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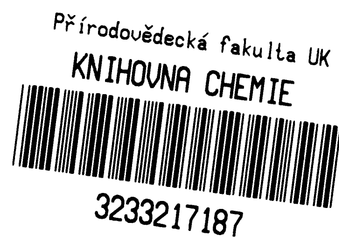

Charles University Prague

Faculty of Science

Department of Biochemistry

DIPLOMA THESIS

Eva Martínková

Supervisor: Doc. RNDr. Marie Stiborová, DrSc.

Prague 2006

Charles University Prague

Faculty of Science

Department of Biochemistry

Oxidation of the Anticancer Drug Ellipticine by Rat and Human Cytochromes P450

Diploma Thesis

Supervisor: Doc. RNDr. Marie Stiborová, DrSc.

Prague 2006 Eva Martínková

Declaration:

I affirm I worked out this diploma thesis alone under the professional direction of my supervisor Doc. RNDr. Marie Stiborová, DrSc. and I presented in all references used.

Prague, April 24, 2006

Eva Martínková
....
Signature

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List of abbreviations

AIDS	acquired immune deficiency syndrome
A_x	absorbance at the wavelength of x nm
BSA	bovine serum albumin
c	concentration
CYP	cytochrome P450
cyt b_5	cytochrome b_5
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
EC	enzyme code
FAD /FADH/$FADH_2$	flavinadeninedinucleotide (quinoid form) /semiquinoid radical/hydroquinone
FMN /FMNH/$FMNH_2$	flavinadeninemononucleotide (quinoid form) /semiquinoid radical/hydroquinone
FMO	flavin containing monooxygenase
GIT	gastrointestinal tract
HIV	human immunodeficiency virus
HPLC	high-performance liquid chromatography
l	optical path
M	mole/l
M1	ellipticine metabolite M1 – 9-hydroxyellipticine
M2	ellipticine metabolite M2 – 12-hydroxyellipticine
M3	ellipticine metabolite M3 – 13-hydroxyellipticine
M4	ellipticine metabolite M4 – 7-hydroxyellipticine
M5	ellipticine metabolite M5 – ellipticine N^2-oxide
MFO	mixed function oxidases
NAD^+ (NADH)	nicotineamideadeninedinucleotide oxidized (reduced)
$NADP^+$ (NADPH)	nicotineamideadeninedinucleotidephosphate oxidized (reduced)
P420	pigment with absorption maximum at 420 nm
PAH	polycyclic aromatic hydrocarbons
PAPS	2′-phosphoadenosine-5′-phosphosulphate
RNA	ribonucleic acid

RP-HPLC	reverse-phase high-performance liquid chromatography
RPM	revolutions per minute
T_3	triiodothyronine
TCDD	2,3,7,8-tetrachlorodibenzo[1,4]dioxin
TRIS	tris(hydroxymethyl)aminomethane
UDP	uridinedinucleotidephosphate
ε	molar absorption coefficient
λ	wavelength

1 Introduction

Since cancer is one of the most serious problems of current clinical medicine, the pathological processes employed attract a lot of scientific interest. In the year 2003, 29,195 people died from cancer, *i.e.* 26.2% of all deaths in the Czech Republic[125]. In addition to this, the patients affected by this civilization disease are becoming younger and younger. These statistics may be partially explained by such extreme changes in the environment as well as in people's lifestyles performed during past several decades - food contains much more fats, sugars and synthetic additives; people are much more stressed and the environment is polluted by a number of carcinogens. These factors cause our immune system to be overburdened and unable to destroy such high amounts of cancerous cells that result from such stressful conditions. These facts are in accordance with the growing incidence of cancer diseases in recent years (for breast cancer see Figure 1).

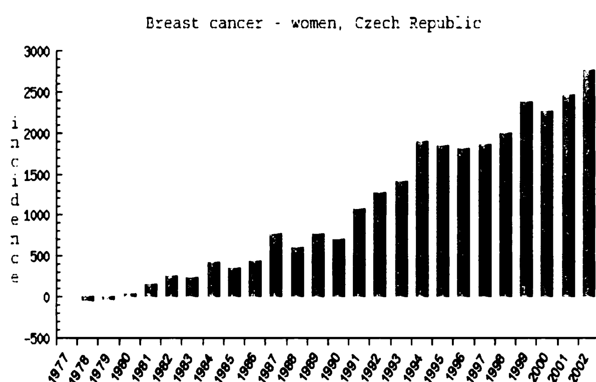

Figure 1. An increase in incidence of breast cancer in the years 1977-2002 in the Czech Republic. Modified from [124].

1.1 *Carcinogenesis*

Carcinogenesis is a multistage process of malign transformation of a normal, healthy cell to a tumorous cell. The genetic changes in oncogenes and/or tumour suppressor genes are the pre-requisite steps of the cell malign transformation[47], because both of these types of genes are responsible for regulation of the cell growth and differentiation. DNA can be damaged either by mistake during the replication process, or by some exogenous factors. These exogenous factors include physical factors: radioactive radiation, cosmic radiation,

UV, X-rays and some particles (*e.g.* asbestos); biological factors: viruses and genetic factors; and chemical carcinogens. Figure 2 shows diverse damages on a DNA molecule. 90% of chemical carcinogens cause formation of DNA adducts. Other types of DNA damages include apurination or apyrimidation, cross-linking, formation of pyrimidine dimers, intercalation, alkylation of bases and single/double strand breaks[3].

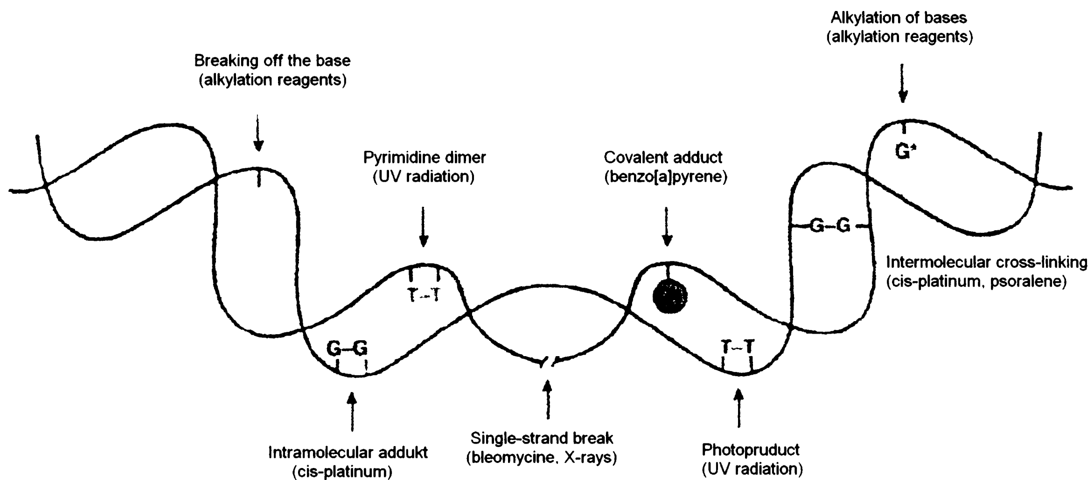

Figure 2. Examples of DNA damages caused by various exogenous factors. Modified from [94].

The transformational process is then followed by dividing and differentiating the cells, and increment of malign potential of these cells[10]. If the proliferation of an abnormal cell is out of control, it will give rise to a tumour (a neoplasm)[4]. As long as the neoplastic cells remain clustered together in a single mass, the tumour is said to be benign. At this stage, it can be cured by removing the mass surgically. A tumour is considered a cancer only if it is malignant – its cells acquire the ability to invade surrounding tissue[4]. The cells that split off from the primary tumour can enter the bloodstream or lymphatic vessels, settle down in various organs or tissues and form there secondary tumours – metastases[3]. Figure 3 shows a scheme of the process of carcinogenesis.

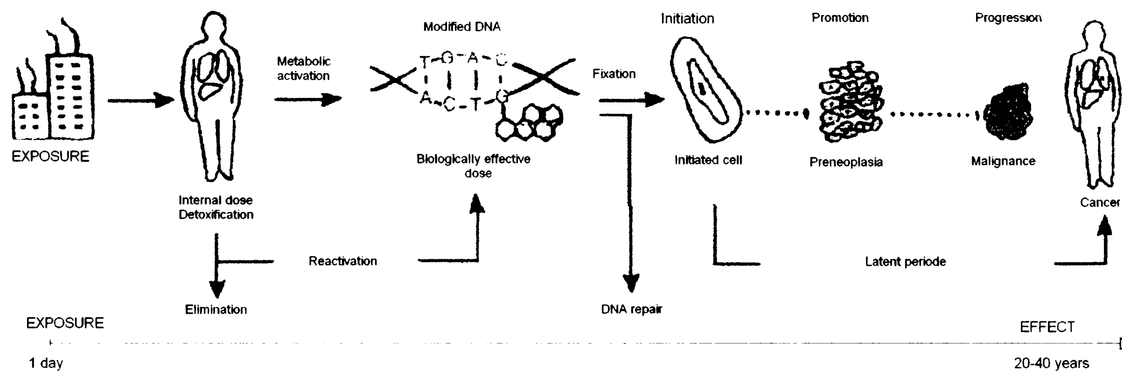

Figure 3. Scheme of a multistage process of carcinogenesis. Modified from [94].

Cancers are classified according to the cell type from which they arise. Cancers arising from the epithelial cells are termed carcinomas; those arising from the connective tissues or muscle cells are called sarcomas. Cancers that do not fit in either of these two categories include leukemias, which are derived from hemopoietic cells, and cancers derived from cells of nervous system[4]. Figure 4 shows cancer incidence and mortality in the United States in the year 2000.

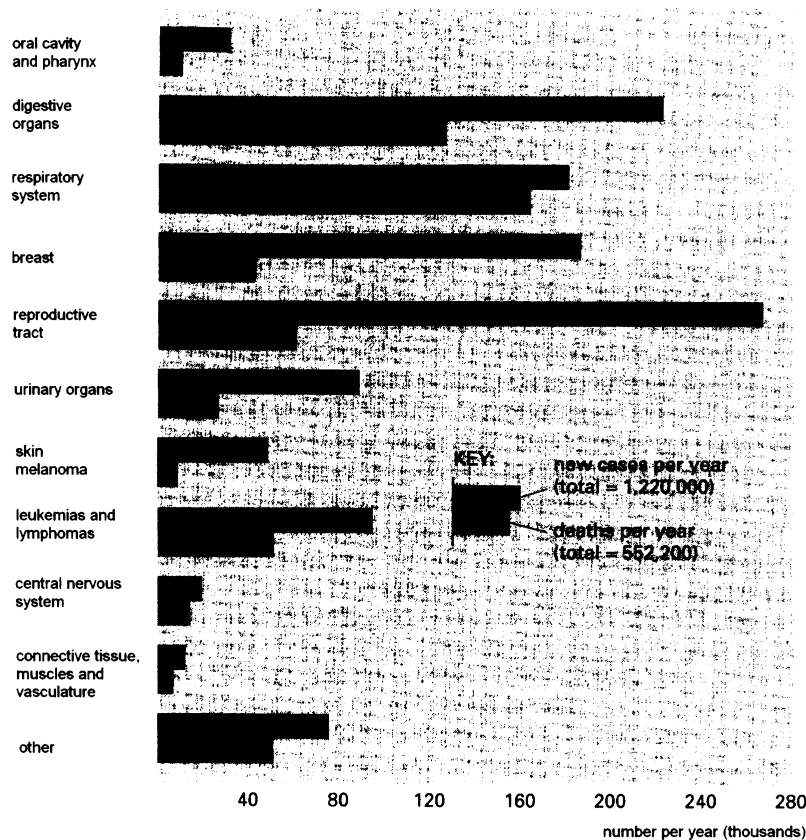

Figure 4. Cancer incidence and mortality in the United States for the year 2000. Total new cases diagnosed in that year in the United States were 1,220,000, and total cancer deaths were 552,200. (Data from American Cancer Society, Cancer Facts and Figures, 2000.) [4].

1.2 Cancer treatment

Cancerous cells are partially familiar to the organism, and thus the treatment of cancer is more complicated than the treatment of infections caused by bacteria, for example. Cancerous cells differ from the normal ones, but the differences are either too small to enable the immune system to recognize them, or the cancerous cells use diverse mechanisms to paralyse the immune system.

In most cases, cancer is not curable by a single method. Complex procedures, combining the basic therapeutic methods: surgery, radiotherapy and chemotherapy (including the hormonal therapy), are much more effective. Large tumours that do not invade into vitally important structures can be removed surgically. Residual tumours can be eliminated by radiotherapy. However, chemotherapy is the only method that can be used for the treatment of disseminated cancer[54]. In some special cases, immunotherapy can be also applied[46].

