THESIS IN PHARMACOLOGY FOR THE DEGREE

CANDIDATA PHARMACIAE

CYP3A5 MEDIATED METABOLISM OF MIDAZOLAM *IN VITRO*

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Nataša Nikolić

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ABBREVIATIONS

Bp	Base pairs
BPE	Bovine pituitary extract
BSA	Bovine serum albumin
cDNA	Complementary DNA
CYP	Cytochrome P450
DMSO	Dimethyl sulfoxyde
E.coli	Escherichia coli
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
FBS	Foetal bovine serum
HBSS	Hanks` Balanced Salt Solution
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HLM	Humane liver microsomes
I.S.	Internal standard
LC	Liquid chromatography
MS	Mass spectrometry
MDZ	Midazolam
P/E	Phosphoethanolamine
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
RA	Retinoic acid
RT	Reverse transcriptase
SNP	Single nucleotide polymorphism
SV40	Simian virus 40
T3	Triiodthyronine
T4	Thyroxine
THLE	Transfected human liver epithelial cells

ABSTRACT

INTRODUCTION: CYP3A subfamily of enzymes is involved in the metabolism of more than 50% of all currently used drugs in humans. Clinically most important CYP3A enzymes are CYP3A4 and CYP3A5. CYP3A5 is polymorphically expressed at approximately 20% of individuals and it shows overlapping substrate specificity with CYP3A4, but the extent of known CYP3A4 substrates metabolized by CYP3A5 at individuals polymorphically expressing it, is under investigation. *In vitro* studies using recombinant systems are extensively applied to predict pharmacokinetic properties of drugs *in vivo,* where enzyme kinetic parameters from the *in vitro* experiments are extrapolated to *in vivo* values.

AIM: Establishment of a stable THLE cell line specifically expressing human CYP3A5 enzymes was one of the aims of the present thesis. Another aim was to investigate and compare metabolism of midazolam, which is a standard *in vitro* probe for characterization of the CYP3A activity, in different *in vitro* systems expressing human CYP3A5 enzymes.

METHODS: Stable transfection of THLE cells with a DNA coding for human CYP3A5 was performed in order to obtain human cell line specifically expressing CYP3A5 enzymes. The metabolism studies of midazolam by CYP3A5 were performed in two *in vitro* systems: microsomes isolated from baculovirus infected insect cells specifically expressing human CYP3A5 enzymes (Supersomes®), the difference between the two preparations applied being coexpression of cytochrome b_5 in only one of them.

RESULTS: The transfection of THLE cells with human DNA coding for CYP3A5 enzymes was achieved, but the cells did not express functional CYP3A5 proteins, which resulted in no metabolite formation after incubation with midazolam. The formation of the main metabolite of midazolam, 1′-hydroxy-midazolam (1′-OH-MDZ), followed Michaelis-Menten like kinetics in Supersomes® with coexpressed cytochrome b_5, while the formation of the same metabolite in the Supersomes® without cytochrome b_5 demonstrated substrate inhibition. Surprisingly, the formation rate of 1′-OH-MDZ appeared to be higher in the Supersomes® without cytochrome b_5. Formation of the minor metabolite of midazolam, 4-hydroxy-midazolam (4-OH-MDZ), had sigmoid shape independently of the presence of cytochrome b_5, but the formation rate was lower in the Supersomes® with coexpressed cytochrome b_5.

CONCLUSION: Cytochrome b_5 appears to have an impact on midazolam metabolism catalyzed by CYP3A5 *in vitro.* For further evaluation, more experiments are necessary, and the conditions need to be optimalized in order to obtain functional CYP3A5 proteins from the THLE cells, so that the comparisons of the CYP3A5 catalyzed metabolism of midazolam between insect and human microsomes can be performed.

1 INTRODUCTION

1.1 METABOLISM

Pharmacological effects of drugs rely on their ability to pass biological membranes, access site of action and interact with target cells, creating a response. Concentration of a drug at the site of action is determined by its absorption, distribution, metabolism and excretion (pharmacokinetics). In order to be excreted from the body, lipophilic characteristics of drugs, promoting their passage through membranes, have to be transformed to more hydrophilic ones. This is achieved by a series of biotransformations, often classified as either phase I or phase II reactions. In phase I reactions, functional groups are either exposed on, or introduced to the parent compound. In phase II reactions, polar groups (glucuronic acid, sulphate, glutathione, amino acids or acetate) are coupled to the parent compound, increasing their water solubility and promoting excretion.

1.2 CYTOCHROME P450 MONOOXYGENASE SYSTEM (CYP 450)

Cytochrome P450 (CYP) enzymes are classified as phase I enzymes and they play a major role in the oxidative metabolism of many structurally different xenobiotics (drugs, chemicals), as well as endobiotics (steroids, fatty acids, prostaglandins) [1]. CYP enzymes are heme containing proteins bound to membrane of endoplasmatic reticulum (ER), where they are also associated with nicotinamide adenine dinucleotide phosphate (NADPH) cytochrome reductase (Figure 1), required for oxidative activity of CYP enzymes. NADPH - cytochrome reductase is present in a ratio of about ten cytochrome P450 molecules per one reductase. Other cofactors involved in metabolic oxidation catalyzed by CYP enzymes are NADPH, molecular oxygen (O_2) and sometimes cytochrome b_5 [1]. CYP enzymes are primarily expressed in hepatic tissue, but they are also present in extrahepatic tissues, such as gastrointestinal tract, kidney, lung and the placenta [2].

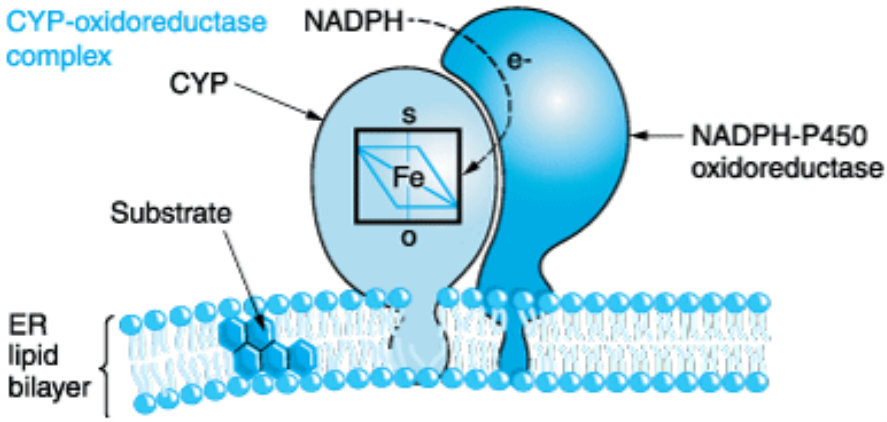


Figure 1: Cytochrome P450 (CYP) hemoprotein in the lipid bilayer of ER. Most part of the CYP enzyme is on the cytosolic surface of ER. The substrate (i.e. drug) often has hydrophobic properties and is dissolved in the membrane [3].

The process of oxidative metabolism of a drug is shown schematically in Figure 2. The drug (D-H) reacts with the oxidized form of the CYP enzyme (CYP-Fe^{3+}), forming a drug-enzyme complex (CYP-Fe^{3+}-D-H). Cytochrome P450 reductase transfers one electron from NADPH to the CYP-Fe^{3+}-D-H complex, reducing it to CYP-Fe^{2+}-D-H, which in turn, reacts with molecular O_2, followed by transfer of a second electron from NADPH. Finally, one atom of O_2 is released as H_2O and the second atom is transferred to the substrate. In some oxidation reactions catalyzed by CYP3A enzymes, cytochrome b_5 has been shown to support the electron transfer from NADPH to CYP3A via the reductase. In that case, the second of the two electrons donated to the CYP (Figure 2), is coming from cytochrome b_5 [4].

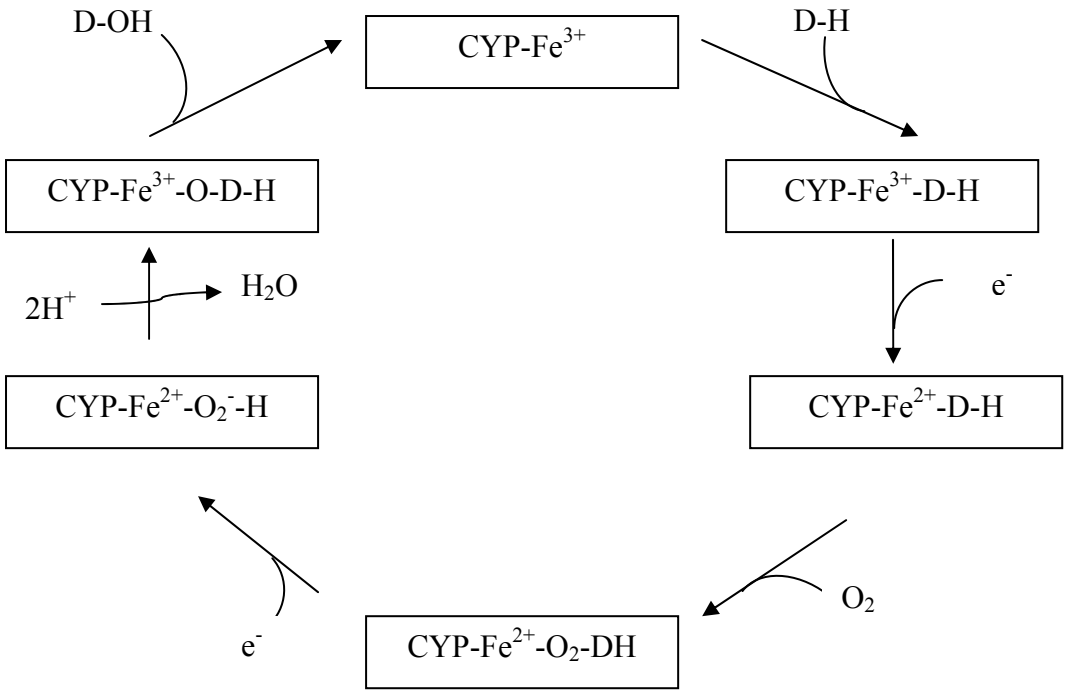


Figure 2: Oxidative metabolism of a drug (D-H) catalyzed by a CYP enzyme. Electrons (e^-) are donated either from NADPH or from cytochrome b_5. Modified from [1].

1.3 CYP GENE SUPERFAMILY

There are approximately 55 different CYP genes in the human genome, and these are further divided into families and subfamilies based on their sequence homology [5]. There have been identified 16 different CYP gene families and 29 subfamilies in humans up to date. Members of one gene family have more than 40% amino acid identity. Members of one subfamily have more than 55% amino acid identity [6].

Recommended nomenclature for CYP genes is italicized root symbol for human CYP (*CYP*), representing "cytochrome P450", followed by an Arabic number denoting the family (*CYP3*), a letter designating the subfamily (*CYP3A*), and an Arabic number representing the individual gene (*CYP3A5*) [7]. Proteins belonging to CYP families 1, 2 and 3 are largely involved in the biotransformations of xenobiotics, and they account for most of the metabolism of pharmaceuticals. Remaining CYP families are responsible for metabolism of endogenous substances, such as steroids and fatty acids [8].

1.4 CYP3A SUBFAMILY

The subfamily of CYP3A is of great importance, mainly because of its high amounts found in organs involved in drug disposition, such as liver, gastrointestinal tract and kidney; as well as because of its extremely broad substrate specificity. Liver is the organ where CYP3A isozymes make the largest proportion of the total CYP pool [9], and where they contribute to the metabolism of approximately 40 - 60% of all known oxidatively metabolized drugs [10]. Four members of the CYP3A subfamily have been described in humans: CYP3A4, CYP3A5, CYP3A7 and CYP3A43 [11]. CYP3A4 is the most abundant CYP 450 expressed in liver and intestine, and it contributes to metabolism of approximately 50% of all used drugs in humans [12].

CYP3A7 is the major isoform of CYP enzymes found in foetal liver, where it accounts for between 30% and 50% of total CYP [13]. It is now established that this isoform is also found in adult liver, with recent studies suggesting that 20% of adult livers express CYP3A7 [14, 15]. In these 20%, this enzyme appears to be responsible for metabolism of approximately 80% of retinoic acid [15, 16]. This means that hepatic expression of CYP3A7 in adults can be of potential interest in physiological pathways regulated by retinoic acid, as well as the outcomes of retinoic therapy. Additionally, CYP3A7 seems to metabolize estrone and dehydroepiandrosterone more efficiently than both CYP3A4 and CYP3A5, and it has also been showed expressed in several different steroid-responsive tissues [11]. This could be related to normal physiological processes, but also to increased susceptibility to cancer.

CYP3A43 has recently been identified as a member of CYP3A subfamily in humans (2000) [17, 18]. This enzyme appears to have role in some physiological processes rather than in drug metabolism, but there is need for more research to understand the function of CYP3A43.

1.4.1 Factors affecting CYP catalyzed biotransformations

It is well known that the outcome of a given drug therapy can vary significantly between individuals. Since CYP enzymes are such major contributors to the metabolism of majority of drugs used in humans, it is clear that there are differences in their activity among individuals in a population. Exposure to some drugs and environmental pollutants can increase synthesis of *de novo* CYP enzymes, leading to increased rate of drugs' biotransformation and lower

bioavailability. More often, CYP enzymes are inhibited during concomitant use of two or more drugs, competing for the same active site.

1.5 CYP 3A5 ABUNDANCE

CYP3A5 is the most studied of the so called minor CYP3A isoforms (other isoforms than CYP3A4) in humans, and it is often considered the second most important CYP3A protein in the liver. CYP3A5 is polymorphically expressed at approximately 20% of adult human livers [19-21], but it is also present extrahepatically; in gastrointestinal tract and the kidney [22]. Major characteristic of CYP3A5, as well as of other CYP enzymes, is the large range of the interindividual variation in the expression of enzymes [2]. Furthermore, there are interracial variations in the level of CYP3A5 expression, 10 to 30% of whites and approximately 50% of African Americans express hepatic CYP3A5 at readily detectable levels [23, 24]. In addition, it seems that the level of CYP3A5 in those expressing it, can approach that of CYP3A4 [24], but this is still questioned, as other investigators suggest lower levels in their experiments [25].

According to previously published research, CYP3A4 does not have defect alleles leading to absence of expression, and its interindividual variations in content are primarily caused by environmental factors [26]. The importance of the genetic polymorphism of CYP3A5 is widely discussed, and it is proposed that CYP3A5, for people expressing it, could be the most important genetic contributor to interindividual and interracial differences in CYP3A dependent metabolism.

Several alleles of *CYP3A5* exist, but only people with at least one *CYP3A5*1* allele express high amounts of CYP3A5 enzyme [5]. Absence of CYP3A5 expression has been related to several different polymorphisms. It is now established that the most common cause of this absence is a single nucleotide polymorphism (SNP) in intron 3 of *CYP3A5*3* allele (A6986G) [23]. This polymorphism leads to alternative splicing and protein truncation. Alleles with A at position 6986 encode a normally spliced CYP3A5 (*CYP3A5*1* allele), but when G is present at this position (*CYP3A5*3* allele), the insertion of material from intron 3 will lead to premature termination of translation. It is interesting to note that, for most CYPs, the first gene allele sequenced is usually the most common one, and is designated *1. This allele is also called “reference” or “wild type” sequence. In the case of CYP3A5, the reference

sequence *CYP3A5*1*, came from a liver sample expressing CYP3A5, meaning that it was a less common variant.

