLC-MS.

- We also indicated that K-48 had a very poor penetration through the BBB because only a low concentration of K-48 was measured in the brain and no metabolites could be found.

7. SUMMARY

Insulin dependent diabetes mellitus (IDDM) is a complex metabolic disorder, which develops changes in the cytochrome P450 (CYP), mediated metabolism in the liver and in the small intestine and it may also produce altered bioavailability. The main goal of this study was to reveal these metabolic changes in experimental diabetic rats and to evaluate their significance in the drug metabolism.

Decreased intestinal CYP3A mediated metabolism in spite of the statistically unaltered total CYP content resulted, which suggests either posttranslational regulation of the enzyme via covalent down-regulation (e.g. phosphorylation) or a change in the intestinal isoenzyme composition. Insulin may be involved in the intestinal CYP3A regulation since inverse correlation was found between the blood glucose concentration (as a marker for insulin level) and the CYP3A function. The hepatic total CYP content and the hepatic CYP2E1 and FMO3 gene expression and function were seen to change remarkably in untreated long-term diabetes and following insulin treatment. Our study concentrated on rat hepatic CYP2C11, CYP2C13, CYP2C22 and CYP2C23 isoforms and reduced gene expressions with the exception of CYP2C23 were found in diabetes, which is explained, by its different physiological role and regulation. The mRNA level of CYP2C11 and CYP2C13 isoforms were sensitive to insulin showing the role of insulin in their regulation. The study resulted in unaltered CL_{int} of the CYP2C substrate; diclofenac in either insulin treated or untreated diabetic rats. Similarly, unchanged biotransformation of the cholinesterase reactivator oxime, K-48 was seen in diabetes. These results suggest no influence of diabetes and particularly compensated diabetes on the metabolism of the two drugs investigated. The *in vitro* and *in vivo* metabolism studies of K-48 resulted in a weak metabolism. None of the *in silico* predicted metabolites but the K-48 was found in serum, CSF and brain while an epoxide metabolite was detected in urine. The presence of K-48 in the brain shows a moderate penetration of K-48 to the CNS.

8. ÖSSZEFOGLALÁS

Az inzulinfüggő diabétesz (IDDM) megváltoztatja a máj és a vékonybél monooxigenáz enzimeinek működését. A megváltozott metabolikus kapacitás megváltozott gyógyszer-biohasznosulást eredményezhet. Vizsgálataink célja patkányban streptozotocinnal (STZ) kiváltott IDDM citokróm P450 enzimrendszerre (CYP) kifejtett hatásának és jelentőségének tanulmányozása volt, egy gyógyszer és egy fejlesztés alatt álló gyógyszerjelölt metabolizmusában.

Kísérletes diabéteszben csökkent intesztinális CYP3A aktivitást mutattunk ki változatlan citokróm P450 tartalom mellett, melyet egy lehetséges poszttranszlációs szabályozással (pl. foszforiláció), illetve az intesztinális CYP izoenzimösszetétel megváltozásával magyarázhatunk. Fordított korrelációt találtunk az intesztinális CYP3A aktivitás és az inzulinszint egyik markere, a vércukorszint között, ebből az inzulin reguláló szerepére következtetünk. 28 napig fennálló diabéteszben és inzulinadást követően a legjelentősebb változást a máj CYP tartalomban illetve a hepatikus CYP2E1 és FMO3 génexpresszióban és aktivitásban mértük. Patkány máj CYP2C11, CYP2C13 és CYP2C22 mRNS szintje csökkent diabéteszben, ez inzulinkezelésre - a CYP2C22 kivételével - szignifikánsan nőtt. A CYP2C23 génexpresszió nem változott kezelt és kezeletlen diabéteszben, amit ezen izoforma eltérő fiziológiás szerepével és szabályozásával magyarázunk. A csökkent CYP2C expresszió ellenére a diclofenac metabolikus klírensze nem változott kísérletes diabéteszben. Nem volt eltérés a piridinium aldoxim kolinészteráz reaktivátor, a K-48 molekula metabolizmusában sem. Feltételezzük tehát, hogy a kiválasztott molekulák biotranszformációja nem változik meg jelentősen kezeletlen és inzulinnal kompenzált cukorbetegségben. A K-48 *in vitro* és *in vivo* vizsgálata a vegyület mérsékelt metabolizmusát mutatta. Patkány szérumban, likvorban és agy homogenátumban csak a K-48 vegyületet, míg vizeletben annak epoxid metabolitját tudtuk kimutatni. Az alacsony K-48 koncentráció az agyban a molekula kismértékű vér-agy gát penetrációjára utal.