1.2.1 Chemotherapy

Significant progress in the treatment of cancer by cytostatics has been made in the last several decades. However, the possibilities of current treatment of cancer are still not satisfactory[54]. Cancers represent a very heterogeneous group of diseases; therefore it is impossible to find some universal drug that would function against all types of tumours. Current medicine is still lacking in substances which would interfere with the disease in its initiation or promotion phase[3].

The most important factor concerning the use of cytostatics against cancer is the tumour's sensitivity to chemotherapy, which is extremely variable for individual types of tumours. Chemotherapy can be primary (curative) in some particularly sensitive cases of cancer; or it can complement another method (radiation or surgery) – in that case it is applied after initial treatment to suppress secondary tumour formation, and is termed adjuvant treatment.

Chemotherapy can also be neoadjuvant – it facilitates the operability of a tumour; or is just palliative (Figure 5)[54].

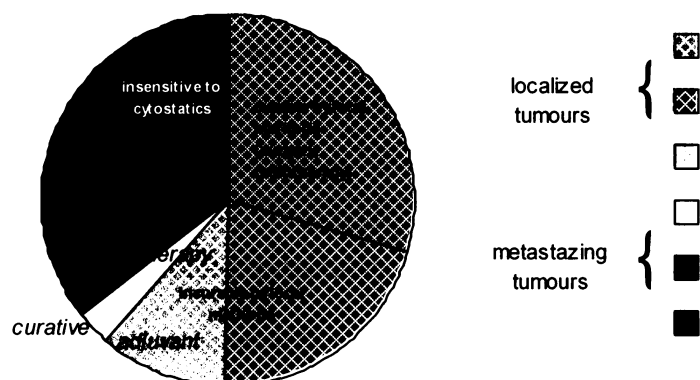

Figure 5. Role of chemotherapy in cancer treatment. Modified from [54].

There are three main methods of developing cytostatics today: synthesis of new substances (most common; cca 15,000 new cytostatics per year); fermentation of moulds with subsequent isolation of anticancer antibiotics; and extraction of natural substances from plants, sponges, etc. (cca 400 substances per year)[54].

Mechanisms of action of conventional cytostatics

Mechanisms of action of most cytostatics have not been fully elucidated yet. Each cytostatic can interfere with various metabolic pathways and block pathological as well as physiological enzymatic systems. The cytotoxic effect of current chemotherapeutics is understood to function due to following mechanisms[3, 54]:

- **Inhibition of enzymes,** which play crucial roles in the synthesis of nucleic acids, and subsequent inhibition of cell division. These substances assigned as antimetabolites are either analogues of metabolites which inhibit reactions of the synthesis of nucleic acids (*e.g.* analogue of folic acid – methotrexate), or analogues of nucleotides which are incorporated into the DNA double-helix – that leads to its incorrect function.
- **Direct damage of nucleic acids** (*e.g.* alkylation, intercalation, breaks, inhibition of topoisomerases), which also results in their incorrect function.
- **Alteration of a microtubular protein,** which results in the inhibition of polymerization and/or depolymerization of microtubules and, therefore, in the inhibition of mitosis.

- **Inhibition of protein synthesis** (rare)
- **Damage of cell membranes** (rare)

While the mechanisms of action and metabolism of anticancer agents should be known before they are used in clinical medicine, these data are often completed later. In such cases (*e.g.* ellipticine, see Chapter 1.2.2), their use is based on empirically acquired data[54].

Specificity of conventional cytostatics

The effect of a drug is dependent on its transport across the cell membrane, its activation and its action. It would be ideal if one of these steps was specific for transformed cells, *i.e.* a specific transport only into transformed cells; activation of the drug only in transformed cells; or interaction with enzymes only in transformed cells. In fact, the selective effect on transformed cells is achieved by distinct kinetics in healthy and in transformed cells – the healthy cells recover from the chemotherapy much earlier than the transformed ones[3].

1.2.2 Ellipticine

Ellipticine (5,11-Dimethyl-6H-pyrido(4,3-b)carbazole) (for the structure see Figure 6) and its derivatives are pyridocarbazoles which were found to possess significant anticancer activity. Ellipticine was first identified in 1959 in the leaves of a small Australian tropical evergreen tree *Ochrosia elliptica* Labill. from the *Apocyanaceae* family (Figure 7)[36, 38]. Subsequently, ellipticines were found also in other *Apocyanaceae* plants, *e.g. Ochrosia moorei, Ochrosia borbonica, Excavatia coccinea, Ochrosia acuminata.* [27, 36, 55, 63, 81, 83]

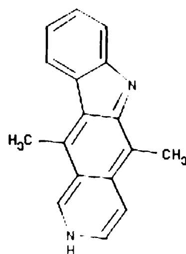

Figure 6. Ellipticine (5,11-Dimethyl-6H-pyrido (4,3-b) carbazole, $C_{17}H_{14}N_2$, MW 246.311)[126]

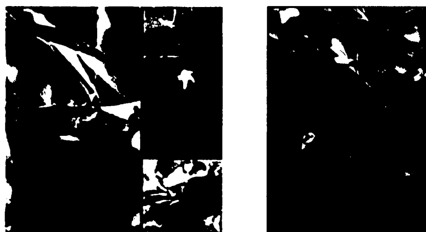

Figure 7. *Ochrosia elliptica*

Ellipticine's more soluble derivatives, 9-methoxyellipticine (Figure 8) and 2-methyl-9-hydroxyellipticine (Figure 9) in the form of acetate, have been utilized pharmacologically since 1970s, particularly in France[56]. The main reason for the interest in ellipticine and its derivatives for clinical purposes is their high efficiency against several types of cancer, their rather limited toxic side effects and their complete lack of hematological toxicity[96]. They are highly efficient against osteolytic breast cancer metastases, acute myeloblastic leukemia, kidney sarcoma and thyroid carcinoma[1, 98]. Ellipticines exhibit also significant anti-HIV activity because of their ability to inhibit retroviral integrase[64, 95, 96]. Most ellipticines are mutagenic to *Salmonella typhimurium* Ames tester strains, bacteriophage T4, *Neurospora crassa* and mammalian cells, and induce prophage lambda in *Escherichia coli*[95, 96].

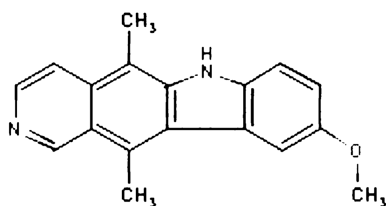

Figure 8. 9-methoxyellipticine (9-Methoxy-5,11-dimethyl-6H-pyrido(4,3-b)carbazole, $C_{18}H_{16}N_2O$, MW 276.337)[126]

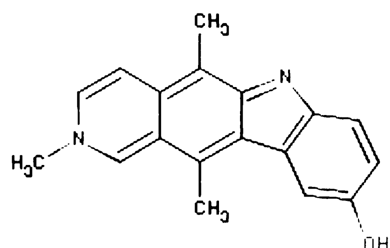

Figure 9. 2-methyl-9-hydroxyellipticine (2H-Pyrido(4,3-b)carbazol-9-ol, 2,5,11-trimethyl, $C_{18}H_{16}N_2O$, MW 276.337)[126]

Ellipticine pharmacokinetics

The physiological disposition of ellipticine was studied by Chadwick and co-workers[20] in the mouse, rat, dog and monkey after administration of [1- ^{14}C]ellipticine at 6 mg/kg intravenously (3 mg/kg to monkey). Ellipticine was widely, but not uniformly, distributed throughout the tissues including the brain, so it seems able to overcome the blood-brain barrier, either because of its lipid-solubility[20], or due to some form of active transport[112]. The rate of elimination, distribution and excretion is species-dependent[20].

The primary site of ellipticine metabolism is the liver, where it is predominantly metabolised to 9-hydroxyellipticine (rat) [20]. The 9-hydroxyellipticine does not accumulate here but is conjugated to its glucuronide and sulphate, which are secreted in bile[14, 20].

Hydroxylation at carbon C9 occurs approximately three times more intensively in animals treated by methylcholanthrene (an inductor of CYP1A and CYP1B1) and twice more intensively in animals treated by phenobarbital (an inductor of CYP2B)[20, 57]. An overview of ellipticine metabolism by human CYP enzymes *in vitro* is shown at Figure 10.

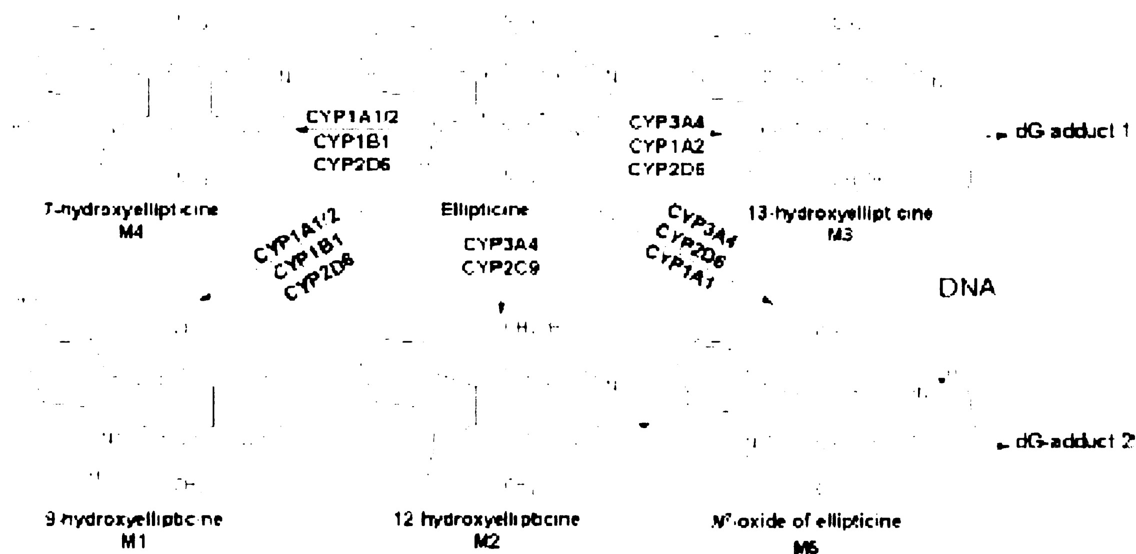

Figure 10. Metabolism of ellipticine by human CYPs showing the metabolites and those leading to DNA adducts[103].

Ellipticine is a weak base ($pK_a = 5.8$), it is ionized in acidic medium of the gastrointestinal tract (GIT) and could not be resorbed from the intestine.

Subcellular distribution of ellipticine is shown in Table 1 (p 17). The majority of both – the ethyl acetate-extractable equivalents, probably ellipticine, and the ethyl acetate-unextractable equivalents, its more polar metabolites - were located in the nuclear fraction (Table 1, p 17). Subcellular distribution of ellipticine is influenced primarily by its interactions with DNA, RNA, proteins and lipids. The ellipticine-lipid complex was detected in all tissues in which toxic effects occurred, blood and bile but it did not appear to influence the pharmacokinetics of ellipticine[20].

Table 1. Distribution of ethyl acetate-extractable and –unextractable ellipticine equivalents in subcellular fractions of dog liver. The distribution of drug equivalents is expressed as a percentage of the total ellipticine equivalents present in the liver homogenate. Liver was removed 4 hr after [1-^{14}C] ellipticine at 6 mg/kg intravenously in 1% lactic acid / 0.9% saline solution (pH 4.3). Ethyl acetate-unextractable equivalents are polar ellipticine metabolites; the parental molecule represents the ethyl acetate-extractable equivalent [20].

Fraction	Distribution of drug equivalents [%]	
	ethyl acetate-extractable	ethyl acetate-unextractable
Liver homogenate	36	64
Nuclear fraction (1)	17	42
Mitochondrial fraction (2)	6	7
Microsomal fraction (3)	2	3
Soluble fraction (4)	3	18
(1) + (2) + (3) + (4)	28	70

Mechanisms of action of ellipticine

The mode of action of ellipticine is considered to be based mainly on:

- **DNA intercalation**[8, 11, 28, 54] caused by weak reversible hydrophobic interactions with DNA bases[9]. Interaction of the methyl of ellipticine and the thymine in DNA is crucial to the ellipticine molecule's orientation at the intercalation site[90];
- **Inhibition of topoisomerase II**[9, 96]; ellipticines interact with DNA or topoisomerase II and form an inactive ternary complex. Thus, they stimulate double-strand breaks in DNA molecules and subsequent cell death[35, 95];
- **Covalent DNA adducts formation** after enzymatic activation by CYPs[13, 34, 95, 96, 103, 104]; ellipticine forms two major covalent DNA adducts through its metabolism to 13-hydroxyellipticine and 12-hydroxyellipticine. The latter one is formed by ellipticine oxidation by CYPs or spontaneously from ellipticine N^2-oxide[103, Stiborová - unpublished results].