1.5.1 Substrate specificity of CYP3A5

CYP enzymes in general, have very low substrate specificity, opening for the possibility of two or more individual enzymes catalyzing the same reaction. CYP3A5 is structurally related to CYP3A4, and they also show overlapping substrate specificity [27]. CYP3A5 has a large hydrophobic active site with the heme part easily available for binding of substrates. This allows a wide variety of substrates to bind to the enzyme, possibly also at the same time (section 1.8) [27]. Although CYP3A5 metabolizes almost all substrates of CYP3A4, several studies have shown different regioselectivity and lower metabolic activity compared to CYP3A4. Up to date, there is discordance in results for the CYP3A5 metabolism of several known CYP3A4 substrates. For example, Wrighton et al. [19] have reported lower metabolic capacity of CYP3A5 compared to CYP3A4 towards testosterone, nifedipine and erythromycin. In contrast, Gillam et al. [28] suggest equal or even higher metabolic capacity than CYP3A4 towards the same substrates. Questions are also asked regarding CYP3A5 metabolism of midazolam, which is a preferred probe substrate for CYP3A metabolism (section 1.7.3). William et al. [29, 30] report equal capacity of midazolam 1′-hydroxylation for CYP3A5 as for CYP3A4, while Patki et al. [31] conclude that CYP3A5 has significantly lower metabolic capacity towards midazolam than CYP3A4. Possible explanation of such differences in results could be different enzyme sources, conditions of incubation and availability of cofactors.

1.6 MODELS FOR *IN VITRO* STUDIES OF CYP3A ACTIVITY

In vitro studies are a useful tool in predicting drug metabolism *in vivo*. Advantage of using *in vitro* approaches in studying drug metabolisms is that the conditions can be more closely controlled and modified than *in vivo*. On the other hand, the chosen conditions may not reflect those *in vivo*, and it can be difficult to make conclusions from the *in vitro* results, especially quantitative.

There are two main strategies for *in vitro* investigation of a certain drug's metabolism by CYP enzymes:

- Incubation with intact cells (i.e. primary cell cultures, encapsulated hepatocytes or tissue slices)
- Incubation with subcellular fractions (i.e. microsomes) [32]

Cultured hepatocytes are usually obtained from liver transplant programmes and surgical waste [33]. Major limitation is that the cells used have to be fully differentiated, and sufficient growth *in vitro* can be difficult to obtain. Since fresh liver samples are not always available, cultured primary hepatocytes have limited use. However, it has been achieved to preserve adult hepatocytes by entrapping them in a polysaccharide matrix ("cryopreservation"), followed by freezing, and these cells are commercially available, used mostly for short-term experiments because of their reduced viability after thawing [34].

Over the past decades, CYP enzymes have been cloned and expressed in various cell lines. Transfected human lever epithelial cells (THLE cells) are an example of human cell lines used for this purpose. THLE cells are non-tumorigenic, immortalized liver epithelial cells, established by Gomez-Lechon [35]. They are obtained from cultured human liver epithelial cells immortalized by infection with the simian virus 40 large T antigen (SV40 T antigen) [36]. While they express some of the enzymes involved in phase II reactions (section 1.1), phase I enzymes, including CYP enzymes, have been detected at very low levels, or not detected at all, and that is why these cells are referred to as "neo-cells". This makes THLE cells suitable for re-expression of individual CYP enzymes by transfection with their respective cDNA [37] .

Liver slices are another example of intact cells used in metabolism studies. They are simple in use, but the cells have relatively low survival within the sliced tissue, even though it has been reported that they can survive for up to 24 hours [38].

1.6.1 Microsomal preparations

Microsomes are subcellular fractions prepared from endoplasmatic reticulum, and they are widely used in metabolism studies. HLM (human liver microsomes) are microsomes isolated from human lever cells. They can be easily prepared from liver tissue by homogenization and

centrifugation, using well-established methods, and can be stored at -80ºC for several years, with little or no loss of CYP enzyme activities [39, 40].

With the development of genetic engineering, the use of recombinant systems as a source of microsomes has increased significantly [32]. Previously mentioned THLE cells can, besides from being used as intact cells in metabolism studies, also serve as a source of microsomes after transfection with cDNA coding for the CYP gene of interest. Their nearly unlimited life-span is a great advantage, and since they are transfected with individual CYP genes, they are especially suitable for studying metabolism by that particular CYP enzyme, originating from a human liver cell. Human cDNA coding for individual CYP genes have also been isolated, reconstituted in vectors and transfected into other expression systems than human cells, such as insects, yeast and bacteria, along with a strong promoter, to achieve high levels of expression of the enzymes [32, 41].These techniques are a useful tool for obtaining large amounts of enzymes of interest.

A clear advantage of intact cells, both primary hepatic cell cultures, transfected THLE cells and liver slices; is that the environment is more similar to the physiological one: plasma membrane, cofactors and genes expressed are all maintained, at least for a certain amount of time. These factors are of great value when results from *in vitro* studies are extrapolated to *in vivo*. Limitation of intact cells is that the metabolism observed reflects not only catalytic activity of the enzymes involved, but also other processes such as transport into the cell.

Microsomes from recombinant systems need to be reconstituted with phospholipids and various cofactors, such as NADPH-P450 reductase, and, in some cases, cytochrome b_5. Alternatively, some of these cofactors can be coexpressed in the used expression system, avoiding these problems. Selecting optimal incubation conditions is also of great importance, as reported by Hermann et al. [42], even though environment *in vivo* can never be duplicated *in vitro*. Finally, microsomes from recombinant systems and *CYP* transfected THLE cells, in contrast to HLM, do not provide quantitative information about the participation of a given isoenzyme in the overall metabolism of the drug.

1.7 MIDAZOLAM AS AN *IN VITRO* PROBE

1.7.1 General characteristics of midazolam

Midazolam (MDZ) is a benzodiazepine with ultra-short duration of action (<6 hours, with great individual variations), mainly used as a hypnotic and an intravenous anaesthetic [43]. It binds selectively to the regulatory site of γ-aminobutyric acid A ($GABA_A$) receptors in the brain, which mediate fast inhibitory synaptic transmission through the central nervous system (CNS). This type of binding, distinct from the GABA binding site, increases the affinity of GABA for the receptors [44].

1.7.2 Metabolism of midazolam

Midazolam is a basic and lipophilic compound with a molecular weight of 325.8 g/mol. It is 94-97% protein-bound and extensively hydroxylated in the liver and the intestine, mainly to 1′-hydroxy-midazolam (1′-OH-MDZ), which is an active metabolite with a half-life of 2 hours, and to an inactive secondary metabolite 4-hydroxy-midazolam (4-OH-MDZ) (Figure 3) [45]. The biotransformation of MDZ is catalyzed by at least three different CYP3A enzymes: CYP3A4, CYP3A5 and CYP3A7 [5, 46]. Up to 80% of midazolam is recovered in the urine as 1′-OH-MDZ-glucuronide [3].

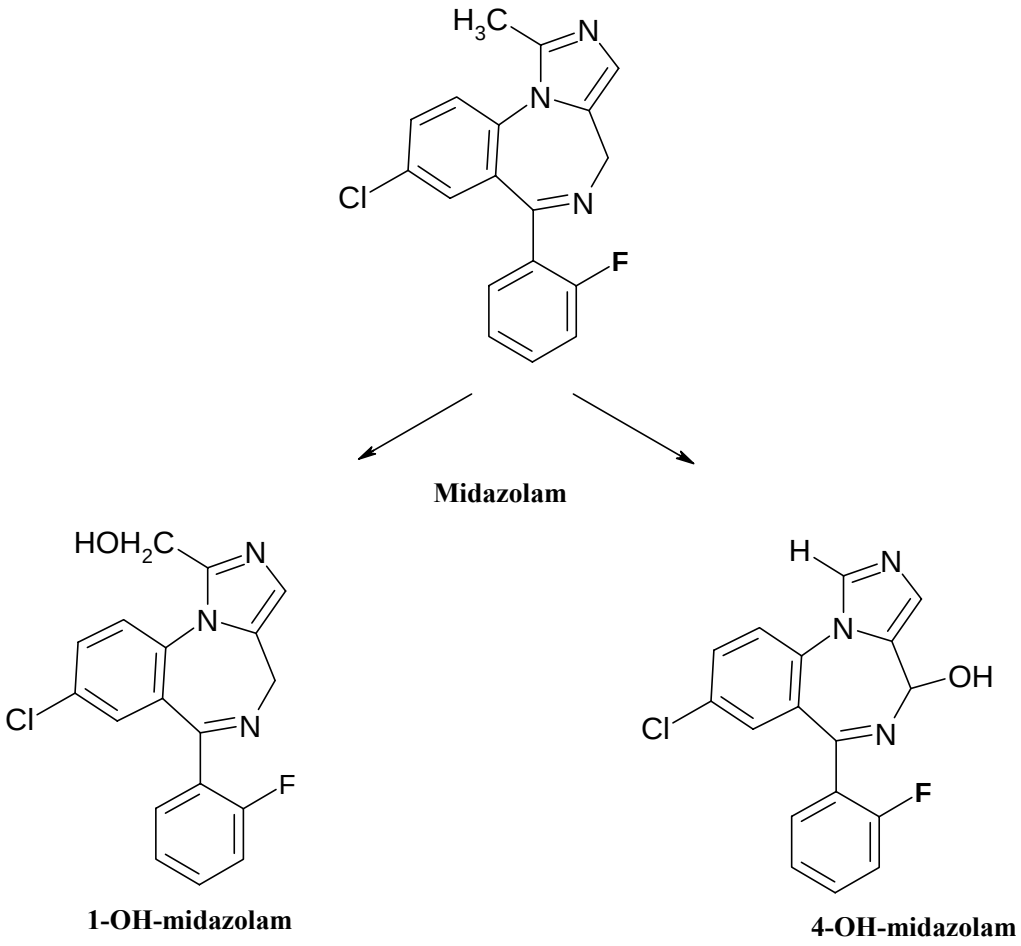


Figure 3*:* Metabolic pathway of midazolam with the formation of the major, active metabolite 1′-OH-midazolam, and the secondary, inactive 4-OH-midazolam**.**

1.7.3 Midazolam as a probe substance

Since CYP3A enzymes are responsible for metabolism of majority of currently used drugs (section 1.3), it is very important to use a standardized CYP3A probe substrate, especially when findings from different studies and drugs are compared. MDZ is the preferred *in vivo* and *in vitro* CYP3A probe recommended by the US Food and Drug Administration (FDA) and the Pharmaceutical Research and Manufacturers of America [47]. Some of several characteristics that make MDZ preferred *in vitro* CYP3A probe are:

- It is exclusively metabolized by CYP3A4/CYP3A5 to a primary metabolite 1′-hydroxymidazolam [46].

- It is highly sensitive to changes in the activity of the CYP3A4/CYP3A5 enzymes [48, 49].
- It is not a substrate for P-glycoprotein [50].
- It is commercially available.
- It is metabolized to an comparable extent by both intestinal and hepatic CYP3A, making it possible to study effects of CYP3A in both the gut and the liver [51, 52].

1.8 ENZYME KINETICS

Substrate affinity and catalytic capacity are kinetic parameters used to quantitatively describe certain enzyme's properties as a catalyst in metabolic reactions. The process of enzymatically catalyzed reaction is often described using following equation:

$$E + S \underset{k_2}{\overset{k_1}{\rightleftarrows}} ES \xrightarrow{k_3} E + P \qquad \text{(Equation 1)}$$

where E is the enzyme, S is the substrate, ES is the formed enzyme - substrate complex, P is the formed metabolite, k_1 and k_2 are the association and dissociation constants, respectively, and k_3 is the catalytic rate constant. According to equation 1, formation of ES =E x S x k_1, while elimination of ES = (ES x k_2) + (ES x k_3). Steady - state is achieved when amount of formed ES equals amount of ES eliminated.

Under the assumption of steady state conditions, Lenor Michaelis and Maud Menten suggested an equation that relates the velocity of a reaction (v) to the substrate concentration (S), Michaelis-Menten equation:

$$v = \frac{V_{max} \times S}{K_m + S} \qquad \text{(Equation 2)}$$

V_{max} is the maximum velocity of the reaction, and it expresses the content of enzymes. The higher the content of enzymes (higher V_{max}), the higher is the catalytic activity. K_m is called the Michaelis-Menten constant, describing the substrate's affinity towards the enzyme, and it is constant for a given substrate. A typical Michaelis-Menten plot is showed in Figure 4. Initially, reaction rate increases hyperbolically until it reaches maximum (V_{max}). Substrate concentration [S] equals K_m when reaction rate (v) is 50% of the maximum velocity (V_{max}), and it will not be affected by the amount of enzymes available.

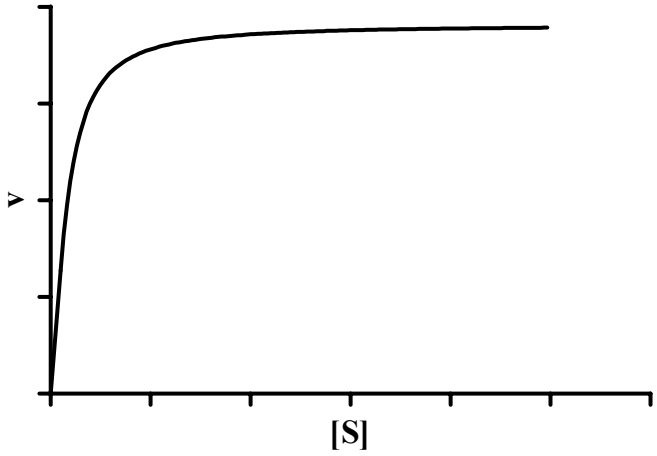


Figure 4*:* Hyperbolic curve representing reaction following classical Michaelis-Menten model**.**

Pharmacokinetic properties of the majority of CYP metabolized drugs can be predicted with the classical Michaelis-Menten kinetic analysis. However, some CYP catalyzed drug reactions show kinetic behaviour that is referred to as atypical, where a plot of substrate concentration versus reaction velocity does not follow a Michaelis-Menten hyperbola, and this unusual type of enzyme kinetics has often been associated with CYP3A metabolism. One important assumption of the Michaelis-Menten model is that substrate - enzyme interactions occur at only one site per enzyme, and that each site functions independently from others. There is evidence suggesting that this is not the case for some CYP3A enzymes, including the clinically most important CYP3A enzyme, CYP3A4. [53]. Several models based on the existence of several binding sites within the enzyme have been proposed to explain the unusual enzyme kinetics in some CYP3A substrates:

- Simultaneous binding of two substrate molecules within the active site of the enzyme [54]

- Binding of multiple substrate molecules to the enzyme due to existence of at least three subpockets in the active site, including one allosteric site too distal from the heme for metabolism to occur [55]
- Existence of multiple conformations of the enzyme [56]
- "Nested model" where the relative proportions of the multiple conformers are determined by allosteric effectors [29]

Atypical enzyme kinetics include activation, substrate inhibition and autoactivation [54]. An important characteristic of CYP3A enzymes is that their catalytic activity for a certain substrate can be activated by the addition of another xenobiotic to the incubation mixture *in vitro*, and this phenomenon is called positive cooperativity/allosterism [53]. Moreover, increasing concentrations of some CYP3A substrates, such as midazolam, have been found to stimulate their own metabolism, a phenomenon referred to as autoactivation. Substrate inhibition on the other hand, occurs when an increase in substrate concentration over a certain value results in a decrease in the metabolism rate, and this is often called negative cooperativity/allosterism [45]. There are two main types of curves for the metabolism of drugs whose kinetics show deviation from standard hyperbolic Michaelis-Menten plots:

1. Sigmoid (Figure 5A), believed to result from activation, and
2. Convex (Figure 5B), believed to result from substrate inhibition.