9. PUBLICATIONS

Publications for the dissertation based on

1. Tímea Borbás, Bernadett Benkő, Imola Szabó, Balázs Dalmadi, Károly Tihanyi: Insulin in flavin-monooxygenase regulation. Flavin-containin monooxygenase and cytochrome P450 activities in experimental diabetes. *European Journal of Pharmaceutical Sciences*, 28(1-2), 2006

2. Bernadett Benkő, Huba Kalász, Krisztina Ludányi, Georg Petroianu, Kamil Kuca, Ferenc Darvas, Kornélia Tekes: *In vitro* and *in vivo* metabolism of K-48. *Analytical and Bioanalytical Chemistry*, 389(4):1243-7, 2007

3. Bernadett Benkő, Rudolf Laufer, Róbert Ohmacht: HPLC Analysis of microsomal metabolism of K-48. *Acta Chromatographica*, 19, 61-72, 2007

Other publications

1. Benkő Bernadett, Zelkó Romána: Minőségi rendszerek a gyógyszerészetben I., *Gyógyszerészet,* 46: 264-271, 2002

2. Benkő Bernadett, Zelkó Romána: Minőségi rendszerek a gyógyszerészetben II. - Minőségi rendszerek áttekintése. Gyógyszerészet, 46: 462-469, 2002

Publication in progress

Bernadett Benkő, Tímea Borbás, István Likó, Zoltán Urbányi, Károly Tihanyi: Metabolism of Diclofenac in streptozotocin induced diabetes

Oral presentations

1. Benkő Bernadett, Borbás Tímea, Dalmadi Balázs, Szeberényi Szabolcs, Leibinger János, Tihanyi Károly: Intesztinális gyógyszermetabolizmus jelentősége és stabil citokróm P450 tartalmú mikroszóma preparálása patkány vékonybélből. *Ph.D. TUDOMÁNYOS NAPOK 2003*, 2003. április 10-11., Budapest
2. Borbás Tímea, Benkő Bernadett, Dalmadi Balázs, Szeberényi Szabolcs, Leibinger János, Beke Gyula, Tihanyi Károly: Koexpresszió CYP és FMO izoformák között enzimatikus szinten, humán és patkány mikroszómán vizsgálva. *Ph.D. TUDOMÁNYOS NAPOK 2003*, 2003. április 10-11., Budapest

3. Benkő Bernadett, Borbás Tímea, és Tihanyi Károly: Streptozotocinnal kiváltott diabétesz hatása az intesztinális gyógyszermetabolizmusra patkányban. *Ph.D. TUDOMÁNYOS NAPOK 2004*, 2004. április 8-9., Budapest
4. Borbás Tímea, Benkő Bernadett és Tihanyi Károly: Streptozotocinnal kiváltott diabétesz hatása a hepatikus gyógyszermetabolizmusra patkányban. *Ph.D. TUDOMÁNYOS NAPOK 2004*., 2004 április 8-9., Budapest
5. Benkő Bernadett, Borbás Tímea, Tihanyi Károly: Extrahepatikus metabolizmus. *"Gyógyszer az ezredfordulón" 2004*, 2004. március 25-27, Sopron
6. Benkő Bernadett, Borbás Tímea, Győrke Imola: Streptozotocin-indukálta diabétesz hatása az intesztinális és hepatikus gyógyszermetabolizmusra patkányban. *VII. CLAUDER OTTÓ EMLÉKVERSENY,* 2004. október 14-15., Visegrád

Posters

1. Borbás Tímea, Benkő Bernadett, Dalmadi Balázs, Győrke Imola, Vastag Mónika és Tihanyi Károly: Humán hepatocita citokróm P450 izoenzimek adatainak statisztikai kiértékelése. GYÓGYSZER AZ EZREDFORDULÓN V. TOVÁBBKÉPZŐ KONFERENCIA, 2004 március 25-27., Sopron
2. Borbás Tímea, Benkő Bernadett, Galgóczy Kornél, Dalmadi Balázs, Győrke Imola és Tihanyi Károly: Streptozotocin-indukálta diabétesz hatása a hepatikus és intesztinális gyógyszermetabolizmusra patkányban. FARMAKOKINETIKAI ÉS GYÓGYSZERMETABOLIZMUS TOVÁBBKÉPZŐ SZIMPÓZIUM, 2004. április 15-17., Mátraháza - **Poszter Díj I. helyezés**
3. Borbás Tímea, Benkő Bernadett, Dalmadi Balázs, Győrke Imola, Vastag Mónika és Tihanyi Károly: Humán hepatocita citokróm P450 izoenzimek adatainak statisztikai kiértékelése. FARMAKOKINETIKAI ÉS GYÓGYSZERMETABOLIZMUS TOVÁBBKÉPZŐ SZIMPÓZIUM, 2004. április 15-17., Mátraháza
4. Rudolf Laufer, Bernadett Benkő: Optimization of HPLC Monitoring of Microsomal Metabolism. BALATON SZIMPÓZIUM, 2007. szeptember 8-10., Siófok

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