Other minor mechanisms of action are as follows:

- **Selective inhibition of p53 phosphorylation**[72, 106]; protein p53 (product of the tumour suppressor gene *p53*) is present at very low concentrations in undamaged cells. DNA damage activates protein kinases that phosphorylate p53. This leads to

the decrease of its degradation, resulting in the induction of apoptosis, which is the physiological function of p53. Mutations of the *p53* gene occur in at least half of all human cancers[4]. Ellipticines seem to be able to regenerate its physiological function[84, 106], probably via inhibition of a specific cyclin-dependent kinase[72].

- **Inhibition of oxidative phosphorylation,** which results in a lethal decrease of ATP concentration[32, 89, 104].
- **Inhibition of telomerase**[84]; telomerase is a ribonucleoprotein enzyme that elongates and/or maintains telomeric DNA. It has been recognized as a potent diagnostic marker in a variety of human cancers because of its prevalence in most tumour cells[84].

Except the covalent DNA adduct formation[13, 34, 95, 104] most of the above mentioned mechanisms of antitumour activity of ellipticine are based on unspecific action. No discrimination between healthy tissues and tumour cells in ellipticine uptake is to be expected because ellipticines are highly hydrophobic and enter cell membranes by diffusion[95]. This fact contrasts with relatively specific antitumour activity of ellipticine against individual types of tumours[98]. A cancer-specific cell-kill is known to be caused by several anticancer drugs, which are almost inactive until metabolised[95]. Thereby, CYP dependent formation of covalent DNA adducts could at least partially explain the antitumour specificity as well as the high efficiency of ellipticine. CYP isoenzymes known to be expressed in higher levels in tumours sensitive to ellipticine (*i.e.* breast cancer, renal cell cancer) than in peritumoral tissues, namely CYP3A4, CYP1A1 and CYP1B1[34], are highly efficient in activating ellipticine to form covalent adducts *in vitro*[95]. Hence, ellipticine is considered to act also as a pro-drug, whose pharmacological efficiency and/or genotoxic side effects are dependent on its enzymatic activation in target tissues[34]. There are significant inter-individual differences during the therapy with ellipticine, which is in accordance with different enzymatic equipment of each person.

1.3 Biotransformation of xenobiotics

Metabolic transformation of xenobiotics entering the organism is partially dependent on their solubility in water or fats. Lipophilic substances pass through membranes and

therefore can cumulate in the organism and cannot be eliminated directly, but they have to be transformed to more polar substances first[65]. These more hydrophilic substances might be either non-toxic (biologically inert) or biologically active, depending on the structure of the xenobiotic and on the enzymatic system transforming that substance. Hence, in spite of detoxification, metabolic activation may also occur. Numerous drugs require such metabolic activation. On the other hand, 98% of genotoxic carcinogens (those generating covalent adducts) are metabolically activated, too[62].

Biotransformation of xenobiotics is different in different species. Integrity and direction of biotransformation of the xenobiotic are important for defining the toxic level of the substance for individual organisms[91].

In animals, biotransformation of xenobiotics is a biphasic process (Figure 11)[108]. In the first phase, the derivatization phase, oxidative and reductive reactions occur to increase the polarity of a lipophilic xenobiotic. Hydrolytic reactions may also be applied to increase the hydrophilicity of some substances (*e.g.* esters)[3]. In the second phase, the conjugation phase, these more polar molecules are conjugated with endogenous compounds (*e.g.* glucuronic acid, glycine, taurine, active sulphate, glutathione, cysteine, acetate)[3].

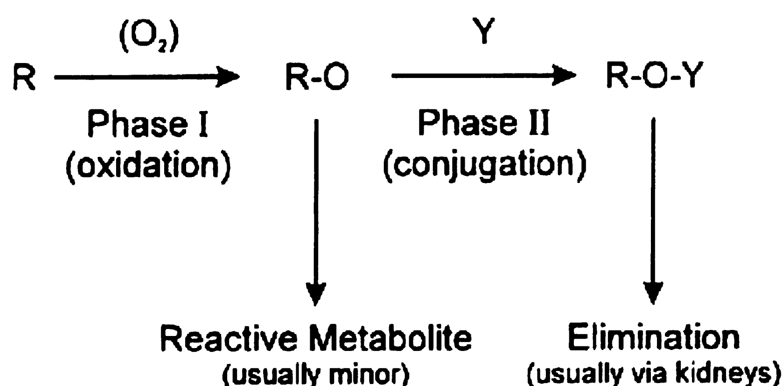

Figure 11. Scheme of the two phases of xenobiotic biotransformation[131].

Phase I of biotransformation

To increase the polarity of a xenobiotic, polar groups are introduced in the molecule or those already present are demasked[51]. The oxidative pathways of biotransformation are the most common ones (C-hydroxylation, N-hydroxylation, N-oxidation, S-oxidation,

dealkylation, deamination, epoxidation, oxidation of alcohol and aldehydes, etc.); other reactions are hydrolytic (hydrolysis of esters and amides) or reductive (nitro- and azo-reductions). For example, aristolochic acids[88, 97, 114] and 2-nitroanisole[102] are carcinogens, which are activated by reductive mechanisms.

Enzymes catalysing the phase I reactions of xenobiotics are those located in the microsomal fraction (subcellular system formed from the endoplasmic reticulum during the homogenization), and in cytosol (cytoplasm diluted in an isolation buffer). A system of mixed function oxidases (MFO system; see Chapter 1.3.1, p 21) plays a crucial role in this phase of biotransformation. It catalyses most of the oxidative reactions. Other enzymes include flavin-containing monooxygenases (Ziegler's enzyme; see Chapter 1.3.3, p 33), DT-diaphorase, heme peroxidases, alcohol- and aldehyde-dehydrogenases, and reductases (cytosolic xanthineoxidase)[3].

Phase II of biotransformation

Small hydrophilic endogenous molecules are bound to the functional groups created during the phase I. This results in further increase in the polarity of the xenobiotic and, therefore, its elimination from the organism is facilitated. Afterwards, it is usually excreted by urine or bile.

O- and N-glucuronates are the most common conjugates excreted by urine. The conjugation of a derivatized xenobiotic with glucuronic acid is catalysed by an enzyme called UDP-glucuronosyltransferase[3].

Glutathione-S-transferase is considered to play an important role in anti-carcinogenic processes, because it is able to bind reactive metabolites, especially epoxides, and catalyse their linkage to glutathione[109]. In kidney, γ-glutamyl is split off; glycyl is split off in liver; cysteyl is then acetylated to form mercapturic acid, which is excreted by urine. Sometimes, xenobiotics form conjugates directly with cysteine. Hence, glutathione and cysteine are also considered as scavengers of xenobiotics[3].

The conjugates of phenolic substances with sulphate and those of carboxylic acids with glycine or taurine are usually excreted by faeces. Products of endogenous steroid compounds biodegradation (*i.e.* cholic acid) are excreted in the same way[3].

In the phase II of biotransformation, xenobiotics may also be converted into more toxic substances. For example, the conjugates of N-hydroxylated compounds, formed from aromatic amines, and active sulphate (PAPS = 2′-phosphoadenosine-5′-phosphosulphate) by sulphotransferases are not stable in acidic conditions (*i.e.* urine) and form nitrene ions. Such electrophiles then react with DNA and proteins[3].

Most enzymes of the phase I of biotransformation are inducible by carcinogens. This is also the case of the phase II enzymes, but these are inducible to a lower extent. Therefore, a certain imbalance between the rate of formation of reactive intermediates and the rate of their conjugation can be expected. On the other hand, there are also substances and complex factors (natural substances in vegetable diet), which predominantly induce enzymes of the phase II of biotransformation. It is obvious, that these factors are of extreme protective importance[109].

1.3.1 Mixed function oxidases system (MFO system)

Monooxygenation is the result of phase I metabolism of xenobiotic molecules. The MFO system is composed of an enzyme catalysing the monooxygenation (cytochrome P450) and enzyme which provides the reducing equivalents (NADPH:cytochrome P450 reductase)[31]. Biological membrane is the third (and essential) part of the system. Membrane lipids, such as phosphatidylcholine, cause conformational changes to increase the affinity of cytochromes P450 (CYPs) to the substrate, accumulate the substrates of CYPs, and stimulate the formation of the *cytochrome P450 - NADPH:CYP reductase* complex, which is essential for the CYP activity[42]. The MFO system may also contain NADH:cytochrome b_5 reductase (EC 1.6.2.2) and/or cytochrome b_5 (see Chapter 1.3.2, p 32)[3].

The MFO system is localized either on the cytosolic side of smooth or rough endoplasmic reticulum (for example in organs highly exposed to xenobiotics - liver, kidney, lung, gastrointestinal tract, skin, brain, where it participates in phase I of biotransformation); or in the mitochondrial membrane (for example in adrenal cortex where it participates the metabolism of endogenous compounds - steroids, fatty acids).

1.3.1.1 Cytochrome P450 (EC 1.14.14.1)

The cytochrome P450 (CYP) is a multi-gene superfamily of constitutive and inducible heme-containing oxidative enzymes, which play an important role in the metabolism of a diverse range of xenobiotics[37, 67, 69, 117]. These enzymes are also considered to play a central role in tumour development and progression and are involved in tumour initiation and promotion, since they can activate or deactivate most carcinogens[37, 67, 116]. Furthermore, CYPs can influence the response of tumorous cells to anti-cancer drugs by metabolising these drugs, both in normal tissues and in tumorous cells[53, 67].

The primary role of cytochromes P450 is the deactivation of a wide variety of chemicals. These enzymes play an important role in metabolism of xenobiotics; most of drugs, environmental chemicals and carcinogens[37]. Apart from the oxidative transformation of xenobiotics, cytochromes P450 are responsible for the oxidative phases of biosynthesis or biodegradation of endogenous compounds – steroids, fatty acids or prostaglandins[61, 76].

Structurally, the CYP proteins consist of several domains: a hydrophobic N-terminal domain, which acts as a membrane anchor; a substrate-binding site; an oxygen-binding site; and a free hydrophilic C-terminal domain[67]. Differential processing of the N-terminal peptide results in targeting of the CYP to either endoplasmic reticulum or mitochondria[2, 5, 67]. Cytochromes P450 contain a single heme *b* prosthetic group[73]. The distal axial ligand of the heme iron is formed by a conserved cysteine residue belonging to the active site of the enzyme molecule[15]. This thiolate anion determines the cytochrome P450 unusual properties. Since the heme is bound to cysteine via a coordination-covalent bond, CYP cannot be regarded as a real cytochrome; it would be more correct to assign it

as a heme-thiolate protein. It usually forms clusters of a molecular weight of 500 kDa embedded in the membrane deeply[39].

Cytochromes P450 exist in two spin forms due to the transient character of the heme iron:

- High-spin form – the iron ion is pentacoordinated, all its valence electrons are unpaired (spin 5/2). This form exhibits an absorption maximum at 390 nm. The iron atom is placed upon the protoporphyrin IX ring plane.
- Low-spin form – the iron ion is hexacoordinated (spin 1/2); the sixth ligand may be, for example, an –OH, -COOH or $–NH_2$ group of an amino acid from the apoprotein[3] or an oxygen atom from a water molecule[33]. The low-spin form exhibits an absorption maximum at 418 nm and the iron atom is placed inside the ring plane[3].

Both spin forms are balanced in the resting state. Binding of substrates in a protein site close to the heme generally shifts the equilibrium between the two forms towards the pentacoordinated complex[61], since the sixth ligand is forced out by the substrate molecule. The absorption increases at 390 nm and decreases at 418 nm – we observe a so-called *substrate spectrum.*

The substrate itself (or another substance not transformed by the enzyme) may become the sixth ligand of the iron ion. In this case, the absorption decreases at 390 nm and the absorption maximum in the range of 418 - 460 nm (depending on the heteroatom) – a so-called *ligand spectrum.*

Functionally, cytochrome P450 is the terminal oxidase in the system of mixed function oxidases that binds molecular oxygen and after its activation stereospecifically incorporates one atom of this molecular oxygen into the substrate. The second oxygen atom is reduced to form water. Cytochromes P450 catalyse many types of reactions besides oxidation, including peroxidation and reduction. The typical reaction of the MFO system is the monooxygenation using NADPH as a cofactor[67] (see p 26). To these monooxygenation reactions belong *C*- and *N*-hydroxylation, epoxidation, *N*- and *O*-dealkylation, *N*- and *S*-oxidation and dehalogenation (Figure 12, p 24)[82].