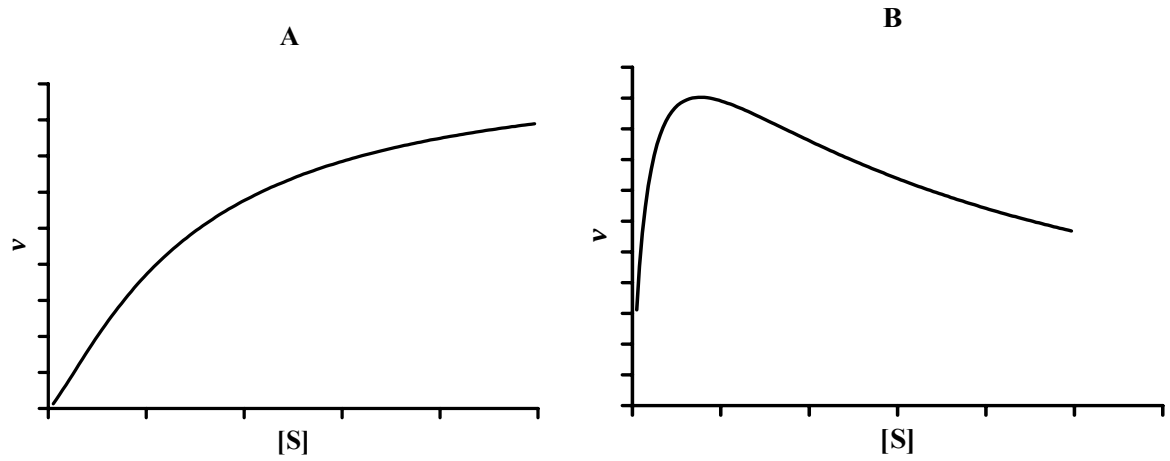


Figure 5*:* Example of plots showing atypical kinetics. (***A***) Sigmoidal curve caused by autoactivation. (***B***) Convex curve resulting from substrate inhibition.

Substrate activation resulting in a sigmoid curve (Figure 5A), with an initial delay, is often described by Hill's equation:

$$v = \frac{V_{max} \times S^n}{S_{50}^n + S^n} \qquad \text{(Equation 3)}$$

where S_{50}^n is analogous to the K_m parameter in Michaelis-Menten equation (Equation 2), and n is Hill's coefficient. Metabolic reactions showing convex curves (Figure 5B) can be quantitatively described using the equation for uncompetitive inhibition:

$$v = \frac{V_{max}}{(1 + (K_m/S) + (S/K_{si}))} \qquad \text{(Equation 4)}$$

where K_{si} is a constant describing the substrate inhibition reaction.

When kinetic results of *in vitro* experiments are extrapolated to *in vivo* metabolic activity, the concept of intrinsic clearance (CL_{int}) is used. The CL_{int} is defined as the velocity (v) of the enzymatic reaction divided by the substrate concentration (S) and can be described by following equation:

$$CL_{int} = \frac{V_{max}}{K_m + S} \qquad \text{(Equation 5)}$$

The *in vitro* CL_{int} value is then scaled-up to reflect CL_{int} *in vivo,* and inserted into a model of hepatic expression [57].

1.9 AIM

CYP3A5 is the most clinically important CYP3A enzyme after CYP3A4. In contrast to CYP3A4, the expression of CYP3A5 in individuals is genetically determined, where only people with at least one *CYP3A5*1* allele express functional CYP3A5 proteins, and the proportion of individuals polymorphically expressing CYP3A5 is approximately 20%. CYP3A5 has showed overlapping substrate specificity with CYP3A4. Genetic polymorphism in the expression of CYP3A5 could be the most important contributor to the interindividual variations in the CYP3A catalyzed metabolism, but the extent of CYP3A5 metabolism of drugs that are known to be metabolized by CYP3A4 is under investigation.

In vitro studies using recombinant systems are extensively applied in order to determine enzyme kinetic parameters such as V_{max}, K_m and CL_{int}, which in turn, are extrapolated to predict pharmacokinetic properties of drugs *in vivo*. In the literature, different enzyme kinetics have been observed using the same substrate for CYP3A5. Additionally, various observations have been related to different laboratories performing the experiments. Therefore, the aim of the present thesis was to investigate and compare metabolism of midazolam, which is a probe substrate for the CYP3A activity, in different recombinant *in vitro* systems expressing human CYP3A5. In addition, human liver epithelial cells were to be transfected with human DNA coding for CYP3A5 enzymes, in order to obtain a stable source of CYP3A5 proteins originating from human cells.

2 MATERIALS AND METHODS

2.1 MATERIALS

1′-hydroxy-midazolam	Sigma-Aldrich, St. Louis, Missouri, USA
4-hydroxy-midazolam	Sigma-Aldrich, St. Louis, Missouri, USA
Acetonitrile, HPLC grade	Merck, Darmstadt, Germany
Acryl-amide/Bis solution	Bio-Rad Laboratories Inc, California, USA
Agarose, electrophoresis grade	Life Technologies, Paisley, UK
Agilent Total RNA Isolation Mini Kit	Agilent Technologies, Palo Alto, California, USA
Alcohol, absolute Prima	Arcus Kjemi AS, Oslo, Norway
Ampli Taq Gold®	Applied Biosystems, Roche Molecular Systems Inc, Branchburg , New Jersey, USA
Anti - CYP3A5 (gift)	Clinical Pharmacology, Imperial College School of Medicine, Hammersmith Hospital, UK
APS	Bio-Rad Laboratories Inc, California, USA
Blasticidin S HCl	Invitrogen, Carlsbad, California, USA
BPE (6mg/mL), Biofluids no.210	Biofluids, Rockville, California, USA
Brom-phenol-blue	Bio-Rad Laboratories, Inc, California, USA
BSA (3%), Sigma no. A-9418	Sigma-Aldrich, St.Louis, Missouri, USA
Deionised water	Easypure UV, Barnstead, Iowa, USA
Diazepam (gift)	Diakonhjemmet Hospital, Oslo, Norway
Distilled water	Aquatron A4S, Bibby Science Products SA, France
ECL Western Blotting Detection reagent	Amersham pharmacia biotech, Cambridge, England
EGF (5µg/mL), Biofluids no.372	Biofluids, Rockville, California, USA
Ethanol	Merck, Darmstadt, Germany
Fat free dry milk (gift)	Normilk AS, Oslo, Norway
FBS	Gibco, Paisley, UK
Fibronectin	PAA Laboratories GmbH, Pasching, Austria

Formic acid, analytical grade	Merck, Darmstadt, Germany
GeneAmp®	Applied Biosystems, Roche Molecular Systems Inc, Branchburg, New Jersey, USA
Gentamycin (10 mg/ML)	Life Technologies, A/S E.Pedersen & Sønn, Oslo, Norway
Glycerol	Sigma-Aldrich, St. Louis, Missouri, USA
Hanks' Balanced Salt Solution	PAA Laboratories Gmbh, Pasching, Austria
HEPES	Sigma-Aldrich, St. Louis, Missouri, USA
Human fibronectin	Sigma-Aldrich, St. Louis, Missouri, USA
Humane lever microsomes	Invitrogen, Carlsbad, California, USA
Hybond-ECL nitrocellulose membrane	Amersham pharmacia biotech, Cambridge, England
Hydrocortisone (10 mM), Biofluids no. 346	Biofluids, Rockville, California, USA
Insuline (0.35 mM), Biofluids no. 350	Biofluids, Rockville, California, USA
Isobutanol	Merck, Darmstadt, Germany
KCl	Merck, Darmstadt, Germany
Kodak AL-4 x-ray fixer	Sigma-Aldrich, St. Louis, Missouri, USA
Kodak BioMax MS Film	Sigma-Aldrich, St.Louis, Missouri, USA
Kodak LX-24 x-ray developer	Sigma-Aldrich, St. Louis, Missouri, USA
L-glutamine (200 mM)	Life Technologies, A/S E. Pedersen & Sønn, Oslo, Norway
Lipofectin® Reagent	Invitrogen, Carlsbad, California, USA
Methanol, HPLC grade	Merck, Darmstadt, Germany
$MgSO_4$	Merck, Darmstadt, Germany
$MgSO_4*H_2O$, analytical grade	Merck, Darmstadt, Germany
Midazolam (gift)	F. Hoffmann-La Roche Ltd, Basel, Switzerland
NADPH	Sigma-Aldrich, St. Louis, Missouri, USA
NaOH	Merck, Darmstadt, Germany
Nitrogen gas	AGA Progas A/S, Oslo, Norway
P/E (0.1 mM), Biofluids no. 353	Biofluids, Rockville, California, USA
Pef6/V5-His-Topo®/*lacZ* vector	Invitrogen, Carlsbad, California, USA
PFMR-4 medium, Biofluids no. 145	Biofluids, Rockville, California, USA

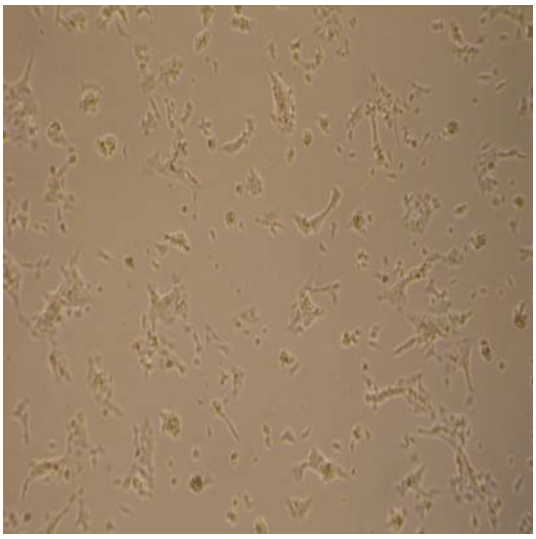
Ponceau S-solution	Sigma-Aldrich, St. Louis, Missouri, USA
Precision Plus protein standard (Dual Colour)	Bio-Rad Laboratories Inc, California, USA
Primers	Invitrogen, Carlsbad, California, USA
RA (3.3 μM), Biofluids no. 348	Biofluids, Rockville, California, USA
SDS	Bio-Rad Laboratories Inc, California, USA
SDS-PAGE standard (Low Range)	Bio-Rad Laboratories Inc, California, USA
Sodium butyrate	Sigma-Aldrich, St. Louis, Missouri, USA
Sucrose	Merck, Darmstadt, Germany
Supersomes® expressing CYP3A5	Larodan Fine Chemicals AB, Malmö, Sweden
TaqMan® Reverse Transcription Reagents	Applied Biosystems, Roche Molecular Systems Inc, Branchbrug, New Jersey, USA
Transferrin (5 mg/mL), Biofluids no. 352	Biofluids, Rockville, California, USA
Triiodthyronine (1 mM), Biofluids no. 354	Biofluids, Rockville, California, USA
Trypsin-EDTA (1x), 0.05 % Trypsin, 0.53 Mm EDTA*4 Na	Gibco, Paisley, UK
Tryptan Blue	Sigma-Aldrich, St. Louis, Missouri, USA
Tween 20	Bio-Rad Laboratories, Inc, California, USA
THLE cells	Nestlé Research Centre, Lausanne, Switzerland
β – Gal Staining Kit	Invitrogen, Carlsbad, California, USA

2.2 EQUIPMENT

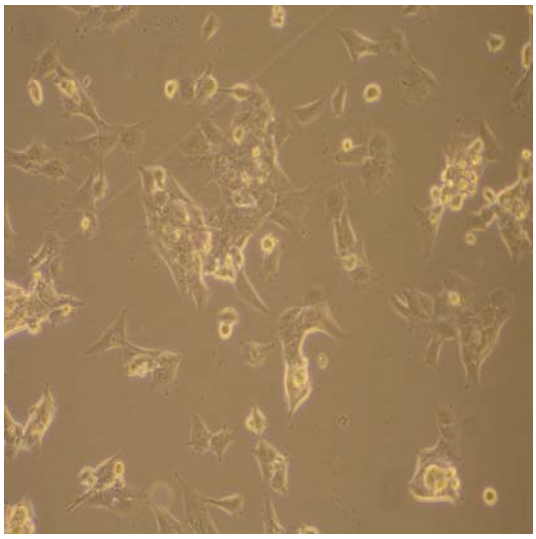
75 cm^2 Flasks, Corning® CellBIND® Surface	Corning Incorporated, Corning, New York, USA
Citenco shaking water bath	Citenco LTD, Horeham Wood, England
Bürker counting chamber	Assistant, Sontheim, Germany
Class VP Chromatography, Datasystem Version 4.3 LC-10AD VP software	Shimadzu, Japan
DGU – 14A degasser	Shimadzu, Japan
Digital camera: Olympus Camedia C-7070	Olympus Norway A/S, Oslo, Norway
FCV-10ALVP mixing chamber	Shimadzu, Japan
GeneAmp PCR System 9700	PE Applied Biosystems, Foster City, California, USA
GeneQuant pro RNA/DNA Calculator	Amersham pharmacia biotech, Cambridge, England
Incubator: Nuaire US Autoflow	NuAire, Plymouth, Maryland, USA
Inertsil C_8, 50*3 mm 5 µM particle size colon	Varian, USA
Kubota 2010 centrifuge	Medinor, Oslo, Norway
LAF bench: Holten LaminAir	Medinor, Oslo, Norway
Multiple Well plate, 6 well Corning® CellBIND® Surface	Corning Incorporated, Corning, New York, USA
Nitrogentank: Locator 8 plus	Barnstead International, Dubuque, Iowa, USA
Phase contrast microscope: Olympus CKX41	Olympus Norway A/S, Oslo, Norway
SCL-10AVP system controller	Shimadzu, Japan
Sil-10 AD VP auto sampler / injector	Shimadzu, Japan
Universal 32R centrifuge	Hettich Zentrifugen, Tuttlingen, Germany
Water bath: thermostat HWT 100	Heto Holten, Oslo, Norway

2.3 CULTURING OF THLE CELLS

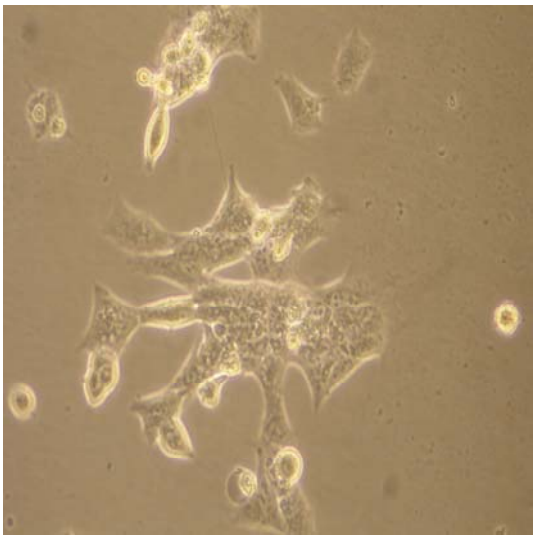
All the work with the THLE cells (Nestlé Research Centre, Lausanne, Switzerland) (section 1.6) was done in a LAF bench using aseptic working procedures. The cells were incubated at 37˚C in a humidified 3.5% CO_2 atmosphere. Culturing of the cells was performed in pre-coated 75 cm^2 flasks, six-well plates and 60 mm plates in a serum free medium (PFMR-4) (section 7.1.1). The growth medium (PMFR-4) was changed every other day. The cells were grown in 12 mL, 2 mL and 4 mL medium per flask, well and plate, respectively.



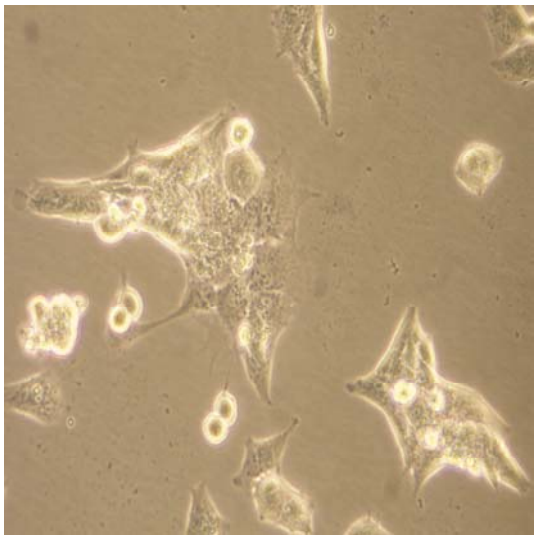
4 x magnification



10 x magnification



20 x magnification



20 x magnification

Figure 6*:* Pictures of THLE cells cultured in 75 cm^2 flasks. The grades of magnifications are marked under each picture.

2.3.1 Thawing of cells

An ampoule of 1 mL THLE cells, clone T5 cl5, was rapidly thawed at 37˚C. The cells were resuspended in a coated 75 cm^2 culture flask containing 11 mL prewarmed (37 ˚C) growth medium (PMFR-4). The cells were incubated at 37˚C in a humidified 3.5% CO_2 atmosphere. Growth medium was changed every other day until the flasks were approximately 90% confluent.

2.3.2 Splitting of cells

The cells were splitted at 90% confluence, which was usually every sixth to seventh day. Each flask then contained approximately $12x10^6$ cells. At that point, the old growth medium was removed and the cells were washed with 5 mL HBSS (Hanks' Balanced Salt Solution). Subsequently, the cells were detached from the flask by adding 2 mL trypsin-EDTA (ethylenediaminetetraacetic acid) diluted with HBSS (1:1, v/v). Trypsin is a proteolytic enzyme, used in combination with chelator EDTA to detach the cells from the surface of the culturing flasks. After 5 minutes incubation at 37˚C, the cells were brought into suspension by tapping the sidewalls of the flask. In order to deactivate trypsin, 5 mL HBSS with 2.5% foetal bovine serum (FBS) was added to the cells. The cell suspension was transferred to a tube and centrifuged for 5 minutes at 200 g (Kubota 2010 centrifuge, Medinor, Oslo, Norway). Afterwards, the supernatant was removed and the cell pellet was resuspended in growth medium. The cells were further seeded into flasks, 60 mm plates and six-well plates, at densities of $1.5x10^6$, $3.5x10^5$ and $2.5x10^5$ cells per flask, plate and well, respectively.

2.3.3 Freezing of cells

The cells were splitted following procedure described in section 2.3.2. Cell pellets from two 75 cm^2 flasks were resuspended in 1 mL growth medium and added 1 mL FBS and 20% (v/v) dimethyl sulfoxide (DMSO), which had been previously diluted with the growth medium. The suspension was mixed gently and transferred to a Nunc vial with a cell density of $2\text{-}5x10^6$ cells per mL. The cells were frozen at -70ºC over the night and then transferred to nitrogen tank (-196°C).

2.4 TRANSFECTION OF THLE CELLS

Molecular cloning is a process where a DNA fragment of interest is recombined with a vector and introduced in a suitable host, which then becomes genetically modified. DNA plasmids are commonly used cloning vectors, obtained in high amounts by transformation of bacteria. Since the term *transformation* in mammalian cells is used to describe conversion of cells to malignant state, the introduction of foreign DNA in mammalian cells is called *transfection*. In stable transfection, the plasmid DNA integrates into the genome, resulting in formation of a cell line carrying and expressing the transgene. Transfection in the present thesis was performed using Lipofectin® Reagent (Invitrogen), which is a cationic lipid compound with a basic structure consisting of a positively charged head group and a hydrocarbon chain. The charged head groups govern the interaction between the lipid and the phosphate backbone of the DNA, leading to the formation of the DNA-lipid cationic complexes. The positive surface charge of the hydrophilic head of the complexes mediates their interaction with the cell membranes, leading to their uptake into the cells through endocytosis. Thus, cationic lipid reagents, such as Lipofectin® Reagent, are thought to facilitate transfection during the early steps of the process by mediating DNA condensation and DNA/cellular interactions.

2.4.1 The plasmid

The vector used for transfection of THLE-cells, pEF6/V5 –His-Topo® vector (Invitrogen), contains ampicillin resistance gene (β-lactamase), which is used for selection of transformants in Escherichia coli (E.coli). The vector also contains blasticidin resistance gene, Blasticidin S deaminase (bsd), which is used for selection of stable transfectants in mammalian cells. Gene coding for CYP3A5 is also inserted in this vector.

2.4.2 Stable transfection of THLE cells

The THLE cells were seeded onto 60 mm plates 2 days before transfection in the PFMR-4 medium without antibiotics. Cells were transfected with pEF6/V5-His-Topo® vector (Invitrogen) with CYP3A5 inserted. For each transfection sample, plasmid DNA was diluted in 100 µL PFMR-4 medium without any additives. Lipofectin® Reagent was diluted with 100 µL PFMR-4 medium without any additives, mixed and incubated for 45 minutes at room temperature. The diluted DNA was combined with the diluted Lipofectin® Reagent, mixed

gently and incubated for 15 minutes at room temperature. The growth medium was removed from the cells. Afterwards, 1.8 mL of PFMR-4 culturing medium without antibiotics was added to the complexes. The suspensions were mixed gently and added to the cells. The cells were incubated for 24 hours at 37˚C in a humidified 3.5% CO_2 atmosphere. Experimental conditions used under transfection are described in Table 1. After the transfection incubation time, the medium was removed and the cells were washed with 4 mL HBSS. Thereafter, 4 mL complete PFMR-4 medium was added to each plate. The cells were incubated at 37˚C in a humidified 3.5% atmosphere. After incubation for 2 days, the cells were splitted as described in section 2.3.2, but this time using 2 mL HBSS, 1 mL Trypsin-EDTA diluted with HBSS (1:1, v/v) and 2.5 mL HBSS with 2.5% FBS per plate. To kill untransfected cells, blasticidin was added to the growth medium to a final concentration of 2 µg/mL three days after the first splitting. The culture medium containing 2 µg/mL blasticidin was changed every other day. After culturing with blasticidin (2µg/mL) for ten days, the cells were splitted as described above and seeded onto new coated six-well plates and further into 75 cm^2 flasks, as the cells were growing. The rest of the cell suspensions were used for total RNA isolation (section 2.5.1).

Table 1*:* Experimental conditions used under transfection of THLE cells with *CYP3A5*

Cell density at seeding	$3.5x10^5$ cells per plate
Day of transfection (day 0 set to the day when seeding of cells was performed)	2
Amount DNA (µg)	1, 2
Lipofectin® Reagent (µL) to DNA (µg) ratio	5:1
Incubation time of Lipofectin® and PFMR-4 medium without additives	45 minutes
Incubation time of Lipofectin® Reagent-DNA complex before adding to cells	15 minutes
BSA present in the culturing medium at the time of transfection	Yes
Antibiotics present in the culturing medium at the time of transfection	No
BSA present at the time of DNA-Lipofectin® Reagent complex formation	No
Transfection incubation time	24 hours

2.5 STUDIES OF GENE EXPRESSION OF CYP3A5

Gene expression of CYP3A5 in the transfected cells was performed using reverse transcriptase- (RT) PCR analysis. In order to be able to apply PCR analysis to the study of mRNA, the mRNA samples were first isolated from the transfected cells, and then reverse-transcribed to complementary DNA (cDNA). Thereafter, amplification of the cDNA sequence was performed by use of the PCR technique. PCR is a method where DNA polymerase is used for exponential amplification of nucleic acids *in vitro* by repeated thermal cycling. The double-stranded DNA is first heat-denatured at high temperature, then annealed at a lower temperature to two oligonucleotide primers, and finally extended at intermediate temperature. The amount of CYP3A5 cDNA was compared to the amount of β-actin (housekeeping gene) cDNA in each sample.

2.5.1 Total RNA isolation and quantification

Isolation of total RNA was performed by use of Agilent Total RNA Isolation Mini Kit®. One day before cell lysis, the cells were stimulated with sodium butyrate, which was added to the growth medium to a final concentration of 5 μg/mL. Splitting of cells was performed as described in section 2.3.2, but this time the cell suspension was centrifuged at 200 g (Kubota 2010 centrifuge, Medinor, Oslo, Norway) for 5 minutes and the supernatant was removed. Further, washing of the cell pellet was performed one time by adding 1 mL x HBSS. Subsequently, 300 μL of prepared (10 μL β-ME /mL) lysis solution was added to each tube. The cell-homogenate was transferred to mini prefiltration column and centrifuged through the column for 3 minutes at 16 000 g (Universal 32R centrifuge, Hettich Zentrifugen, Tuttlingen, Germany). Subsequently, an equal volume (300 μL) of 70% ethanol was added to the filtrate, mixed and incubated for 5 minutes. The ethanol/lysis mixture was added to the mini-isolation column and then centrifuged for 30 seconds at 16 000 g. The flow-through was discarded and the RNA loaded column replaced into the same collection tube. An amount of 500 μL of previously prepared wash solution (to which ethanol has been added) was added to the mini-isolation column in the collection tube, then centrifuged for 30 seconds at 16 000 g. The flow-through was discarded, and the mini-isolation column replaced in the collection tube. Washing process, followed by centrifuging was repeated one more time. Subsequently, the mini-isolation column was centrifuged for 2 minutes at 16 000 g. Elution of purified RNA was performed by adding 10 μL of nuclease-free water. After incubation for at least 1 minute,

the column was centrifuged for 1 minute at 16 000 g. To determine the RNA concentration, 2 µL of the RNA solution was diluted with 68 µL nuclease-free water. Determination of RNA concentration was performed by measuring absorbance at 260 nm with GeneQuant Pro RNA/DNA Calculator (Amersham pharmacia biotech, Cambridge, England). The RNA purity was determined by using a ratio of the absorbance at 260 nm and 280 nm. Pure RNA solution should have a 260/280 ratio of 1.9-2.1.

2.5.2 Reverse transcriptase (RT)

RT was performed by use of TaqMan® Reverse Transcription Reagents. RT-reaction mix was prepared and it consisted of: 2.0 µL 10xTaqMan RT Buffer, 4.4 µL $MgCl_2$ (25 µM), 4.0 µL dNTP mix (10 mM), 1.0 µL Random hexamers (50 µM), 0.4 µL Rnase inhibitor (20 U/µL) and 0.5 µL Multiscribe Reverse Transcriptase (50 U/µL), volumes given per sample. Subsequently, 12.3 µL of the mixture was pipetted to each PCR tube. Thereafter, a total volume of 7.7 µL DEPC-dH2O and mRNA sample (1 µg) was added to PCR tubes. RT was run by use of GeneAmp PCR System 9700 (PE Applied Biosystems, Foster City, California, USA). RT – conditions were set to 10 minutes at 25˚C (hexamer/template priming), 1 hour at 37˚C (reverse transcription) and 5 minutes at 99˚C (reverse transcriptase inactivation).

2.5.3 Polymerase chain reaction (PCR)

PCR was performed by use of AmpliTaqGold® with GeneAmp® reagents. The reaction mix was prepared and it consisted of: 5.0 µL 10xPCR Buffer, 1.5 µL $MgCl_2$ (50 µM), 1.0 µL dNTP mix (10 mM), 1.5 µL Primer R, 1.5 µL Primer F, 0.3 µL Taq DNA polymerase (5 U/µL, AmpliTaq Gold), volumes given per sample. PCR reaction mix was aliqouted to the PCR tubes. Finally, 29.8 µL DEPC-dH2O and 10 µL cDNA sample (RT-product) was added to each tube. PCR was run by use of GeneAmp PCR System 9700 (PE Applied Biosystems, Foster City, California, USA). The amplification program was initially started at 94˚C for 3 minutes, thereafter 35 cycles of the following three steps: 94˚C for 45 seconds, 55˚C for 30 minutes, and finally 72˚C for 5 minutes.

Primers:

CYP3A5:	Fragment length: 75 base pairs (bp)
Forward CYP3A5:	5'-GGT GAT TCC AAC TTA TGC TCT TCA-3'
Reverse CYP3A5:	5'-AAC CTT TCA GGG CGG ACC TC-3'

β – actin (positive control):	Fragment length 62 bp
Forward β – actin:	5`-ACCGAGCGCGGCTACA-3`
Reverse β – actin:	5`- TCCTTAATGTCACGCACGATTT-3`

2.5.4 Agarose gel electrophoresis

DNA fragments obtained in RT-PCR were separated on a 2% agarose gel by electrophoresis. To prepare the gel, 2 g agarose was dissolved in 100 mL 1xTBE buffer by heating up to boiling point in a microwave oven. After the solution was cooled down a bit, 10 µL (5 mg/mL) ethidium bromide was added and stirred well. The warm solution was poured into the electrophoresis chamber. The gel was allowed to set at room temperature and then covered with 1xTBE buffer. Subsequently, 15 µL of each PCR product was mixed with 3 µL loading dye buffer and applied to the wells. For determination of the sizes of the DNA fragments, 15 µL of standard low molecular weight DNA ladder (0.1 µg/µL) was applied to one well. The gel was run at 100 V for approximately 1.5 hours. The DNA was visualized by use of UV-light.

2.6 STUDIES OF CYP3A5 PROTEIN EXPRESSION

To investigate expression of CYP3A5 proteins in the THLE cells transfected with *CYP3A5*, electrophoresis was performed. Electrophoresis is a method whereby charged molecules in solution, e.g. proteins, migrate in response to an electrical field; the rate of migration being determined primarily by the strength of the field and the net charge, size, and shape of the molecules. Since an anionic detergent sodium dodecylsulfate (SDS) preparation was used, which denatures proteins by conferring a net negative charge proportional to their length, the migration rate was determined only by the molecular weight of the proteins. Migration of proteins was carried out in polyacrylamide gels, mounted between two buffer chambers containing separate electrodes. The buffer used was Laemmli system, which is a discontinuous buffer system. In a system like that, a non restrictive large pore gel, the

stacking gel, is layered on top of the separating gel. The advantage of Laemmli gel is greater resolution because the treated proteins are stacked in a stacking gel before entering the separating gel. The proteins in the samples were analyzed by transferring them to a membrane, where they were detected chemiluminescently after being bound to the specific antibodies.

2.6.1 Preparation of the samples from the *CYP3A5* transfected THLE cells for protein electrophoresis

CYP3A5 transfected THLE cells were cultured in a 60 mm plate with 4 mL complete PFMR-4 culturing medium. Before harvesting for the protein analysis, the cells were rinsed twice with HBSS. To detach them from the culturing plate, 300 µL of sample buffer (section 7.1.5) was added to the cells. The cells suspended in sample buffer were then transferred to a heat-resistant vial and cooked on a water bath for 5 minutes. Beta - mercaptoethanol (10%, v/v) and brom - phenol - blue (5%, v/v) was added to the vial and cooked on a water bath for additional 5 minutes, before it was frozen at -20ºC.