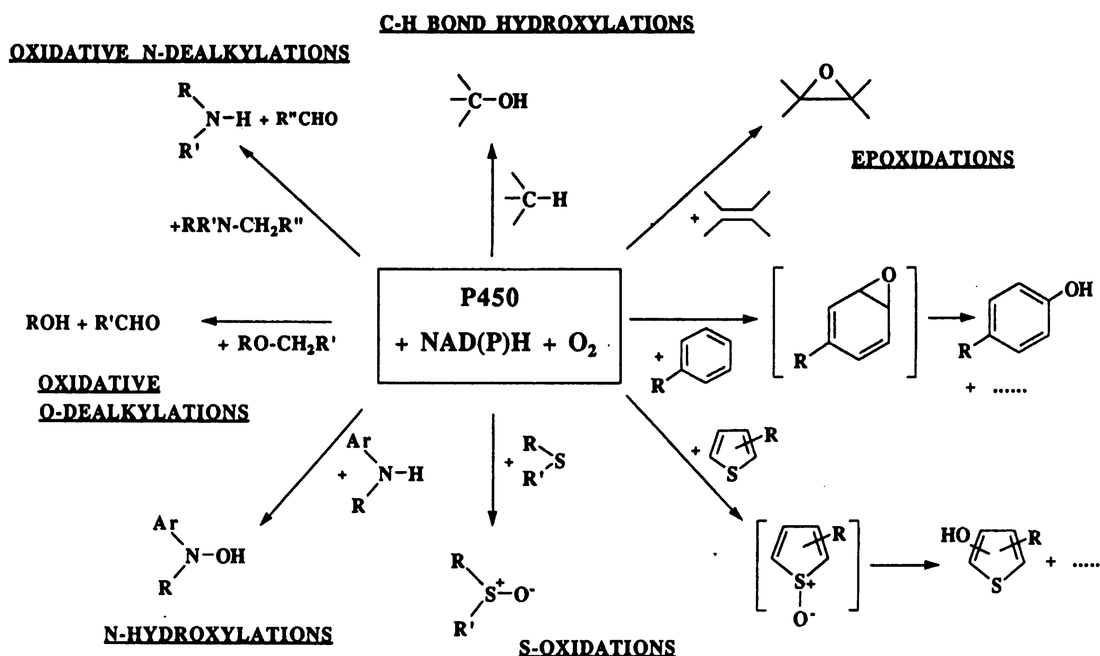

Figure 12. Monooxygenation reactions catalysed by cytochromes P450[61].

During the CYP-dependent monooxygenation of many substrates, partial decoupling occurs between the electron transfer from NADPH to dioxygen and the transfer of an oxygen atom to the substrate. This results in an oxidase-type activity with formation of $O_2^{\cdot-}$, H_2O_2 and H_2O from reduction of dioxygen[61]. This phenomenon is particularly important in the metabolism of xenobiotics, which do not perfectly fit in the CYP active site to receive an oxygen atom[61]. Some of the oxidative reactions catalysed by CYPs are shown in Figure 13 (p 25).

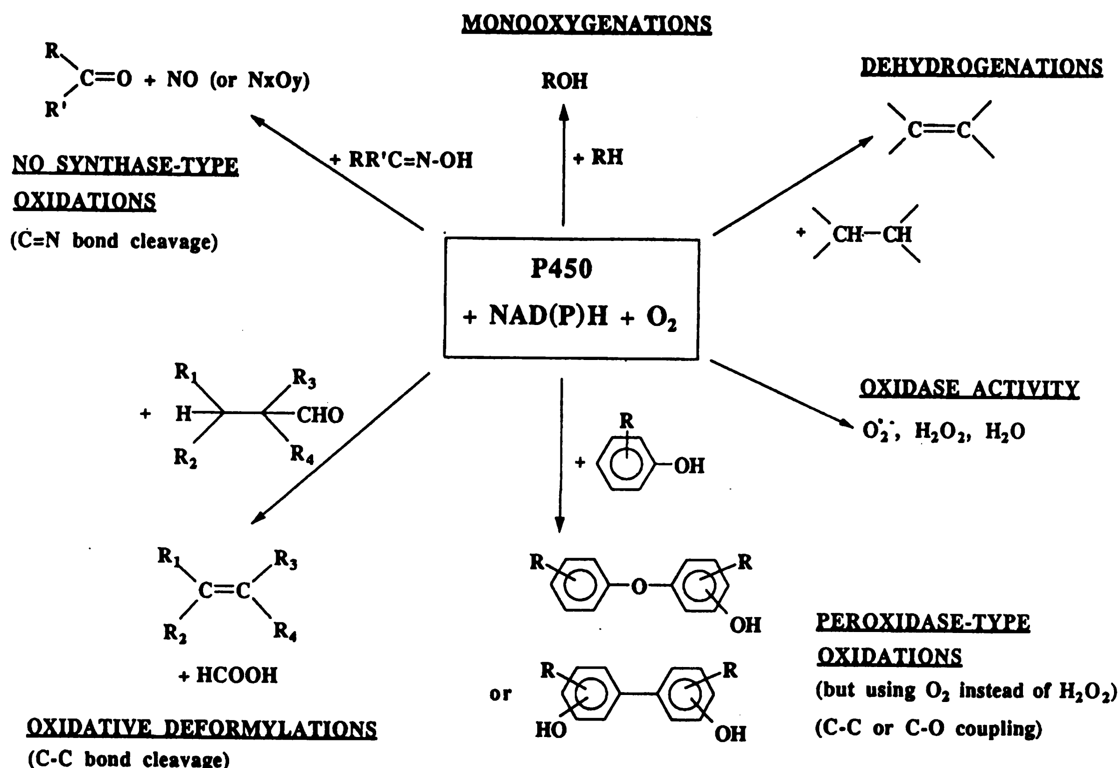

Figure 13. Oxidative reactions catalysed by cytochromes P450[61].

Cytochromes P450 also exhibit peroxidase activity if the second substrate is represented by an organic peroxide (or H_2O_2) instead of oxygen[6]. For this type of reaction NADPH is not necessary as an electron donor[24]. The product of the reaction is the same as in the case of the typical reaction mechanism – a hydroxylated substrate[3].

Figure 14 (p 26) shows some of the non-oxidative reactions of cytochromes P450. Especially the reductions are important in the metabolism of xenobiotics. The electrons provided by NADPH:CYP reductase can reduce the substrate directly and are not utilized for the activation of oxygen. This situation comes up if the hydrophobic substrate binds as a sixth ligand to an iron ion instead of oxygen[3].

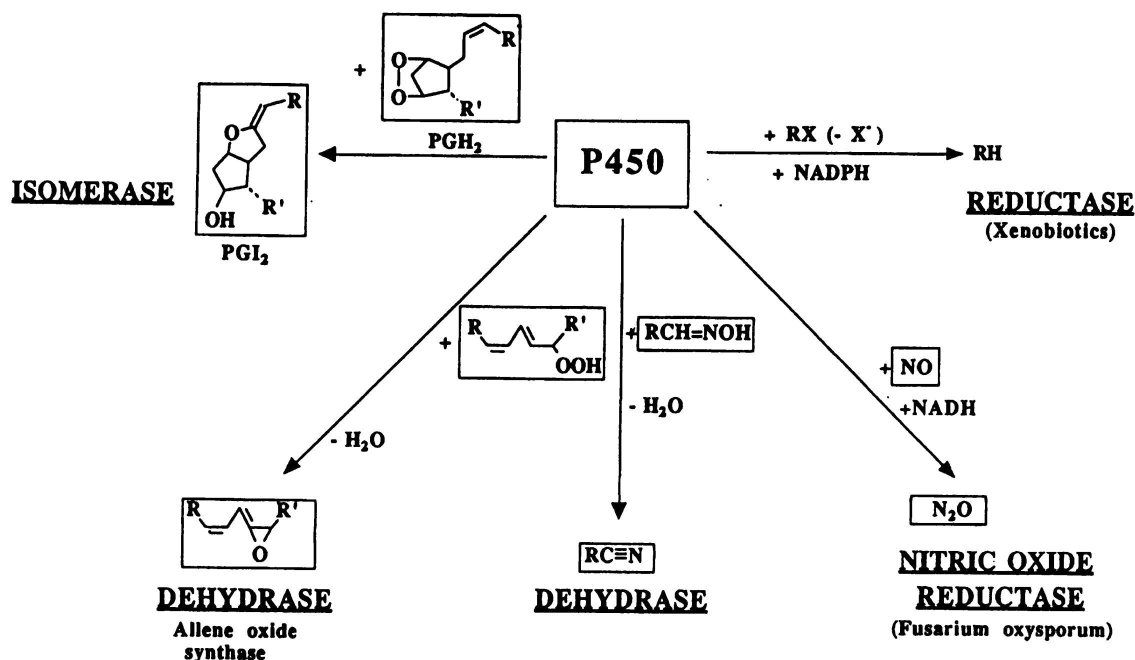

Figure 14. Non-oxidative reactions catalysed by cytochromes P450[61].

Reaction mechanism of the monooxygenation reaction catalysed by CYPs

Figure 15 (p 27) shows the reaction mechanism of CYP-dependent monooxygenase reaction. In the resting state CYP is in its low-spin form, *i.e.*: heme iron is hexacoordinated while the sixth ligand is represented by a water molecule or some amino acid of the apoprotein (*I* in Figure 15, p 27). The binding of a substrate to ferri form of cytochrome P450 causes a lowering of its redox potential[82] that makes the transfer of an electron from its redox partner favorable. The binding of a substrate brings about a conformational change in the enzyme (heme iron changes from hexacoordinated to pentacoordinated form since the substrate forces the sixth ligand out) (*II* in Figure 15, p 27)[82]. An electron transferred from NADPH:cytochrome P450 reductase reduces the Fe^{3+} ion to Fe^{2+} (*III* in Figure 15, p 27). An O_2 molecule binds rapidly to the Fe^{2+} ion, forming an Fe^{2+}-O_2 complex that undergoes a slow conversion to the more stable Fe^{3+}-O_2^- complex (*IV* in Figure 15, p 27)[7]. The ferri-superoxide complex is reduced to the ferro-superoxide (Fe^{2+}-O_2^-) complex by NADPH:cytochrome P450 reductase or NADH:cytochrome b_5 reductase. This reduction is the rate-determining step of the whole reaction cycle[48]. Fe^{2+}-O_2^- complex is converted to Fe^{3+}-O_2^{2-} complex that is energetically more favorable (*VI* in Figure 15, p 27). The O_2^{2-} reacts with two protons from the surrounding solvent, the O-O

bond is split and one oxygen atom is reduced to water. The second oxygen atom remains in the $(Fe\text{-}O)^{3+}$ complex (*VII* in Figure 15), which is stabilized by a mesomeric shift from the thiolate sulphur towards oxygen[3]. The Fe-ligated oxygen atom is transferred to the substrate forming the hydroxylated derivative of the substrate. Initially, the hydrogen atom is released from the substrate and the substrate radical and hydroxyl radical, which is bound to the Fe atom, are formed. Thereafter, both radicals join together to produce the hydroxylated substrate. The reaction product is released from the active site of the enzyme, which returns to its initial state[23, 44, 82].

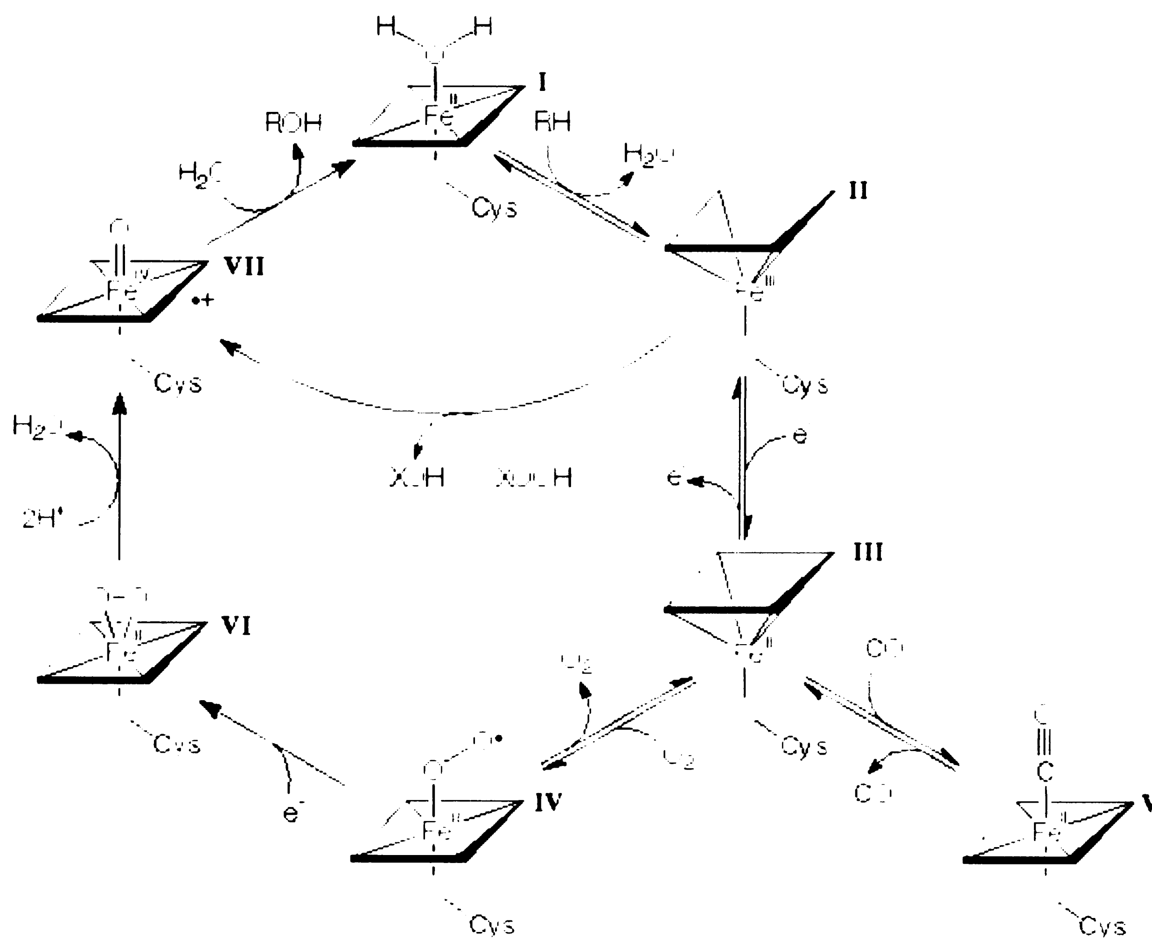

Figure 15. Scheme of a reaction mechanism of the monooxygenation[120].