2.6.2 Electrophoresis

Two pieces of 10% separating gel solutions were prepared as described in section 7.1.4. Ammonium persulfate and TEMED were left out and added last, simultaneously. The solution was gently swirled and pipetted to the glass sandwich to a level about 1.0 cm from the top. The glass sandwich was made of two glass plates and two metal plates making two cassettes, separated by two spacer strips at the edges, and clamped together to make a tight seal. To prevent formation of meniscus on the top of the gel, which could cause distortion of the banding pattern, 200 µL of isobutanol was applied to the gel-containing cassettes before polymerization. The gels were allowed to polymerize for 45 minutes, when isobutanol was poured from the surface, and gels were rinsed with distilled water. Stacking gel (4%) was prepared according to section 7.1.4 and added to the sandwich. Combs were inserted into each gel. The gels were allowed to set for another 40 minutes. After that, the two gel-containing cassettes were rinsed with distilled water and placed in an electrophoresis chamber, which was then filled with Tank buffer until the cassettes were immersed in buffer. The combs were carefully removed from the gels. Volumes of 5 µL of protein standard and 15 µL of samples were applied to the wells and separated by electrophoresis at 200 V for 1 hour.

2.6.3 Western-blot

Proteins separated on gel by electrophoresis were transferred to nitrocellulose membranes by a technique called blotting. After the electrophoresis was run, the cassettes were disassembled and stacking gel was removed from the separating gels and discarded. Gels, blotter papers, nitrocellulose membranes and cellophane sheets were all soaked in blotting buffer (section 7.1.4), and then arranged in a "transphor sandwich" (Figure 7). Gels were blotted at 100 mA for 1 hour, with electric field applied perpendicular to the gels, making the proteins move out to the membrane. To verify successful protein transfer after blotting, membranes were stained for 1 min with Ponceau S-solution and rinsed with distilled water. Membranes were placed in tris - tween - buffered - saline - solution (T-TBS solution) (section 7.1.4) for 10 minutes. T-TBS was discarded, 15 mL of blotto - solution (section 7.1.4) was added to each membrane and they were allowed to set for 60 minutes before addition of 15 mL of 1:10 000 primary CYP3A5 antibodies to each membrane, allowing them to incubate in a cold room over the night. Membranes were then washed at room temperature for 3x10 minutes in blotto prior to addition of the secondary antibodies, goat – anti – mouse diluted 1:3000 in blotto, followed by additional 1 hour of incubation at room temperature. Membranes were then washed 3x10 minutes in blotto and 2x10 minutes in T-TBS. Equal amounts of chemiluminescense (ECL) - reagents (6 mL total volume) were mixed and added to the membranes, which were then packed in plastic folium with protein side facing down. The membranes were transferred to a film cassette and exposed to film for 5 minutes. The film was developed and fixated (section 7.1.4).

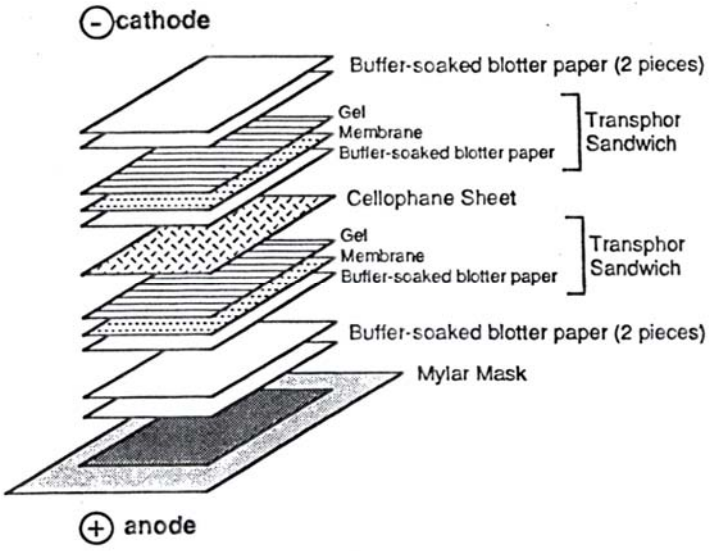


Figure 7: Arrangement of the filter (blotter) paper, membranes, cellophane sheet, and gels for transphor. A transphor sandwich consists of one piece of filter paper, one membrane and one gel.

2.7 METABOLISM STUDIES

Metabolism studies of midazolam were performed in different recombinant microsomal preparations: the microsomes from baculovirus infected insect cells specifically expressing CYP3A5 (Supersomes®), with or without coexpressed cytochrome b_5, and the microsomes isolated from the *CYP3A5* transfected THLE cells.

2.7.1 Metabolism studies of midazolam in Supersomes® as a function of time

The experiments of midazolam metabolism as a function of time (5-20 minutes) were conducted to establish reaction conditions that would ensure linearity of product formation with incubation times. Two substrate concentration were used, 3 μM and 50 μM midazolam. Incubations contained Supersomes® with or without coexpressed cytochrome b_5. Incubation conditions used were established by Hermann et al. [42]. Midazolam solved in methanol was added to a vial, and the methanol was evaporated to dryness under nitrogen gas. A previously prepared incubation buffer, containing Tris-H_2SO_4 (pH 7.4), $MgSO_4$ and NADPH was added to the vial with midazolam and mixed. Additional incubation buffer was added to each vial, so that the final volume was 170.5 μL / vial and the final concentration of the incubation buffer was 118 mM Tris-H_2SO_4 (pH 7.4), 0.5 mM $MgSO_4$ and 1.6 mM NADPH. Vials containing midazolam and incubation buffer were preincubated in a 37˚C water bath for 5 minutes, while the Supersomes® with or without coexpressed cytochrome b_5, diluted 1:64 in a buffer consisting of 10 mM HEPES, 2 mM EDTA and 0.25 M sucrose, were preincubated in a 37˚C water bath for 2 minutes. The metabolism was initialized by adding 50 μL of the microsomes solution to each vial with 30 seconds intervals. The mixtures were incubated in a 37˚C shaking (grade 7.5) water bath for 5, 10, 15 and 20 minutes, respectively, for both concentrations of midazolam. Incubations were terminated by adding 150 μL ice cold acetonitrile (ACN) with 0.044 μM internal standard diazepam (DIA) to each incubation vial. Vials were vortex-mixed for at least 10 seconds and immediately placed on ice. They were allowed to set for a minimum of 30 minutes and then centrifuged for 5 minutes at 1600 g, 4ºC (Universal 32R centrifuge, Hettich Zentrifugen, Tuttlingen, Germany). The supernatant (300 mL) was transferred to new vials and the formations of both 1′-OH-MDZ and 4-OH-MDZ were analyzed using LC-MS.

2.7.2 Metabolism studies of midazolam in Supersomes® as a function of substrate concentration

The aim of these experiments was to estimate and compare kinetic parameters of the formation of the metabolites of midazolam, 1′-OH-MDZ and 4-OH-MDZ, in two different recombinant microsomal preparations of CYP3A5, and with various concentrations of MDZ. Metabolism of midazolam in Supersomes®, with or without coexpressed cytochrome b_5, was evaluated over a range of substrate concentrations (0.5-50 μM). Incubations were prepared as described in section 2.7.1. The metabolisms were initiated by addition of 50 μL of either Supersomes® with or without coexpressed cytochrome b_5, diluted 1:64 in a buffer solution consisting of 10 mM HEPES, 2 mM EDTA and 0.25 M sucrose. The incubation time was 7.5 minutes for both enzyme preparations. The analysis of the two metabolites of MDZ was performed using LS-MS. Velocity of the metabolism as a function of substrate concentration was expressed as the amount of either 1′-OH-MDZ or 4-OH-MDZ per pmol CYP3A5 protein per minute. Enzyme kinetic analysis of the data was preformed by nonlinear regression using Graphpad Prism version 4.03 (GraphPad.software, San Diego, USA).

2.7.3 Preparation and isolation of microsomes from *CYP3A5* transfected THLE cells

After the thawing (section 2.3.1), the *CYP3A5* transfected THLE cells were allowed to go through three passages. On the day before harvesting (seven days after the last splitting), the fresh PFMR-4 culturing medium was added to the cells. At this point confluence was approximately 90%. On the day of harvesting, the cells grown in 75 cm^2 culturing flasks were suspended in 5 mL buffer solution of 0.154 M KCl and 50 mM Tris-HCl, pH 7.4. The cell suspension was centrifuged at 4ºC, 710 g for 5 minutes (Hettich centrifuge, Universal 32R). The pellet was resuspended in hypotonic (0.05 M) sucrose solution (500 μL per 12 million cells). Homogenisation of the cells was performed by 10 strokes with a 7 mL, tight fitting Dounce homogenisator. Subsequently, 445 μL of a hypertone sucrose solution (2.5 M) was added to make environment isotonic. The cell-suspension was stroke three more times with the pestle. The mixture was then centrifuged at 4ºC in three subsequent steps, 10 minutes at 1700 g, (Hettich Zentrifugen, Universal 32R), 10 minutes at 12 000 g (Beckmann, Avanti J-25) and 60 minutes at 105 000 g (Sorvall Ultra Pro 80), with transferral of the supernatant to the next step each time. After these centrifugations, the pellet, containing the microsomes, was homogenised in a buffer solution consisting of 10 mM HEPES, 2mM EDTA and 0.25 mM sucrose (200 μL per 12 million cells). The microsomes suspension was stored at -70ºC.

2.7.4 Metabolism studies of midazolam in microsomes isolated from the *CYP3A5* transfected THLE cells

The metabolism of midazolam was studied in the microsomes isolated from the *CYP3A5* transfected THLE cells (section 2.7.3). Incubation buffer was prepared as described in section 2.7.1. Metabolism of midazolam was studied at three concentrations: 10, 25 and 50 µM. The metabolism was initialized by adding 100 µL of undiluted microsomes isolated from the *CYP3A5* transfected cells. In addition, three vials containing only the incubation buffer and midazolam were used as a negative control. All the mixtures were incubated in a 37˚C shaking (grade 7.5) water bath for 10 minutes, when they were terminated by addition of 150 µL ice cold ACN with 0.044 µM DIA to each incubation vial. Vials were vortex for at least 10 seconds and immediately placed on ice. They were allowed to set for a minimum of 30 minutes and then centrifuged for 5 minutes at 1600 g, 4ºC (Universal 32R centrifuge, Hettich Zentrifugen, Tuttlingen, Germany). The supernatant (300 µL) was transferred to new vials and the formations of both 1′-OH-MDZ and 4-OH-MDZ were analyzed using LC-MS.

2.8 LC-MS

LC-MS is high-performance liquid chromatography coupled to mass spectrometry. It is a highly sensitive analytical method. In the MS step, the ions of interest are selected according to their mass - to - charge ratio (m/z). This ion is fragmented. Separation of the analysts by use of HPLC precedes the MS detection. Some of the advantages of LC-MS are low detection limit (pg-level), short run time, high specificity, precision and accuracy.

2.8.1 LC-MS analysis method

LC-MS analysis of the hydroxylated metabolites of MDZ was performed using a method validated by Postvoll [58], with modifications. The hydroxylated metabolites of MDZ were eluated by a gradient mobile phase system containing mobile phase A (ammonium - concentrated formic acid - deionised water) and mobile phase B (acetonitrile - methanol) (section 7.1.6). A pre-run switch in duration of 3 minutes, where the mobile phase A was sent to waist, was added to the validated method [58], in order to eliminate sucrose interfering with the analysis. Analysis run time per sample was 17 minutes. The relative contribution of mobile phase B increased linearly from 25% to 37.5% during the first five minutes after the sample injection. This mobile phase mixture was held constant for 2.5 minutes. During the

following 2.5 minutes, the mobile phase B content was increased to 46.7% and then quickly to 80%. From 11^{th} to 17^{th} minute, the colon was re-equilibrated by reversing the mobile phase B to the start concentration of 25%. Flow rate of the mobile phase was 0.5 mL/min during the analysis and 1 mL/min during the re-equilibration of the colon. All these steps were carried out before injection of the next sample. The analysis of the chromatograms of the separated metabolites was performed using the software LCMSsolution Version 2.05 (LabSolutions, Shimadzu, Japan).

2.8.2 Validation of the linearity of the analysis method

In order to test the linearity of the analysis method described in section 2.8.1, standard curves for 1′-OH-MDZ and 4-OH-MDZ were made. Seven concentrations of each of these two metabolites, ranging from 0.013-0.15 μM were analyzed, and 2 parallels of each concentration were measured using the LC-MS analysis method described in section 2.7.1. Each standard sample contained 118 mM Tris-H_2SO_4 (pH 7.4) buffer, 150 μL acetonitrile with 0.044 μM internal standard diazepam and both metabolites at their respective concentrations, making the total volume of 370.5 μL, equal to the volume used in metabolism studies, section 2.9.1. The obtained standard curves were used to calculate the amount of metabolites formed in the metabolism studies (section 2.7).

3 RESULTS

3.1 STUDIES OF GENE EXPRESSION OF CYP3A5

To study gene expression of CYP3A5, RT-PCR was performed, and the products of RT-PCR were separated on a 2% agarose gel (Figure 8). CYP3A5 gene has 75 base pairs (bp). Low molecular weight ladder was used as a reference (Figure 8). The amount of CYP3A5 cDNA was normalized to the amount of β-actin (housekeeping gene) cDNA in each sample (Figure 8, lanes 5 - 7). The *CYP3A5* transfected THLE cells (Figure 8, lane 3) showed gene expression of CYP3A5, in contrast to non-transfected THLE cells (Lane 2), where no gene expression of CYP3A5 was observed. This indicates that stable transfection with *CYP3A5* was successfully obtained by use of Lipofectin® Reagent under conditions described in section 2.5.1, Table 1, using 1 µg plasmid DNA per plate.

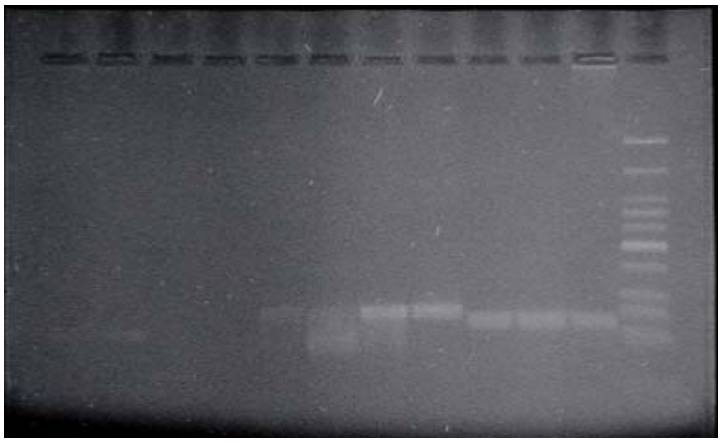


Figure 8*:* RT-PCR products separated on a 2% agarose gel*.* **1**: Blank sample (only CYP3A5 primers), **2**: non-transfected THLE cells (negative control), **3**: *CYP3A5* transfected THLE cells, **4**: *CYP3A5* (positive control), **5-7**: β-actin.

3.2 STUDIES OF PROTEIN EXPRESSION BY WESTERN BLOTTING

Results from protein expression studies of CYP3A5 in the transfected THLE are shown in Figure 9. A protein standard containing proteins with various known molecular weights was added to one of the wells in the gel to be used as a reference. CYP3A5 protein has a molecular weight of 57109 Da [31]. The bands from the insect microsomes (Figure 9, bands 2-5) and HLM (Figure 9, band 6), which have been incubated with CYP3A5 specific

antibodies, were positioned approximately at the same level as the standard protein with molecular weight of 50 Da, showing that they were expressing CYP3A5 proteins. The bands for the *CYP3A5* transfected THLE cells (Figure 9, bands 7-9) were positioned higher than the standard protein with molecular weight of 50 Da, and the bands for the CYP3A5 proteins from insect microsomes. This indicates that the molecular weight of the proteins expressed in the *CYP3A5* transfected THLE cells was higher than 50 Da, and they possibly did not have the same form as the reference proteins (Figure 9, bands 2-6).

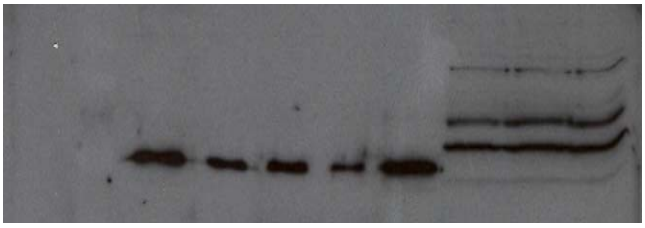


Figure 9*:* Picture of a film developed after addition of CYP3A5 antibody. **Well 1**: Protein standard**,** wells **2-5** contain dilutions of insect CYP3A5 microsomes with declared amounts of CYP3A5 proteins**. 2**: insect (i) CYP3A5 1:7500, **3**: iCYP3A5 1:10000, **4**: iCYP3A5 1:15000, **5**: iCYP3A5 1:20000. Well **6**: HLM. Wells **7 - 9**: samples from *CYP3A5* transfected THLE cells. Well **1** contained standard proteins, but the bands for these are not shown in the picture since they do not bind to CYP3A5-specific antibodies.

3.3 METABOLISM STUDIES WITH MIDAZOLAM

3.3.1 Metabolism of midazolam as a function of time

Formation of the two metabolites of midazolam, 1′-OH-MDZ and 4-OH-MDZ, was studied as a function of time in CYP3A5 expressing insect microsomes (Supersomes®) (Figure 10A and B, respectively) and in Supersomes® with coexpressed b_5 (Figure 10C and D, respectively), in order to assure linear conditions. Curves describing formation of 1′-OH-MDZ at both concentrations of MDZ, 3 µM (blue) and 50 µM (red) in Supersomes® showed tendency of bending after approximately 10 minutes of incubation (Figure 10A). On the other hand, when Supersomes® coexpressed with b_5 were used, formation of 1′-OH-MDZ was more linear with increasing incubation time, both at 3 µM and 50 µM MDZ (Figure 10C). When it comes to the formation of 4-OH-MDZ, curves were almost linear with time at both

microsomes preparations, and at both concentrations of midazolam used (Figures 10C and D). Based on these experiments it was concluded that incubation time of 7.5 minutes would assure linear conditions in the experiments of midazolam metabolism as a function of substrate concentration in both microsomal preparations.

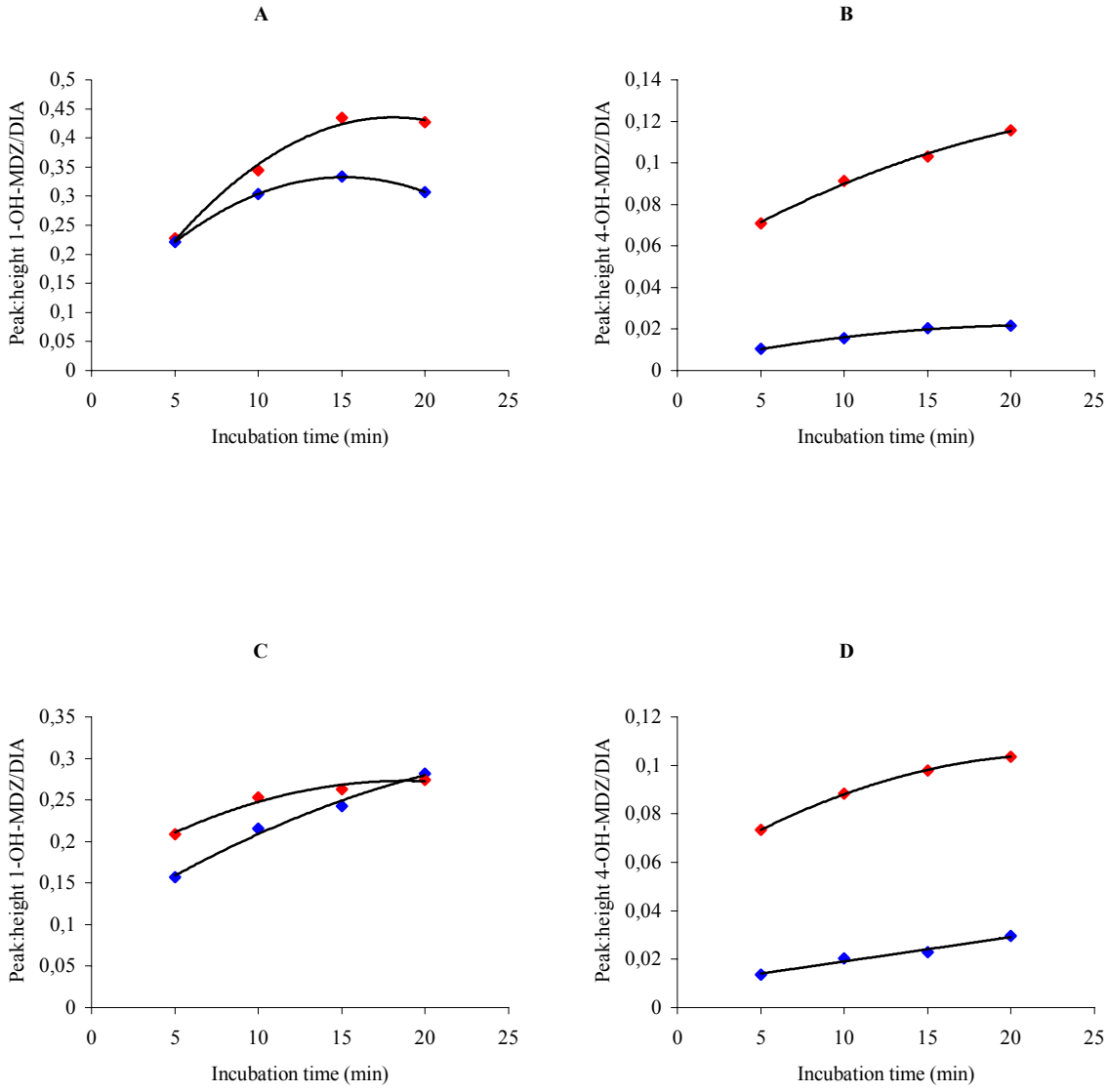


Figure 10: Time curves showing formation of 1′-OH-MDZ (***A*** and ***C***) and 4-OH-MDZ (***B*** and ***D***) after incubation with 3 µM MDZ (blue) and 50 µM MDZ (red). All points represent averages of duplicate measurements. Figures ***A*** and ***B*** show metabolite formations in Supersomes®, while figures ***C*** and ***D*** demonstrate incubations in Supersomes® with coexpressed cytochrome b_5.

3.3.2 Metabolism of midazolam in Supersomes® as a function of substrate concentration

The LC-MS chromatograms of the formed metabolites, 1′-OH-MDZ and 4-OH-MDZ, were integrated with respect to peak height. Correlation factors from the standard curves of the metabolites (section 2.8.2) were used to calculate velocity of the metabolism expressed as pmol of either 1′-OH-MDZ or 4-OH-MDZ per pmol CYP3A5 protein per minute. Examples of standard curves used for these calculations are showed in Figure 11.

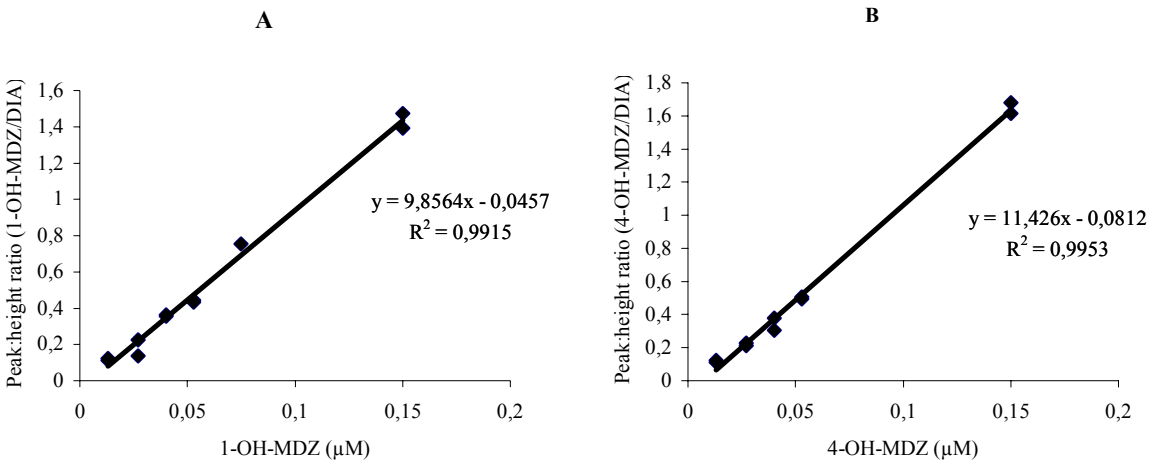


Figure 11*:* Examples of standard curves for 1′-OH-MDZ (***A***) and 4-OH-MDZ (***B***)

Kinetic analysis of the metabolite formation was performed using GraphPad Prism 4.03 (GraphPad software, San Diego, CA, USA). The dose-response curves showing formation of 1′-OH-MDZ as a function of midazolam concentration from two separate experiments in the two insect CYP3A5 microsomal preparations are shown in Figure 12. Both curves describing formation of 1′-OH-MDZ in Supersomes® without cytochrome b_5 (Figures 12A and C) showed substrate inhibition (section 1.8); and the kinetic parameters of these reactions were described using the equation for uncompetitive inhibition (Equation 4). On the other hand, the curves observed after incubations with Supersomes® with coexpressed b_5 (Figures 12B and D) showed Michaelis-Menten like kinetics and have a hyperbolic shape. However, non-linear regression gave a slightly better fit with the Hill's equation (Equation 3) than with the Michaelis-Menten equation (Equation 2).

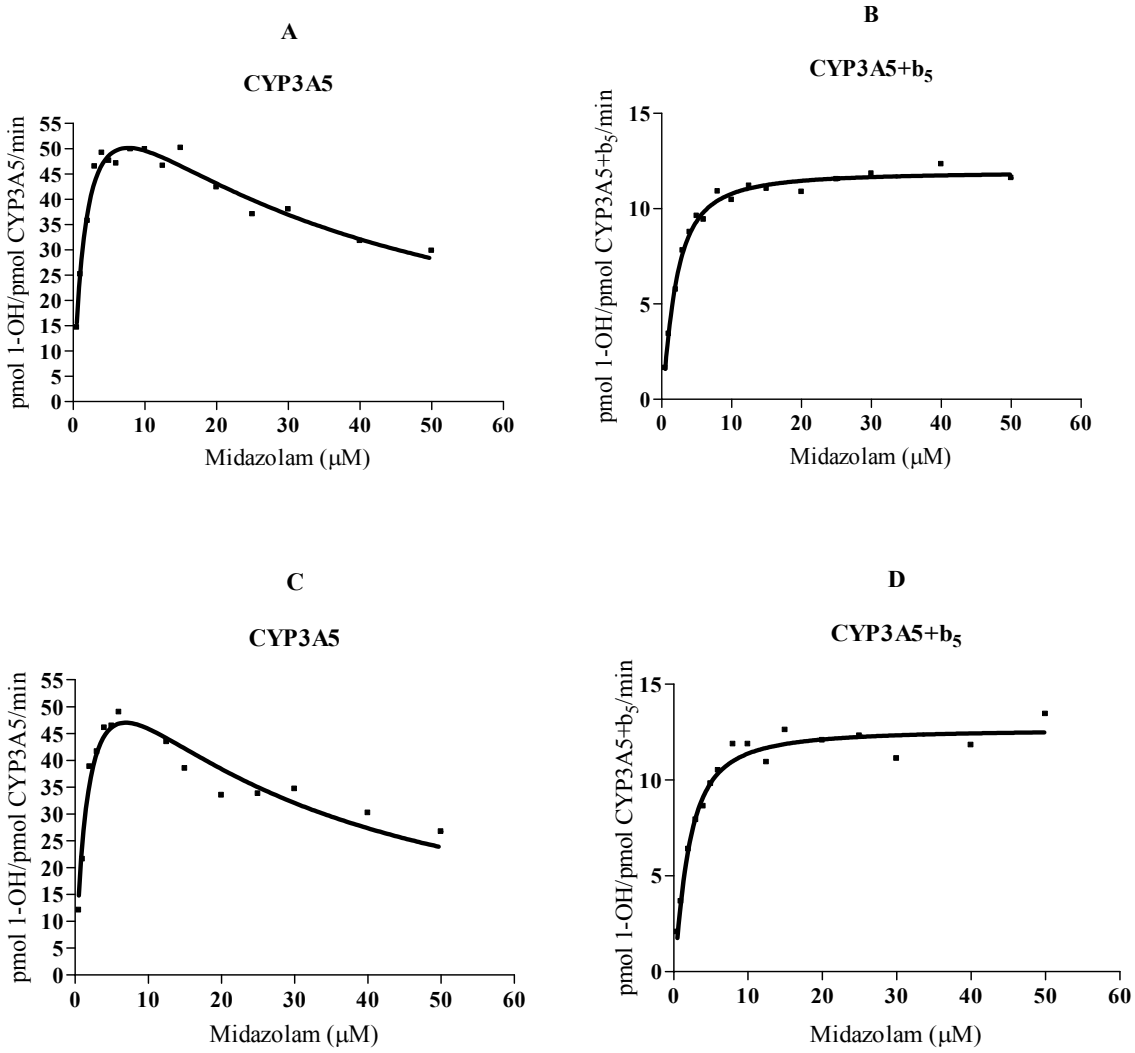


Figure 12*:* Dose-response curves showing formation of 1′-OH-MDZ Supersomes® (***A*** and ***C***), and in Supersomes® with coexpressed cytochrome b_5 (***B*** and ***D***) Non linear regression of the analysis data was performed using GraphPad Prism 4.03 (GraphPad software, San Diego, USA).

The estimated kinetic parameters for the formation of 1′-OH-MDZ in the two microsomal preparations are presented in Table 2. V_{max} values were 6-fold higher in Supersomes® without cytochrome b_5 (74.50 and 75.49 pmol 1′-OH-MDZ/pmol CYP3A5/min) compared to those where cytochrome b_5 was coexpressed (12.39 and 13.20 pmol 1′-OH-MDZ/pmol CYP3A5/min). K_m values were in the same range, but slightly higher in the preparations where cytochrome b_5 was coexpressed with the CYP3A5 enzymes (2.5 μM) than in the Supersomes® without cytochrome b_5 (1.9 μM). Consequently, the CL_{int} values were approximately 7-fold higher in Supersomes® without cytochrome b_5 than in those without it (Table 2).

Table 2: Kinetic data with 95 % confidence interval for the formation of 1′-OH-MDZ in two different microsomal preparations of CYP3A5. Data from two separate experiments for each preparation are shown. n is Hill's coefficient (section 1.8, equation 3). K_{si} is the coefficient describing the grade of the inhibition (section 1.8, equation 4). U.I. is uncompetitive inhibition.