In the presence of oxidative agents such as organic peroxides, *VII* in Figure 15 may arise directly from complex *II* in Figure 15 (peroxidase activity of CYPs).

Cytochrome P450 enzyme families and subfamilies

The group of cytochromes P450 is one of the largest known mammalian gene families (over 1,000 genes)[120]. The CYPs are classified into families, sub-families and individual enzymes based on amino acid sequence homology[69]. CYP family is marked with a number after the abbreviation "CYP": *e.g.* CYP3; CYPs from one family exhibit at least 40% sequence homology. After the number of a family, there is a letter representing the subfamily (at least 60% sequence homology): *e.g.* CYP3A. Afterwards, the number of an individual CYP enzyme follows, *e.g.* CYP3A4 (Figure 16). Current CYP nomenclature has been introduced by Nebert and co-workers in 1996[69]. Since the number of known CYPs and *CYP* genes continues to grow, there has been established an official website applied to the standardized CYP nomenclature, where newly discovered CYP forms are continuously added[120].

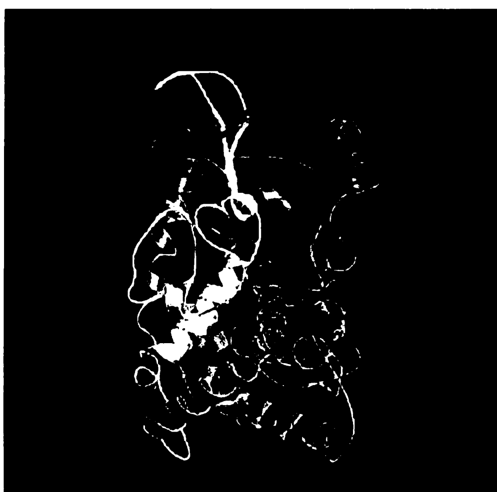

Figure 16. Structure of CYP3A4[128].

There are two broad groups of mammalian cytochromes P450 (57 known *CYP* genes and 58 pseudogenes in humans[70, 49]): a large group whose primary role is the metabolism of xenobiotics (CYP1, CYP2, CYP3, and to a lesser extent CYP4), and much smaller group of CYPs which are constitutively expressed in endocrine glands, where they are specifically involved in steroid hormone synthesis (CYP11, CYP17, CYP19 and CYP21)[67].

There are great inter-individual differences among the activities of individual forms of cytochrome P450 caused predominantly by the two following factors:

- Genetic polymorphism (changes in genotype). Intrinsic changes in the DNA can result in the absence of some CYP form, in the alteration of CYP inducibility or a CYP form with altered catalytic activity[40].
- Changes in gene expression (changes in phenotype). The regulation of CYPs is complex and involves both transcriptional and post-transcriptional mechanisms[66].

The CYPs that are constitutively expressed are predominantly regulated by basal transcription factors, while other CYPs, which show inducible expression, are regulated by ligand-activated receptor-mediated mechanisms. Many of the receptor ligands are also substrates for CYPs. Post-trascriptional mechanisms include mRNA stabilization and protein stabilization[67].

The following overview summarizes known facts about enzymatic activities of the human CYP forms participating in the biotransformation of xenobiotics (Figure 17):

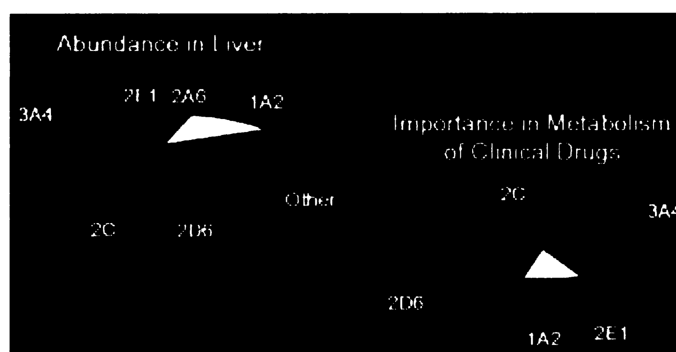

Figure 17. Abundance of individual CYPs in human liver with emphasis on those improtant in the metabolism of xenobiotics. Modified from [127].

CYP1A subfamily

Cytochromes P450 1A1 and 1A2 are the most important CYP enzymes in activation of procarcinogens. They can activate almost 90% of known carcinogens[79], especially chemicals of cigarette smoke. Both enzymes are very similar: they exhibit more than 70% homology in their amino acid sequences and they catalyse similar reactions[41]. Nevertheless, they differ in organ localization. CYP1A1 is present mainly in lung, GIT, kidney, placenta and skin. It is also expressed in human liver, but in much lower levels (<0.7% of the total hepatic CYP content)[100, 101]. CYP1A1 expression can be induced even 100-times by several compounds, *e.g.* TCDD, benzo[a]pyrene, 3-methylcholanthren, 5,6-benzoflavone or smoking[99]. The major substrates of CYP1A1 are large planar molecules like polycyclic aromatic hydrocarbons (PAHs). It was shown that the genetic polymorphism of CYP1A1 might influence the development of tumours in lung, breast and skin[41].

CYP1A2 is a typical hepatic enzyme, forming cca 13% of total hepatic CYP. It is also inducible up to 40-times. Its inducers are similar to those of CYP1A1. CYP1A2 metabolises hydroxylation mainly of heterocyclic and aromatic amines and PAHs. High levels of CYP1A2 are associated with an increased risk of colon and bladder cancer[41].

CYP2A6

It is an inducible enzyme present in liver and lung. It forms cca 4% of the total CYP content in human livers. CYP2A6 metabolises nicotine and coumarin and activates several procarcinogens, *e.g.* nitrosamines from tobacco smoke, aflatoxin B1, hexamethylphosphoramide or butadiene[99].

CYP2B6

This is a hepatic, pulmonary and GIT enzyme taking part in activation of carcinogens as aflatoxin B1, nicotine and dibenzo(a,h)anthracene. Moreover, it plays a significant role in metabolism of anticancer drugs[12, 21]. It is inducible almost 50-fold by barbiturates and dexamethasone[99].

CYP2C subfamily

There are four major isoforms of human CYP2C subfamily – CYP2C8, 2C9, 2C18 and 2C19, present in liver, GIT, lung and pharynx. All of them are moderately inducible by barbiturates and ethanol. Due to their large genetic polymorphism, they play an important role in metabolism and effectiveness of several drugs[99]. CYP2C8 and 2C9 also activate carcinogenic benzo[a]pyrene but with lesser potency than other cytochrome P450 isoforms[79].

CYP2D6

This hepatic and GIT CYP isoform is responsible for oxidation of more than 70 different drugs. Similarly to CYP2C, it exhibits large genetic polymorphism affecting drug metabolism in humans.

CYP2E1

It is present mainly in liver (cca 7% of total hepatic CYP), lung and placenta. Its levels are increased by ethanol, acetone, isoniazide or starving. CYP2E1 metabolise small molecules such as benzene, vinyl chloride, styrene, acrylonitrile, *N*-nitrosodimethylamine, etc. During reactions catalysed by CYP2E1 oxygen radicals are sometimes released[99].

CYP3A4

CYP3A4 is the most abundant CYP enzyme in human liver (about 30% of total hepatic CYP content), and is also present in many other organs. Furthermore, it can be induced more than 30-times by barbiturates, erythromycin and rifampicin. Its substrate specificity is very wide. CYP3A4 metabolises carcinogenic xenobiotics as aflatoxins, PAHs or aromatic amines, as well as endogenous steroid compounds such as 17ß-estradiol, testosterone, progesterone and cortisol.

1.3.1.2 NADPH:cytochrome P450 oxidoreductase (EC 1.6.2.4)

NADPH:cytochrome P450 oxidoreductase (NADPH:CYP reductase) is a membrane protein which catalyses the electron transfer from NADPH to all known forms of CYPs[85], to cytochrome c[45], cytochrome b_5[30], heme oxygenase, ferricyanide, etc.[123]. It is termed a "yellow protein" and is an unusual flavoprotein in that it contains both FMN and FAD[74].

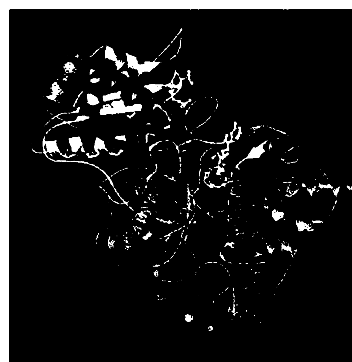

Figure 18. NADPH:cytochrome P450 oxidoreductase[128].

Structurally, NADPH:CYP reductase (Figure 18) consists of two functional domains: a hydrophobic N-terminal domain (6 kDa), which acts as a membrane anchor[52], and a hydrophilic C-terminal domain (72 kDa)[115]. A pancreatic protease trypsin is able to solubilize the C-terminal domain, which stays partially functional - it is able to transfer the electrons to cytochrome c and some other artificial electron acceptors, but it is no more capable to reduce cytochrome P450[115]. The FAD-binding domain, which is a part of this

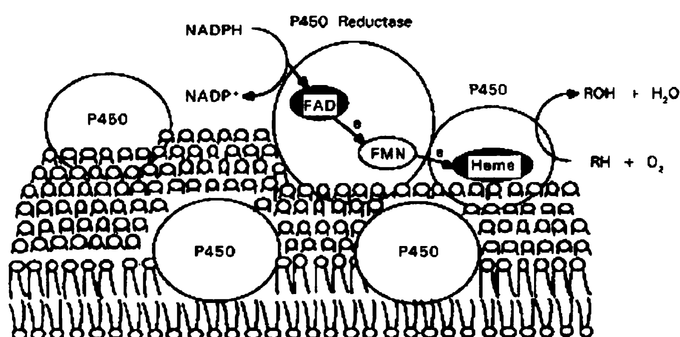

Figure 19. Cooperation of NADPH:CYP reductase with CYP within the MFO system [129].

functional C-terminus, is crucial for the non-covalent binding of the NADPH molecule. The FMN-binding domain is responsible for the electron transfer to the acceptor molecule – *e.g.* cytochrome P450 (Figure 19, p 31).

The interactions between NADPH:CYP reductase and cytochrome P450 are predominantly electrostatic: the positively charged surface of CYP (lysines, arginines) interacts with the negatively charged surface of the NADPH:CYP reductase (aspartates, glutamates). In the surrounding of membrane domains, hydrophobic interactions among non-polar amino acids also play important roles[68, 107].

Since the NADPH:CYP reductase contains two prosthetic groups (FAD and FMN) with different redox potentials, it acts as an electron pair divider[74, 110, 111]. The FAD prosthetic group accepts the electrons (hydrogen atoms) from NADPH, which then transfers these to FMN. Finally, the reduced hydroquinone $FMNH_2$ reduces the CYP molecule (Figure 20)[3, 122].

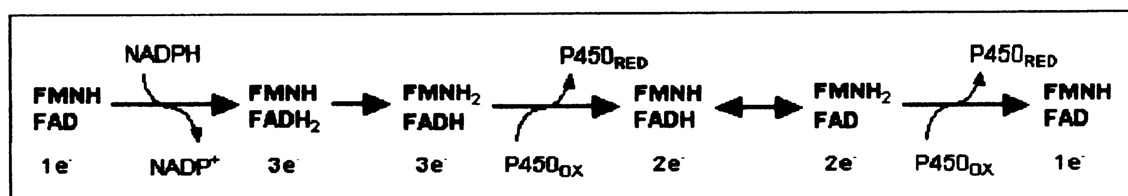

Figure 20. Scheme of the electron transfer cycle in NADPH:CYP reductase[3].