Supersomes®	Exp.	V_{max}*	K_m/ S_{50}**	K_{si}	n	CL_{int}	R^2	Model
Without b_5	**1**	74.5 (65.4-83.6)	1.90 (1.31-2.49)	31.1 (21.8-40.3)		39.2	0.967	U.I.
	2	75.5 (57.7-93.3)	1.98 (0.90-3.06)	24.2 (12.2-36.2)		38.1	0,931	U.I.
With b_5	**1**	12.4 (12.0-12.8)	2.52 (2.04-2.99)		1.37 (1.17-1.57)	4.93	0.990	Hill
	2	13.2 (12.4-14.0)	2.50 (1.67-3.33)		1.36 (1.01- 1.71)	5.27	0.971	Hill

*Unit is pmol 1′-OH-MDZ/pmol CYP3A5/min

**Unit is μM

Figure 13 shows the observed formation curves of the minor metabolite of MDZ, 4-OH-MDZ, in the two microsomal preparations of CYP3A5, in two separate experiments. Figures 13A and C represent metabolite formation in Supersomes® without cytochrome b_5, while figures 13B and D represent incubations with coexpressed cytochrome b_5. All of the curves were sigmoid (section 1.8) and were best fitted using Hill's equation (Equation 3).

The estimated kinetic parameters for the formation of 4-OH-MDZ in the two microsomal preparations are presented in Table 3. Values for V_{max} in the microsomal preparations without cytochrome b_5 were 8.9 and 6.9 pmol 4-OH-MDZ/pmol CYP3A5/min, while K_m values were 14.3 and 15.3 μM for the formation of 4-OH-MDZ. Greater uncertainty was associated with the parameters describing 4-OH-MDZ formation in the microsomal preparations with coexpressed cytochrome b_5 (Figures 13B and D) since these curves barely reached saturation (Figure 13B) or, did not appear to reach it at all (Figure 13D). The estimated CL_{int} values in the Supersomes® without cytochrome b_5 were twice as high as those in the Supersomes® with coexpressed cytochrome b_5 (Table 3).

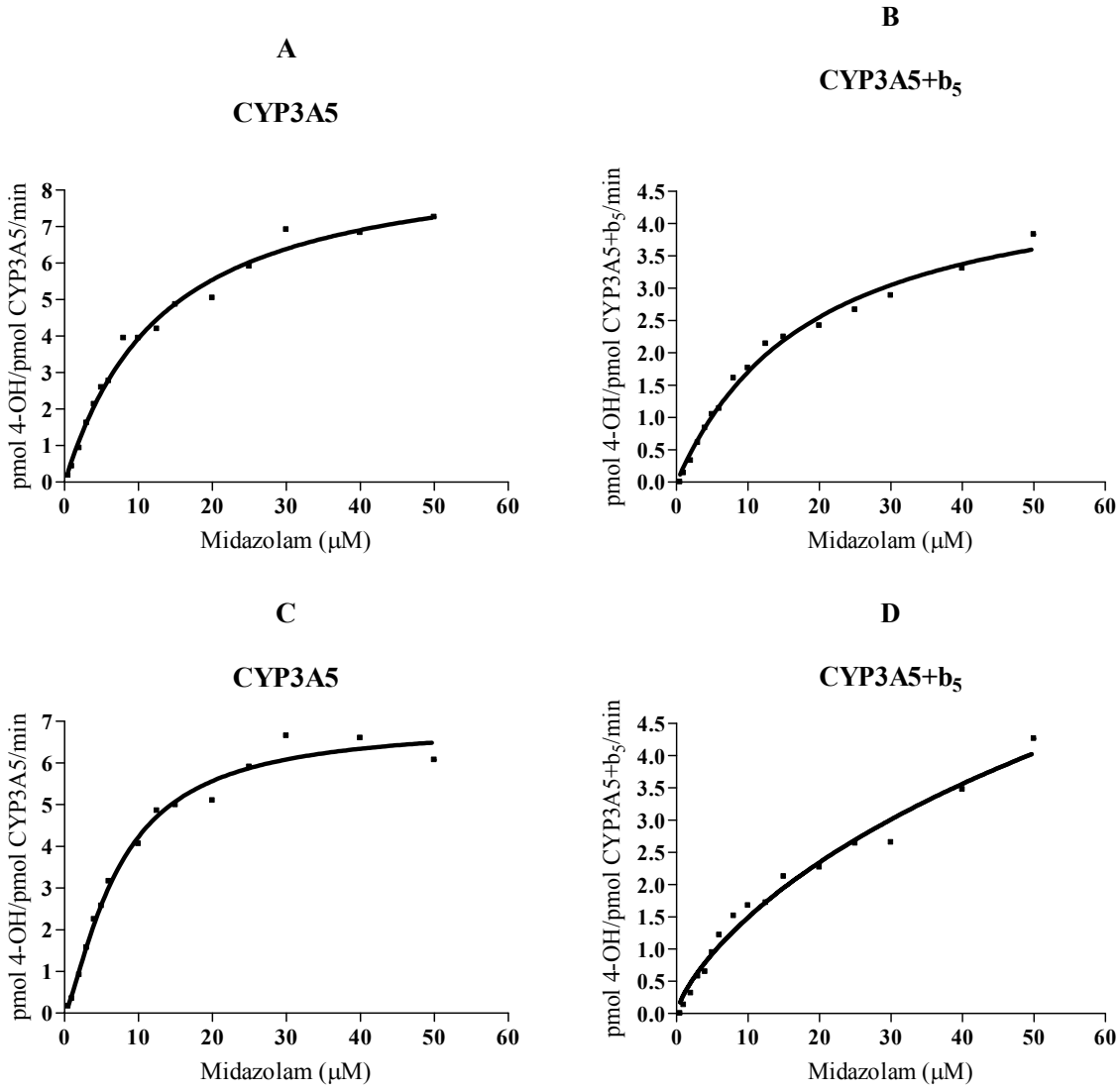


Figure 13*:* Dose-response curves showing formation of 4′-OH-MDZ in insect CYP3A5 microsomes (***A*** and ***C***) and insect CYP3A5 microsomes with coexpressed cytochrome b_5 (***B*** and ***D).***

Table 3: Kinetic data with 95 % confidence interval for the formation of 4-OH-MDZ in two different microsomal preparations of CYP3A5. Data from two separate experiments for each preparation are shown.

Supersomes®	Exp.	V_{max*}	K_m/ S_{50}**	N	CL_{int}	R^2	Model
Without b_5	**1**	8.98 (7.25-10.7)	14.3 (10.1-18.4)	1.05 (0.80-1.29)	0.62	0.988	Hill
	2	6.89 (6.16-7.62)	15.3 (8.36-22.3)	1.38 (1.06-1.70)	0.45	0.988	Hill
With b_5	**1**	4.88 (3.63-6.12)	20.0 (14.3-25.7)	1.03 (0.78-1.27)	0.24	0.990	Hill
	2	17.1 (-23.0-57.2)	54.6 (54.4-163)	0.74 (0.45-1.02)	0.31	0.980	Hill

*Unit is pmol 4-OH-MDZ/pmol CYP3A5/min

**Unit is μM

Figure 14 shows a formation ratio of the two midazolam metabolites (1′-OH-MDZ/4-OH-MDZ), expressed in peak heights as a function of substrate concentration in the two microsomal preparations, Supersomes® with and without coexpressed cytochrome b_5. Both preparations showed a concentration-dependent change in the ratio of the major and minor oxidation product, with higher 1′-OH/4-OH-MDZ product ratio at low substrate concentrations, and a decrease in the ratio at higher concentrations of MDZ. The difference between the formation ratios in the two preparations was also highest at low substrate concentrations, with the Supersomes® without coexpressed cytochrome b_5 (red colour) showing higher ratio (approximately 50), compared to Supersomes® with cytochrome b_5 (blue colour) (approximately 30). Formation ratios of the two metabolites in the two microsomal preparations were overlapping at higher midazolam concentrations.

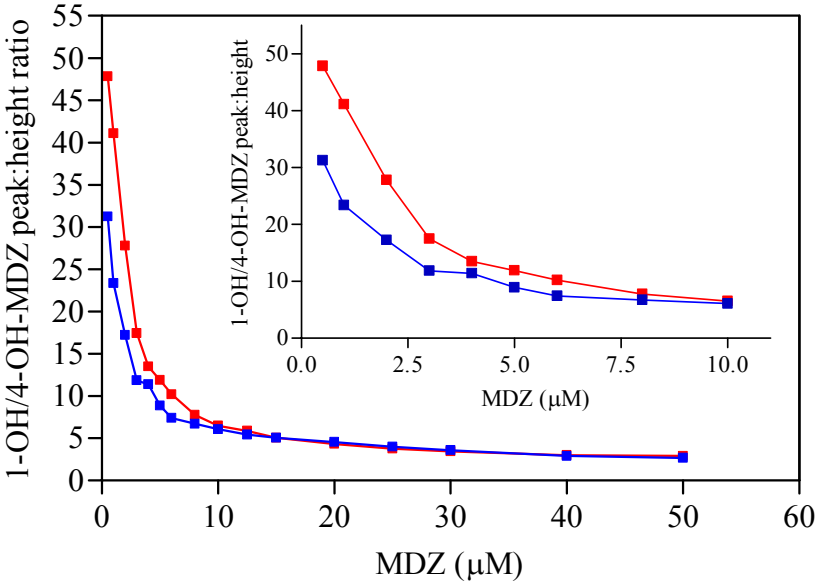


Figure 14: Curve showing formation ratio of 1′-OH-MDZ to 4-OH-MDZ as a function of MDZ concentration in two Supersomes® preparations. Red colour represents 1′-OH/4-OH-MDZ ratio in the Supersomes® without cytochrome b_5, while the blue colour represents the same ratio in the Supersomes® with cytochrome b_5. The inserted curve shows the formation ratio in the two preparations at lower concentrations of MDZ.

3.3.3 Metabolism of midazolam in the *CYP3A5* transfected THLE cells as a function of substrate concentration

LC-MS analysis of midazolam metabolism in microsomes from *CYP3A5* transfected THLE cells showed no formation of the two metabolites, 1′-OH-MDZ and 4-OH-MDZ. There was no difference between LC-MS chromatograms of the samples containing microsomes and those not containing microsomes. It was concluded that microsomes isolated from the *CYP3A5* transfected THLE cells did not metabolize midazolam.

4 DISCUSSION

4.1 METHODOLOGICAL CONSIDERATIONS

The aim of a stable trasfection is to obtain a cell clone with the gene of interest permanently integrated into the genome. To achieve this, the transgene must spontaneously integrate by recombination of the plasmid into the host genome and replicate in synchrony with the cell. In this thesis, the plasmid coding for the CYP3A5 enzymes was successfully integrated into the genome of the THLE cells (section 3.1). However, studies of protein expression and pilot metabolism studies with the microsomes isolated from these cells (section 3.1 and 3.3.3, respectively), gave negative results. This suggests that even though the CYP3A5 DNA had become integrated into the genome of the THLE cells, the conditions were not optimal for obtaining functional CYP3A5 proteins.

Efficiency of a transfection is affected by a variety of experimental conditions, which had been taken into account in the present thesis, and the conditions used are summarized in Table 1. For example, the cell density at the time of transfection is an important factor, but it varies with different cell types. For adherent cells, such as the ones used in this thesis, confluence between 70-90% is recommended in order to provide high yields. Another important issue is the phase of the cell cycle, as the cells should not be in the stationary phase under the transfection. Thirdly, presence of serum (BSA) during the transfection process was once considered to decrease the efficiency, but now it is established that serum can be present in the growing medium, as performed in this thesis, as long as the DNA-lipid cationic complexes (section 2.4) are formed in the absence of serum, since some proteins can interfere with their formation. The DNA-cationic lipid ratio is also of importance, and in this thesis it was showed that a ratio of 5:1 results in a successful transfection.

Despite the fact that the performed transfection seemed to have succeeded, the expression of functional CYP3A5 proteins did not occur. The quantity of the transfected CYP3A5 genes is uncertain and, assuming that this was low, approaches directed towards increased transfection efficiency, besides those summarized in Table 1, could be an alternative. Stable transfection, as conducted in this thesis, takes at least several weeks to perform. Cells containing integrated DNA are rare and must be amplified by selection for a drug resistance (blasticidin treatment,

section 2.5.1). However, cell line resulting after stable transfection should be a stable source of protein production, which yet, turned out not to be the case in this thesis, where CYP3A5 proteins were not expressed. A potential factor which could be further evaluated in order to increase the efficiency of transfection is to perform transient transfection. The level of enzyme expression in transient expression systems is usually far higher than that obtained with stable systems [9]. Detection of transient expression of the transgene generally lasts for 1 to 7 days. Only a fraction of DNA delivered to the cell makes it to the nucleus for transcription with eventual export of the message to the cytoplasm for protein production. Within a few days, most of the foreign DNA is degraded by nucleases or diluted by cell division, and after a week, its' presence can no longer be detected. Less time and work is required to perform transient expression than stable expression, but since the efficiency of DNA uptake and level of expression varies from one assay to the next, experiments must be carefully controlled. However, considering the fact that the *CYP3A5* transfected THLE cells did not express functional CYP3A5 proteins, transient transfection might be worth trying in the future.

Film developed in Western analysis of the proteins expressed in the *CYP3A5* transfected THLE cells (Figure 9) showed formation of bands after addition of the CYP3A5 antibodies. Since these antibodies are specific for CYP3A5, it indicates that at least protein fragments recognized by these antibodies are present. However, position of the bands, which is higher than references with declared CYP3A5 protein content, demonstrates that the molecular weight of the proteins from the transfected cells is higher, and they possibly do not have the same form as the reference proteins. Possible explanation to the higher molecular weight is that the appropriate post-translational modifications have not occurred. Protein synthesis is one of the most complex biosynthetic processes, starting with the activation of amino acids in the cytosol and terminating with the proteins folding to their active, three-dimensional forms. After synthesis, many proteins are directed to particular locations in the cell. In eukaryotic cells, the so called targeting signals are often bound to the polypeptides, transferring them to their location in the cell, ER in the case of CYP enzymes. Uncompleted protein processing could result with the targeting signal still being attached to the polypeptide, resulting in higher molecular weight. In conclusion, even though the observed results from the transfection indicate that the CYP3A5 gene was inserted, the reason why there was no expression of CYP3A5 proteins requires further evaluation.

4.2 CYP3A5 CATALYZED MIDAZOLAM METABOLISM

Pharmacokinetic parameters such as bioavailability and half-life are of major importance in defining the pharmacological and toxicological profile of drugs, and they can be calculated from clearance intrinsic, obtained from *in vitro* studies. Extrapolations of the *in vitro* data to *in vivo* are highly debated and quantitative predictions of *in vivo* clearance have, for many compounds, shown to be poor in retrospective studies [59]. Recombinant CYP enzymes, as the ones presented in this thesis, are used for these predictions, and variations in these expression systems can affect the accuracy of the prediction.