The NADPH:CYP reductase activity is regulated hormonally, namely by triiodothyronine (T_3), which is a thyroid gland hormone.

1.3.2 Cytochrome b_5

Cytochrome b_5 is a small, cylindrical membrane protein (15 kDa) localized on the cytosolic side of a membrane of endoplasmic reticulum[60]. It contains one or two heme *b* molecules[113]. It participates on the electron transfer in a number of diverse reactions included in metabolism of, for example, lipids, steroids or xenobiotics[86].

Cytochrome b_5 consists of 6 α-helixes and 5 β-sheets. Its hydrophobic C-terminal domain acts as a membrane anchor, and its hydrophilic N-terminal domain contains the heme(s) molecule(s). The heme iron is coordinated by the side chains of two histidines ([68]His and [44]His). Figure 21 shows that the catalytic site includes a slot where the heme is placed. Mammalian cytochrome b_5 contains approximately 16% of negatively charged amino acids (aspartates, glutamates) in the catalytic domain. These amino acids participate on ionic interactions of cytochrome b_5 with other electron transporters and other proteins[25, 26, 78, 80, 106].

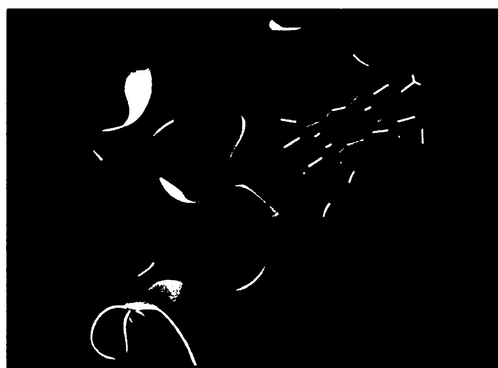

Figure 21. Cytochrome b_5[128].

Since both the fifth and the sixth coordination positions of iron are occupied by the histidines, direct interaction of cytochrome b_5 with oxygen is not possible. Nevertheless, cytochrome b_5 may act as an intermediate electron transporter between reductase and CYP[86]. For example, it is able to accept an electron from NADH:cytochrome b_5 reductase and deliver it to the cytochrome P450[50, 71].

Cytochrome b_5 is also considered to stimulate many reactions catalysed by cytochromes P450 as it is another heme protein of the microsomal membrane. This fact is being explained by two interpretations: it directly transfers an electron to the CYP, or it mediates some conformational changes of the CYP[118].

1.3.3 Flavin-containing monooxygenase 3 (FMO3; EC 1.14.13.8)

The flavin-containing monooxygenases (FMOs) comprise a family of FAD-, NADPH- and molecular oxygen-dependent microsomal enzymes[19]. FMOs are arguably the second most important human monooxygenase system, after CYPs, and catalyse the oxygenation of nucleophilic heteroatom-containing xenobiotic chemicals and drugs[18, 19].

Generally, FMO converts lipophilic materials to more polar molecules that are more readily excreted. Often, this is a detoxication reaction whereby a chemical is transformed into an N-oxide or S-oxide that possesses sufficient polarity to terminate the pharmacological activity of the molecule[18]. However, analogous to cytochromes P450, FMO can also bioactivate molecules to reactive metabolites[18]. The fundamental difference between FMO and CYP consists in their reaction mechanisms. The FMO utilizes a two-electron oxygenation mechanism (Figure 22) while the CYP utilizes a sequential one-electron oxidation mechanism[18].

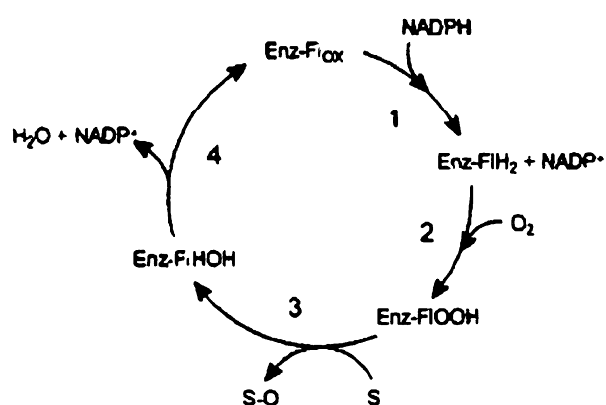

Figure 22. Schematic representation of the catalytic cycle of pig FMO1. Abbreviations used: S = substrate; S-O = oxygenated substrate; Enz = enzyme. Modified form [17].

Of the five functional human FMOs known, FMO3 (Figure 23) of a molecular weight of 60 kDa appears to be the most important FMO present in adult human liver. FMO3 is expressed at levels approaching 60% of the expression levels of CYP3A4 (most abundant CYP present in human liver)[75].

Figure 23. Human flavin-containing monooxygenase 3[128].

2 Aims

The present thesis was aimed at improving our knowledge of biotransformation of the anticancer drug ellipticine by rat and human cytochromes P450 (CYPs). Microsomes isolated from tissues of both organisms contain extremely diverse mixtures of numerous CYP forms. Therefore, Supersomes^{TM} containing individual rat and human recombinant CYPs were used to enable us to compare the efficiency of rat and human CYP forms to oxidize ellipticine. Within the thesis following tasks were being solved:

- The efficiency of individual rat recombinant CYP enzymes in Supersomes^{TM} to oxidize ellipticine
- The efficiency of individual human recombinant CYP enzymes in Supersomes^{TM} to oxidize ellipticine
- The effect of cytochrome b_5 on ellipticine oxidation by both rat and human recombinant CYPs

The efficiency of other enzymes of the biotransformation phase I, namely human flavin-containing monooxygenase 3, to oxidize ellipticine was investigated.

3 Materials

3.1 Drugs and reagents

Drugs and reagents used in the experiments were purchased from:

Chemopetrol, Czech Republic	solid carbon dioxide (dry ice)
Fluka, Switzerland	DMSO, methanol, ethyl acetate
Lachema, Czech Republic	glycerol, sodium dithionite, acetic acid, ammonium acetate, KOH, KH_2PO_4, $MgCl_2$, KCl, $Na_4P_2O_7.10H_2O$
Linde, Czech Republic	liquid nitrogen, carbon monoxide (*g*)
Sevapharma, a. s., Czech Republic	bovine serum albumin (BSA)
Sigma, USA	ellipticine, $NADP^+$, D-glucose 6-phosphate, D-glucose 6-phosphate dehydrogenase, phenacetin, heptane sulfonic acid (sodium salt), TRIS
Velaz, Czech Republic	male Wistar rats
Gentest Corp., USA	Human FMO3 Supersomes™, CYP Supersomes™ (see below)

Supersomes™ are microsomes isolated from insect cells transfected with *Baculovirus*, in which individual recombinant enzymes from various organisms are expressed (*e.g.* human flavin-containing monooxygenase 3, rat or human cytochromes P450).

CYP Supersomes™

- Rat CYP1A1 + P450 Reductase
- Rat CYP1A2 + P450 Reductase
- Rat CYP2A1 + P450 Reductase + Cytochrome b_5

- Rat CYP2A2 + P450 Reductase + Cytochrome b_5
- Rat CYP2B1 + P450 Reductase + Cytochrome b_5
- Rat CYP2C6 + P450 Reductase + Cytochrome b_5
- Rat CYP2C11 + P450 Reductase + Cytochrome b_5
- Rat CYP2C12 + P450 Reductase + Cytochrome b_5
- Rat CYP2C13 + P450 Reductase + Cytochrome b_5
- Rat CYP2D1 + P450 Reductase
- Rat CYP2D2 + P450 Reductase
- Rat CYP2E1 + P450 Reductase + Cytochrome b_5
- Rat CYP3A1 + P450 Reductase + Cytochrome b_5
- Rat CYP3A2 + P450 Reductase + Cytochrome b_5
- Human CYP1A1 + P450 Reductase
- Human CYP1A2 + P450 Reductase
- Human CYP1B1 + P450 Reductase
- Human CYP2A6 + P450 Reductase
- Human CYP2B6 + P450 Reductase
- Human CYP2B6 + P450 Reductase + Cytochrome b_5
- Human CYP2C8 + P450 Reductase
- Human CYP2C9*1 (site mutation at [144]Arg) + P450 Reductase
- Human CYP2C19 + P450 Reductase
- Human CYP2D6*1 (site mutation at [374]Val) + P450 Reductase
- Human CYP2E1 + P450 Reductase
- Human CYP2E1 + P450 Reductase + Cytochrome b_5
- Human CYP3A4 + P450 Reductase
- Human CYP3A4 + P450 Reductase + Cytochrome b_5

Cytochome b_5 was isolated from rabbit (*Oryctolagus cuniculus*) liver microsomes by Mgr. Kateřina Janouchová and Mgr. Kateřina Kukačková.

3.2 *Instruments*

Centrifuges

- MSE MicroCentaur, Sanyo, UK
- K-23, Janetzki, Germany (pivoted rotor 4 x 70 ml)
- K-24, Janetzki, Germany (angular rotor 6 x 35 ml)
- Optima LE-80K Ultracentrifuge, Beckman Coulter, USA (rotors: Ti45 6 x 65 ml, Ti70 8 x 39 ml)
- T 52.2, MLW, Germany

Spectrophotometers

- Specord M-40, Carl Zeiss Jena, Germany

Balance

- PESA 40SM-200A (analytical balance), Switzerland
- KERN EW600-2M (electrobalance), Germany

Incubators

- Thermomixer compact, Eppendorf AG, Germany
- G24 Enviromental Incubator Shaker, New Brunswick, Scientific Co. Inc., Edison, USA

HPLC system, Dionex, USA

- P580 pump
- ASI-100 Automated Sample Injector
- UV/VIS Detector UVD 170S/340S
- columns: Nucleosil 120-5 C-18, 250 x 8 mm (Watrex, USA); 250 x 4,6 mm, 5 µm (Beckman, USA); CC 250/4 Nucleosil 100-5 C18 (Macherey-Nagel, Germany)

- precolumn: C 8/4 Nucleosil 100-5 C18 (Macherey-Nagel, Germany)

Other instruments used

- pH-meter ATI Orion370 with combined electrode, USA
- vacuum evaporator SpeedVac DNA110, Savant, USA
- sonicator Ultrasonic compact cleaner, Teson1, Tesla, Czech Republic
- automatic micropipettes Biohit, Finland; Nichipet EX, Nichiryo, Japan

4 Methods

4.1 Oxidation of ellipticine by rat and human recombinant cytochromes P450 in Supersomes™

Incubations, in which the efficiency of rat and human recombinant CYPs was investigated, contained in a final volume of 250 µl: 100 mM potassium phosphate buffer (pH 7.4), NADPH-generating system (1 mM $NADP^+$, 10 mM D-glucose 6-phosphate, 1 unit/ml D-glucose 6-phosphate dehydrogenase, 10 mM $MgCl_2$), 10 µM ellipticine dissolved in 1 µl methanol and from 10 to 200 nM CYP in Supersomes™.

The reaction was started by adding NADPH-generating system. After the incubation (37°C, 30 min), the reaction was stopped by adding 750 µl ethyl acetate and 2.5 µl phenacetin (1 mM solution in methanol) was added as an internal standard. The ellipticine metabolites were extracted with ethyl acetate (2 x 750 µl, 5 min, 1,400 RPM, Thermomixer compact, Eppendorf AG, Germany) and then centrifuged 7 minutes at 13,000 RPM (centrifuge MSE MicroCentaur, Sanyo, UK). The organic phase was collected and evaporated to dryness.

4.2 Oxidation of ellipticine by rat and human recombinant cytochromes P450 in Supersomes™ with addition of cytochrome b_5

Incubations, in which the influence of cytochrome b_5 on ellipticine oxidation by rat and human recombinant CYPs was followed, contained in a final volume of 250 µl: 100 mM potassium phosphate buffer (pH 7.4), NADPH-generating system (see Chapter 4.1), 10 µM ellipticine dissolved in 1 µl methanol, from 10 to 50 nM CYP in Supersomes™ and from

30 to 150 nM purrified rabbit cytochrome b_5 (CYP:cyt b_5 1:3). At first, cytochrome b_5 was incubated with Supersomes™ for 5 minutes at the laboratory temperature before starting the reaction with ellipticine to let the cytochrome b_5 integrate to the Supersomal system. Then, the incubation mixture was completed and the reaction was started by adding the NADPH-generating system. After the incubation (37°C, 30 min) the reaction was stopped by adding 750 µl ethyl acetate and 2.5 µl phenacetin (1 mM solution in methanol) was added as an internal standard. The ellipticine metabolites were extracted with ethyl acetate (2 x 750 µl, 5 min, 1,400 RPM, Thermomixer compact, Eppendorf AG, Germany) and then centrifuged 7 minutes at 13,000 RPM (centrifuge MSE MicroCentaur, Sanyo, UK). The organic phase was collected and evaporated to dryness.

4.3 Oxidation of ellipticine by human recombinant flavin-containing monooxygenase 3 (FMO3)

Incubations used for the study of ellipticine metabolism by human recombinant flavin-containing monooxygenase-3 (FMO3) contained in a final volume of 500 µl: 100 mM potassium phosphate buffer (pH 7.4), NADPH-generating system (see Chapter 4.1, p 40), 10 µM ellipticine dissolved in 1 µl methanol and 25, 50 and 100 µg protein (protein content in FMO3 Supersomes™ was 5.0 mg/ml).

After incubation (37°C, 30 min) the reaction was stopped by adding 1 ml of ethyl acetate and 5.0 µl phenacetin (1 mM solution in methanol) was added as an internal standard. The ellipticine metabolites were extracted with ethyl acetate (2 x 1 ml, 5 min, 1,400 RPM, Thermomixer compact, Eppendorf AG, Germany) and then centrifuged 7 minutes at 13,000 RPM (centrifuge MSE MicroCentaur, Sanyo, UK). The organic phase was collected and evaporated to dryness.

4.4 High-performance liquid chromatography – separation of ellipticine metabolites

The evaporated extracts were dissolved in 30 µl methanol and ellipticine metabolites were separated at 35°C using reverse-phase high-performance liquid chromatography (RP-HPLC).

HPLC conditions:

Column:	C18, 250 x 4.6 mm, 5 µm (Beckman, Fullerton, CA)
Mobile phase:	64% methanol and 36% of 0.005 M heptane sulfonic acid and 0.032 M acetic acid in distilled water
Flow rate:	0.7 ml/min
Pressure:	9.2 MPa (26°C)
Injection volume:	25 µl
Detection:	313 nm

4.5 Isolation of microsomal fraction from rat liver[58]

Male Wistar rats (120-180 g; Velaz, Czech Republic) were bred under standard laboratory conditions for 5-7 days[Stiborová-personal communication]. The animals were fed with pellet food (Velaz, Czech Republic) and starved on the last day to decrease the glycogen level.

The isolation of microsomes was carried out at 4-6°C. The livers were washed three times in buffer **B1** (0.05 M TRIS, 0.15 KCl, pH 7.4) and homogenized in 4 volumes of the same buffer using the Potter-Elvehjem homogenizer. The homogenate was filtrated over four layers of gauze and the filtrate was centrifuged at 600 x g for 10 minutes (K-23, pivoted rotor 4 x 70 ml, Janetzki, Germany; 2,000 RPM; 4°C). The pellet was rehomogenized in a small volume of the buffer **B1**, and the supernatant fraction obtained upon centrifugation was combined with the original supernatant fraction and centrifuged at 15,000 x g for 20 minutes (K-24, angular rotor 6 x 35 ml, Janetzki, Germany; 13,500 RPM; 4°C). The supernatant was centrifuged at 105,000 x g for 60 minutes (Optima LE-80K

Ultracentrifuge, rotor Ti45 6 x 65 ml, Beckman Coulter, USA; 45,000 RPM; 4°C). The pellet was resuspended in a minimum volume of buffer **B2** (0.1 M $Na_4P_2O_7.10H_2O$, pH 7.2) and centrifuged at 105,000 x g for 60 minutes (Optima LE-80K Ultracentrifuge, rotor Ti70 8 x 39 ml, Beckman Coulter, USA; 70,000 RPM; 4°C). The supernatant was removed and the microsomal pellet was rehomogenized in 1/20 volume of the original homogenate of buffer **B3** (0.05 M TRIS, 0.15 KCl, 20% glycerol, pH 7.4), frozen with liquid nitrogen and stored at -80°C.

4.6 Cytochrome P450 determination

Reduced form of cytochrome P450 in a complex with carbon monoxide gives a differential spectrum with a sharp absorption maximum at 450 nm[73].

50 µl microsomes were suspended in 2 ml 0.1 M phosphate buffer, containing 2% glycerol, pH 7.4, and reduced by a few milligrams of solid sodium dithionite. Such microsomal preparations were placed in both the sample and the reference cells. After recording the base-line, carbon monoxide was carefully bubbled through the content in the sample cell for about 50 seconds – this was sufficient to saturate the sample with the gas. Then, the difference spectrum was measured (spectrophotometer Specord M-40, Carl Zeiss Jena, Germany; quartz cuvettes of 1 cm optical path). CYP content was determined according to the following equation:

$$\mathbf{c}_{P450} = (A_{450} - A_{490}) / \varepsilon / l$$

$\mathbf{c}_{P450}$	concentration of cytochrome P450
A_x	absorbance at $\underline{x}$ nm
ε	molar absorption coefficient ($\varepsilon = 0.091\ dm^3.\mu mol^{-1}.cm^{-1}$)
l	optical path

Cytochrome P450 can be converted into a form assigned as cytochrome P420 according to its absorption maximum if reduced and saturated by CO. This conversion occurs after the treatment by some detergents, enzymes (*e.g.* phospholipases), alcohols or other substances.

Cytochrome P420 is enzymatically ineffective. Such a form of CYP was also present in the microsomes isolated from rat liver.

4.7 Preparation of the standard of 13-hydroxyellipticine

Incubations used for preparation of 13-hydroxyellipticine in large quantities contained in a final volume of 10 ml: 100 mM potassium phosphate buffer (pH 7.4), NADPH-generating system (see Chapter 4.1, p 40), 10 µM ellipticine dissolved in 20 µl DMSO and 0.4 µM CYP in microsomes isolated from rat liver.

After incubation (37°C, 45 min) the reaction was stopped by adding approximately 10 ml ethyl acetate. The ellipticine metabolites were extracted by ethyl acetate (2 x 10 ml, 5 min) and then centrifuged 5 minutes at 4,000 RPM (T 52.2, MLW, Germany). The organic phases from 24 incubations were pooled and evaporated under vacuum.

The evaporated extracts were dissolved in 110 µl methanol and 13-hydroxyellipticne was isolated from the mixture of ellipticine metabolites using the reverse-phase high-performance liquid chromatography by the following procedure:

Step 1: rough separation of ellipticine and its metabolites - HPLC conditions

Column: Nucleosil 120-5 C-18, 250 x 8 mm (Watrex, USA)

Mobile phases: A: 80% methanol and 0.07 M ammonium acetate in distilled water
B: 0.07 M ammonium acetate in 100% methanol

Program: elli grad (see Table 2)

Table 2. HPLC program „elli grad" used for the separation of ellipticine and its metabolites

time [min]	% B (mobile phase)	flow rate [ml/min]
0	0	1.2
18	0	1.2
19	100	1.6
27	100	1.6
28	0	1.2
31	stop sample	

Pressure: 4.5 – 6.2 MPa (26°C)

Injection volume: 100 µl

Detection: 313 nm

The fraction containing 13-hydroxyellipticine eluted at 15.1 minute was evaporated. The sample was dissolved in 35 µl methanol and 13-hydroxyellipticine was further purified by RP-HPLC:

Step 2: purifying 13-hydroxyellipticine – HPLC conditions

Column: C18, 250 x 4,6 mm, 5 µm (Beckman, USA)

Mobile phase: 64% methanol and 36% of 0.005 M heptane sulfonic acid and 0.032 M acetic acid in distilled water

Flow rate: 0.7 ml/min

Pressure: 9.2 MPa (26°C)

Injection volume: 30 µl

Detection: 313 nm

The fractions containing 13-hydroxyellipticine eluted at 8.2 minute were collected from multiple HPLC runs and evaporated. The sample was dissolved in 30 µl methanol and 13-hydroxyellipticine was finally repurified and the heptane sulfonic acid was removed by HPLC:

Step 3: final purification of 13-hydroxyellipticine and removal of the heptane sulfonic acid – HPLC conditions

Precolumn: CC 8/4 Nucleosil 100-5 C18, Macherey-Nagel, Germany

Column: CC 250/4 Nucleosil 100-5 C18, Macherey-Nagel, Germany

Mobile phase: 70% methanol

Flow rate: 0.7 ml/min

Pressure: 13.5 MPa (26°C)

Injection volume: 25 µl

Detection: 296 nm

13-hydroxyellipticine eluted at 10.6 minute was collected from multiple HPLC runs, evaporated and used as a standard.

5 Results

5.1 Oxidation of ellipticine by rat recombinant cytochromes P450

Oxidation of ellipticine by rat cytochromes P450 (CYPs) was studied using microsomes from *Baculovirus* transfected insect cells (Supersomes^{TM}) expressing rat recombinant CYP enzymes. The pilot study was performed with commercially available Supersomes^{TM} containing rat recombinant CYP, NADPH:CYP reductase and, except those expressing CYP1A1/2 and CYP2D1/2, cytochrome b_5. The incubations contained 50 nM CYP, 10 μM ellipticine and NADPH-generating system.

All of the rat recombinant CYP enzymes employed in the study oxidized ellipticine (Figure 24, p 47). Up to five metabolites were separated by HPLC (see Figure 25, p 48 for oxidation of ellipticine by rat CYP2C13) after the incubation of ellipticine with rat CYPs in Supersomes^{TM}. The elution times of 9-hydroxyellipticine (M1), 12-hydroxyellipticine (M2), 13-hydroxyellipticine (M3), 7-hydroxyellipticine (M4) and ellipticine N^2-oxide (M5) were: 6.2, 6.8, 7.4, 7.8 and 9.8 minutes, respectively (Figure 25, p 48). The structures of all five ellipticine metabolites were identified on the basis of co-chromatography with standards (for the structures see Figure 10, p 16).

Because of the differences in the efficiency of individual CYPs to oxidize ellipticine, its oxidation by individual CYP isoforms was studied in detail.

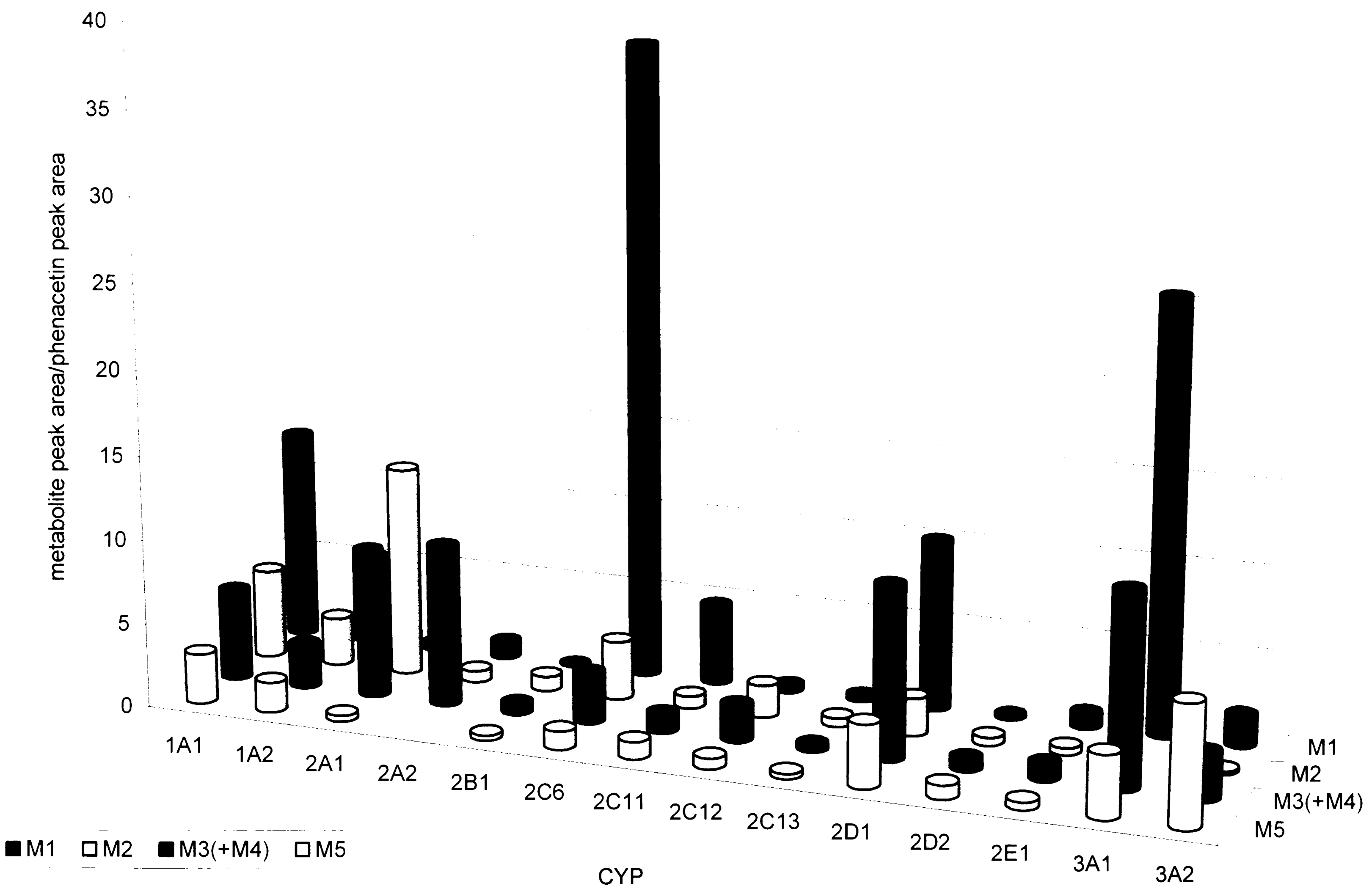

Figure 24. Ellipticine oxidation by rat recombinant CYP enzymes using Supersomes^{TM}. 50 nM CYP and 10 μM ellipticine were used in all of the experiments.