4.2.1 Formation of 1′-OH-MDZ in the two microsomal preparations

One of the primary aims of the present thesis was to investigate CYP3A5 catalyzed formation of 1′-OH-MDZ, which is the main metabolite of midazolam, in different microsomal preparations. Since the CYP3A5 transfected THLE cells showed no metabolism, the remaining two preparations to be investigated were recombinant insect microsomes specifically expressing CYP3A5 enzymes; the only difference between them being the presence of cytochrome b_5 in one of the two preparations. There are two main observations in the present study, both somewhat different from the previously published data. The first important observation is that the two preparations exerted different enzyme kinetics; metabolism of midazolam in microsomes with coexpressed cytochrome b_5 showed Michaelis-Menten pattern, even though the Hill's equation gave a slightly better fit; while metabolism in microsomes without b_5 showed substrate inhibition pattern. The fact that the two preparations demonstrated different kinetics suggests that cytochrome b_5 might have an impact on the CYP3A5 catalyzed metabolism of midazolam *in vitro*. Substrate inhibition observed in the absence of cytochrome b_5 is quite the opposite from previously published results [4, 29, 60], where midazolam metabolism in insect CYP3A5 microsomes followed classical Michaelis-Menten kinetics, independently of the presence of cytochrome b_5. The observed substrate inhibition in absence of cytochrome b_5 is on the other hand, in accordance with the work published by Galetin et al. [61], where the formation of both 1′-OH-MDZ and 4-OH-MDZ mediated by insect CYP3A5 without cytochrome b_5 demonstrated substrate inhibition. As mentioned in the introduction (section 1.1), substrate inhibition is a phenomenon that has been described previously *in vitro* experiments involving CYP3A enzymes, where the relatively large size of the active site of CYP3A enzymes with possibilities for several substrates binding simultaneously, has been suggested as an explanation. Effects of

cytochrome b_5 on drug oxidation activities of several human CYP3A enzymes have been investigated, and several investigators have reported enhanced enzymatic activity by addition of cytochrome b_5 [62, 63], with CYP3A7 as an exception, where addition of cytochrome b_5 appeared to have no effect on the enzymatic activity [4]. The requirement of cytochrome b_5 for oxidations has been shown to depend on the substrates employed, but the relationship between chemical structures of the substrates and effects of cytochrome b_5 is still not clear [64]. The mechanisms involved in the enhancing effect of cytochrome b_5 on the CYP-catalyzed metabolism have been extensively investigated, and donation of the second of two electrons in the oxidative metabolism of drugs (Figure 2) was demonstrated for the first time in 1971 by Hildebrandt et al. [65]. After that, several studies have shown that this donation prevents the oxygenated CYP complex (Figure 2) from decomposition, which is thought to stimulate the enzymatic effect of CYP, but increased affinity of CYP to substrates and enhanced activity between CYP and the reductase have also been proposed as stimulating effects of cytochrome b_5 on CYP enzymes [66]. In 2003 Yamaori et. al. [4] investigated 14 different CYP3A5 substrates, among them also midazolam, and reported an enhancement in V_{max} about twice by addition of cytochrome b_5, which is approximately the same enhancement as the one reported for CYP3A4, which has been studied more extensively with respect to effects of cytochrome b_5 on the enzymatic activity [4].

The second important observation in the present thesis is that the quantitative data describing the observed metabolism, somewhat surprisingly, gave lower formation rate in the microsomes expressing CYP3A5 with cytochrome b_5. Possible explanation for the apparently lower formation rate in the presence of cytochrome b_5 could be that the metabolic turnover actually is much faster, so that the formed 1′-OH-MDZ “disappears” faster, being subsequently metabolized during the incubation time of 7.5 minutes. As a matter of fact, a third minor metabolite of midazolam, the 1′,4-diOH-MDZ, has been described previously [67, 68], formation of which had been observed in human liver microsomes incubated with both 1′-OH-MDZ and 4-OH-MDZ as substrates. On the other hand, the higher formation rate observed in the absence of cytochrome b_5 could then be a consequence of potential accumulation of the formed 1′-OH-MDZ resulting from unavailability of the cytochrome b_5 that would contribute to further metabolism.

Interestingly, cytochrome b_5, which has been one of the main focuses of this thesis, has also been addressed in a study published in September 2007, where several prediction methods

were compared in order to determine Cl_{int} value using data from recombinant CYP enzymes, and which demonstrated that recombinant CYP microsomes coexpressed with cytochrome b_5 in insect cells might be suitable for use in these predictions [69].

4.2.2 Formation of 4-OH-MDZ in the two microsomal preparations

The observed formation rate for 4-OH-MDZ was slower than for 1′-OH-MDZ in the same microsomal preparations, which is in concordance with the previously published work [29, 70]. On the other hand, unlike the hyperbolic formation curves reported previously [29, 70], the patterns of 4-OH-MDZ formation in this thesis were sigmoid. As was the case for 1′-OH-MDZ, the reaction velocity was slightly lower when microsomes expressing cytochrome b_5 were applied, in addition to the fact that saturation was not reached.

4.2.3 The relative proportions of the formation of the midazolam metabolites

The relative ratio of the formation of the two metabolites of midazolam, 1′-OH-MDZ and 4-OH-MDZ was also investigated. Product ratio 1′-OH/4-OH-MDZ was higher in CYP3A5 preparation without cytochrome b_5 than in the one with cytochrome b_5, at low concentrations, while a decrease in the ratio was observed for both microsomal preparations at higher concentrations of midazolam. This is in concordance with the previously published results, where, in addition, metabolic switching to the minor metabolite has been observed at substrate concentrations over 50 μM MDZ [62]. For further evaluation, investigations using higher concentrations of midazolam would have to be performed. The observed difference in the formation ratio of the two metabolites at low concentrations additionally supports the possible impact of cytochrome b_5 on the CYP3A5 catalyzed metabolism of midazolam *in vitro*.

4.3 FUTURE CONSIDERATIONS

More experiments are necessary in order to characterize enzymatic activity of CYP3A5. THLE cells expressing specifically CYP3A5 enzymes resemble *in vivo* environment more closely than the insect cells applied in the present thesis, and studies should be carried out in them as well, especially since the comparison of the kinetics in these two *in vitro* systems has not been extensively studied, while there are suggestions that there might be differences in the metabolism of midazolam in CYP3A4 depending on the *in vitro* system applied [58]. In order to perform this comparison, the experimental conditions need to be optimalized to obtain functional CYP3A5 proteins following *CYP3A5* transfection of the THLE cells.

As the presence of cytochrome b_5 appeared to be of relevance for the enzyme kinetics observed in this thesis, the quantification of cytochrome b_5 in human liver cells using specific antibodies for cytochrome b_5, which are commercially available, might be of importance for the extrapolations of the *in vitro* data to *in vivo.* Whether the expression of cytochrome b_5 is genetically determined is another important aspect that should be addressed in the future.

Performing depletion studies is also an approach that could be used to evaluate midazolam metabolism catalyzed by CYP3A5, as opposed to the metabolite formation method used in the present thesis. In the metabolite formation studies, as showed here, intrinsic clearance is obtained by determination of enzyme kinetic parameters (V_{max} and K_m). In depletion studies, CL_{int} is determined by measuring the first-order rate constant for consumption of the substrate at a low concentration. The substrate depletion method is useful in the early drug-discovery stage, when the main metabolites and the metabolic pathways have not been identified. There are two assumptions in the depletion model that are not required if intrinsic clearance were determined from enzyme kinetic data (V_{max}/K_m), and these are: 1) the substrate concentration employed is well below the apparent K_m, and 2) there is no significant product inhibition, nor is there any mechanism based inactivation of enzyme. The latter assumption might represent a limitation in applying depletion studies to the systems presented in this thesis, as substrate inhibition has been observed. However, depletion studies would be valuable in further investigations, especially in order to evaluate the hypothesized subsequent metabolism of midazolam to 1, 4-diOH-MDZ presented in this thesis. Another possibility to evaluate the subsequent metabolism is to include this third metabolite in the LC-MS analysis of the metabolite formation, which was not done in the present thesis.

Since genetic polymorphism is a characteristic of CYP3A5 and not CYP3A4, and these two enzymes are the clinically most important CYP3A enzymes, with overlapping substrate specificities, further studies comparing their metabolic capacities should be conducted. Studies of this kind have been performed, as mentioned in section 1.5.1, but there is great discordance in the results, which can partly be explained by the difference in enzyme preparations employed and how these were supplemented with coenzymes, specifically cytochrome b_5 [62]. For example, cytochrome b_5 has been coexpressed in the preparations with CYP3A4 and supplied in preparations with CYP3A5, and these preparations have then been used for comparison. Later, it has been shown that the metabolic capacity of an enzyme in a given preparation actually can vary dependently of the source of the cytochrome b_5; with the preparations coexpressing it showing higher metabolic activity than preparations where cytochrome b_5 had been supplied to the same ratio [62]. This again stresses the importance of standardizing *in vitro* systems, especially when results are to be compared across studies.

5 CONCLUSION

The metabolism studies of midazolam in two *in vitro* systems, the CYP3A5 expressing Supersomes® with or without cytochrome b_5, with respect to the formation of two metabolites, 1′-OH-MDZ and 4-OH-MDZ, demonstrated different kinetics depending on the presence of cytochrome b_5. Formation of the main metabolite, 1′-OH-MDZ, demonstrated Michaelis-Menten like kinetics in the presence of cytochrome b_5, and atypical substrate-inhibition in the absence of cytochrome b_5, while enzyme kinetic parameters calculated from the non-linear regression analysis showed higher formation rate of the primarily metabolites in the preparations without cytochrome b_5 than in those with coexpressed cytochrome b_5. This indicates that cytochrome b_5 might have an impact on the CYP3A5 catalyzed metabolism of midazolam *in vitro*, but more experiments are needed to evaluate its role, *in vitro* as well as *in vivo.* In order to obtain expression of functional CYP3A5 proteins in the transfected THLE cells, the experimental conditions need to be optimalized, since this expression system will be of value for future comparisons of different *in vitro* systems.

6 REFERENCES

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7 APPENDIX

7.1 RECIPES

7.1.1 Solutions for culturing of THLE cells

Growth medium		Trypsin-EDTA diluted with HBSS	
PFMR-4 medium	500 mL	1:1, v/v	
L-glutamine (200 mM)	5.0 mL	Trypsin-EDTA (1x)	25 mL
Gentamycin (10 mg/mL)	2.5 mL	HBSS	25 Ml
Insulin (0.35 mM)	2.5 mL		
Hydrocortisone (10 mM)	0.01 mL		
EGF (5 µg/mL)	0.5 mL		
Transferrin (5 mg/mL)	1.0 mL	HBSS with 2.5% FBS	
Triiodthyronine (1 mM)	0.025 mL	HBSS	97.5 mL
P/E (0.1 mM)	2.5 mL	FBS	2.5 mL
RA (3.3 µM)	0.05 mL		
BSA (3%)	50 mL		

7.1.2 Solutions for PCR analysis

10xPBS, Sodium citrate buffer		1xPBS	
$NaH_2PO_4 \cdot H_2O$	1.57 g	10xPBS	
$NaH_2PO_4 \cdot 2H_2O$	9.8 g	DEPC-dH_2O	
$Na_3C_6H_5O_7 \cdot 2H_2O$	60 g		
NaCl	81.8 g	DEPC-dH_2O	
Distilled H_2O	ad 100 mL	DEPC	1 mL
pH 7.4		Distilled H_2O	ad 1000 mL
Autoclave		Autoclave	

7.1.3 Solutions for agarose gel electrophoresis

5XTBE, Tris-borate buffer		1xTBE	
Tris		5xTBE	
HBr		Distilled H_2O	
0.5 M EDTA pH 8.0			
Distilled H_2O			
Autoclave			
Ethidium bromide (10 mg/mL)		Loading dye buffer	
Ethidium bromide	0.2 mg	Bromophenol blue	0.25 g
Distilled H_2O	20 mL	Xylene cyanol FF	0.25 g
		Ficoll	15 g
		Distilled H_2O	ad 100 mL
DNA standard (0.1 µg/µL)			
Low molecular weight DNA			
Ladder, 100 bp, 1 µg/µL	20 µL		
Loading dye buffer	33 µL		
Distilled H_2O	147 µL		

7.1.4 Solutions for Western electrophoresis

10% separating gel		4% stacking gel	
Distilled water	5.23 mL	Distilled water	6.10 mL
1.5 M Tris-HCl pH 8.8	3.25 mL	0.5 M Tris-HCl pH 6.8	2.50 mL
Monomer	4.33 mL	Monomer	1.30 mL
10% SDS	130 µL	10% SDS	100 µL
10% ammoniumpersulfate (APS)	65 µL	10% ammoniumpersulfate (APS)	50 µL
TEMED	6.5 µL	TEMED	10 µL
Electrophoresis buffer pH 8.3		Blotting buffer pH 8.3	
0.025 M Tris- HCl	7.5 g	0.025 M Tris- HCl	12 g
SDS	2.5 g	SDS 0.02%	0.8 mL
0.192 M Glycin	35 g	0.192 M Glycin	57.6 g

Distilled water 2.5 L	20% MeOH 800 mL Distilled water 2.5 L
Sample buffer 0.5 M Tris-HCl pH 6.8 12.5 mL Glycerol 10 mL 10 SDS 20 mL	
2xLaemmLi buffer 0.0625 M Tris.HCl 0.1% SDS 15% Glycerol	1xLaemmLi buffer 50% 2 X LaemmLi buffer 50% distilled water
T-TBS 100 mM Tris.HCl pH 7.5 100 mM NaCl 0.05 % Tween	Blotto (125 mL/membrane) 25 g fat-free dry milk solved in 0.5 L T-TBS. Heated, filtered and stored at refrigerator for about a week.
Fixation solution 120 mL Fix 280 mL water	Developing solution 120 mL developer 280 mL water

7.1.5 Solutions for microsomal studies

Buffer solution for microsomal studies	
200 mM Tris-H_2SO_4 (pH=7.4)	130 µL/vial
20 mM $MgSO_4$	5.5 µL/vial
10 mM NADPH	35 µL/vial

7.1.6 Solutions for LC-MS

Mobile phase A:		Mobile phase B:	
NH_3 25%	4.036 mL	Acetonitrile	950 mL
HCOOH (conc.)	2.125 mL	Methanol	50 mL
Deionised water	1 L		

7.2 RAW DATA

Table 4: Peak height values for the two metabolites of MDZ (1′-OH and 4-OH) and internal standard DIA in the two experiments performed in Supersomes® without cytochrome b_5**.**

	Experiment 1			Experiment 2		
MDZ(µM)	**1-OH**	**4-OH**	**DIA**	**1′-OH**	**4-OH**	**DIA**
0.5	30212	564	18786	24975	522	18875
1	54205	1432	19646	45094	1097	19045
2	73748	2921	18804	76979	2768	18081
3	90675	4814	17796	87949	5038	19270
4	96825	6376	17953	92685	6870	18348
5	97702	8077	18708	86741	7302	17039
6	93440	8367	18104	89330	8756	16626
8	98917	11876	18091	88866	11463	24511
10	98917	11876	18091	80266	12366	18312
12.5	92967	12701	18179	82291	13932	17258
15	92316	13595	16792	77845	15322	18438
20	86427	15640	18610	70001	16173	19055
25	69167	16757	17029	66207	17555	17866
30	70657	19538	16964	65984	19188	17333
40	61891	20211	17774	60808	20149	18353
50	61034	22552	18658	56417	19473	19268

Table 5: Peak height values for the two metabolites of MDZ (1′-OH and 4-OH) and internal standard DIA in the two experiments performed in Supersomes® with coexpressed cytochrome b_5.

	Experiment 1			Experiment 2		
MDZ(µM)	**1′-OH**	**4-OH**	**DIA**	**1′-OH**	**4-OH**	**DIA**
0.5	9732	0	36779	11557	370	34585
1	18641	864	33636	20271	868	34159
2	29374	1889	31564	32033	1860	31007
3	38090	3324	30199	44044	3722	34392
4	44682	4767	31545	45604	3998	32681
5	45672	5564	29417	52144	5865	32894
6	47712	6442	31328	57425	7761	33835
8	52425	8637	29759	57083	8506	29774
10	50950	9604	30168	62151	10222	32408
12.5	50027	10679	27677	55496	10199	31421
15	52414	11908	29393	64014	12597	31419
20	55274	13739	31458	60770	13322	31159
25	55708	14373	29890	63353	15832	31842
30	61699	16815	32284	59329	16507	32999
40	58665	17571	29460	60595	20775	31714
50	64845	23892	34580	71024	26281	32703