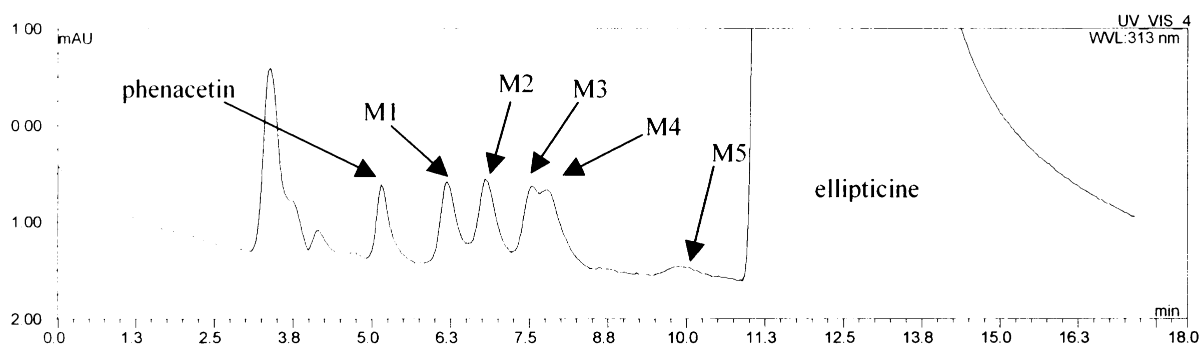

Figure 25. HPLC of ellipticine and its metabolites after its incubation with rat recombinant CYP2C13. 200 nM CYP2C13 and 10 μM ellipticine were used. Phenacetin was added after the incubation as an internal standard at a final concentration of 10 μM.

5.1.1 Oxidation of ellipticine by rat recombinant CYP1A1/2

Rat recombinant CYP1A1 and 1A2 oxidized ellipticine to up to five metabolites: 9-hydroxyellipticine (M1), 12-hydroxyellipticine (M2), 13-hydroxyellipticine (M3), 7-hydroxyellipticine (M4) and ellipticine N^2-oxide (M5). The retention times of 13-hydroxyellipticine and 7-hydroxyellipticine are very close – 7.4 and 7.8 minutes, respectively. Hence, the quantification of each ellipticine metabolite separately was impossible and they were quantified together. An increase in CYP concentration correlated with an increase in the formation of 9-hydroxyellipticine, 13-hydroxyellipticine and 7-hydroxyellipticine (Figures 26 and 27, p 49; Tables 3 and 4, p 49-50). Likewise, the formation of 12-hydroxyellipticine increased with an increase of CYP1A1 concentration. In the case of CYP1A2, the formation of 12-hydroxyellipticine was difficult to be quantified because it was generated at a low level. Both CYP enzymes from 1A subfamily also generated low amounts of ellipticine N^2-oxide.

An increase in CYP1A2 concentration produced an increase in the percentage levels of 9-hydroxyellipticine, while a decrease in the production of 12-hydroxyellipticine and ellipticine N^2-oxide (Table 4, p 50).

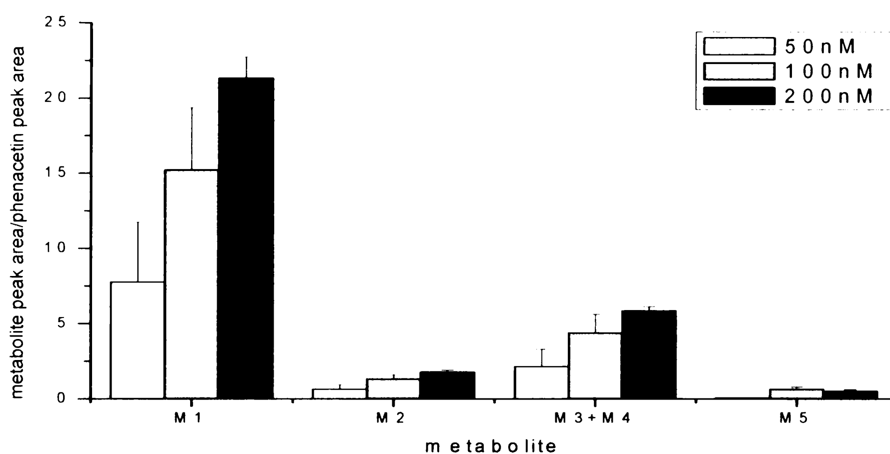

Figure 26. Ellipticine oxidation by rat recombinant CYP1A1. Fifty to 200 nM CYP and 10 μM ellipticine were used in all of the experiments.

Table 3. Percentage levels of individual ellipticine metabolites generated by rat recombinant CYP1A1. Fifty to 200 nM CYP and 10 μM ellipticine were used in all of the experiments.

c CYP	[illegible]	[illegible]	[illegible]	[illegible]
50 nM	72.8	6.1	20.3	0.8
100 nM	70.4	6.2	20.3	3.0
200 nM	72.3	6.1	19.9	1.8

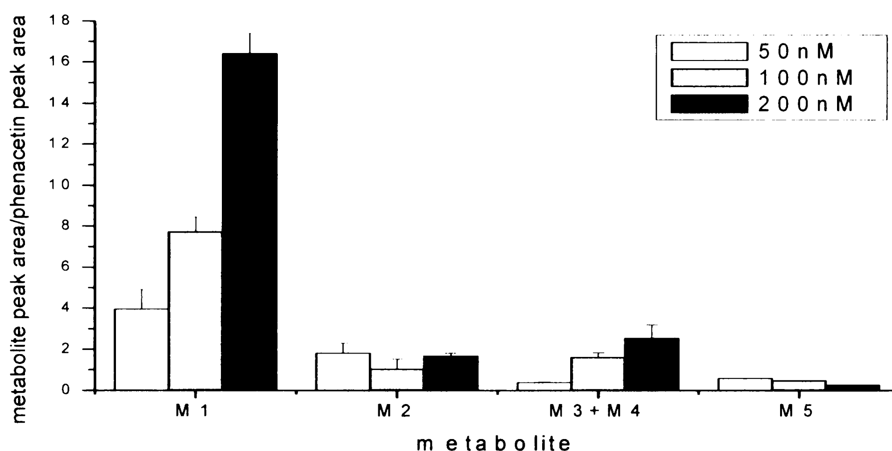

Figure 27. Ellipticine oxidation by rat recombinant CYP1A2. Fifty to 200 nM CYP and 10 μM ellipticine were used in all of the experiments.

Table 4. Percentage levels of individual ellipticine metabolites generated by rat recombinant CYP1A2. Fifty to 200 nM CYP and 10 μM ellipticine were used in all of the experiments.

c CYP	M1	M2	M3+M4	M5
50 nM	58.6	26.9	5.7	8.8
100 nM	71.3	9.5	14.8	4.4
200 nM	78.5	8.1	12.2	1.3

5.1.2 Oxidation of ellipticine by rat recombinant CYP2A1/2

CYP2A1 generated four of the ellipticine metabolites. The major one was 12-hydroxyellipticine (Figure 28; Table 5, p 51). Its formation increased with an increase in CYP concentration. On the contrary, CYP2A2 formed this metabolite only in very low amounts. CYP2A2 generated predominantly 13-hydroxyellipticine (Figure 29, p 51; Table 6, p 51), which was formed twice as effectively as by CYP2A1. 9-hydroxyellipticine and ellipticine N^2-oxide were formed by CYP2A1 only in minority. There was no ellipticine N^2-oxide detected in the incubations containing CYP2A2.

The proportional formation of 12-hydroxyellipticine decreased with an increase in CYP2A2 concentration, whereas the percentage levels of 9-hydroxyellipticine increased when CYP2A2 concentration increased (Table 6, p 51).

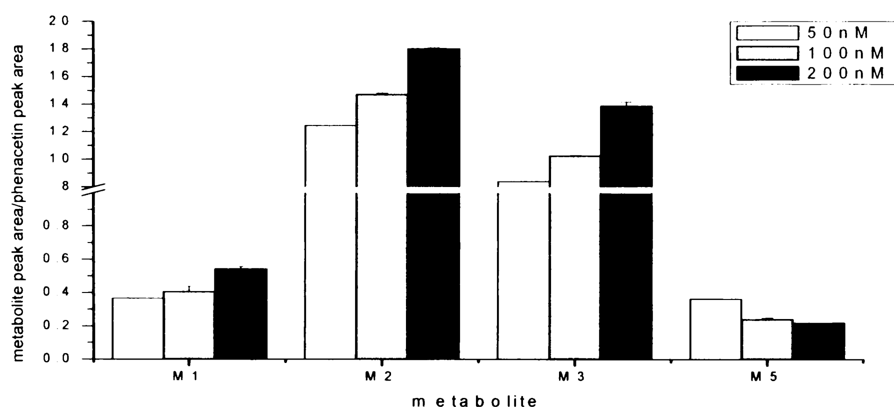

Figure 28. Ellipticine oxidation by rat recombinant CYP2A1. Fifty to 200 nM CYP and 10 μM ellipticine were used in all of the experiments.

Table 5. Percentage levels of individual ellipticine metabolites generated by rat recombinant CYP2A1. Fifty to 200 nM CYP and 10 μM ellipticine were used in all of the experiments.

c CYP	M1	M2	M3	M5
50 nM	1.7	57.7	38.9	1.7
100 nM	1.6	57.4	40.0	1.0
200 nM	1.6	55.2	42.5	0.7

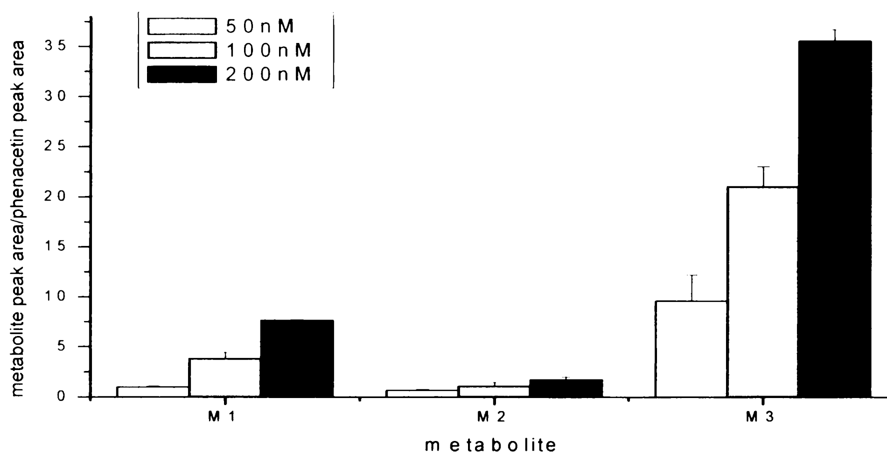

Figure 29. Ellipticine oxidation by rat recombinant CYP2A2. Fifty to 200 nM CYP and 10 μM ellipticine were used in all of the experiments.

Table 6. Percentage levels of individual ellipticine metabolites generated by rat recombinant CYP2A2. Fifty to 200 nM CYP and 10 μM ellipticine were used in all of the experiments.

c CYP	M1	M2	M3
50 nM	8.9	6.0	85.1
100 nM	14.8	4.1	81.1
200 nM	17.0	3.9	79.1

5.1.3 Oxidation of ellipticine by rat recombinant CYP2B1

Rat recombinant CYP2B1 exhibited low efficiency to oxidize ellipticine (Figure 30, p 52; Table 7, p 52). The formation of 12-hydroxyellipticine, 13-hydroxyellipticine and ellipticine

N^2-oxide was decreasing with an increase of CYP concentration. 9-hydroxyellipticine was generated only in minority.

An increase in CYP2B1 concentration resulted in a decrease in the percentage levels of 12-hydroxyellipticine, while an increase in the production of 9-hydroxyellipticine (Table 7).

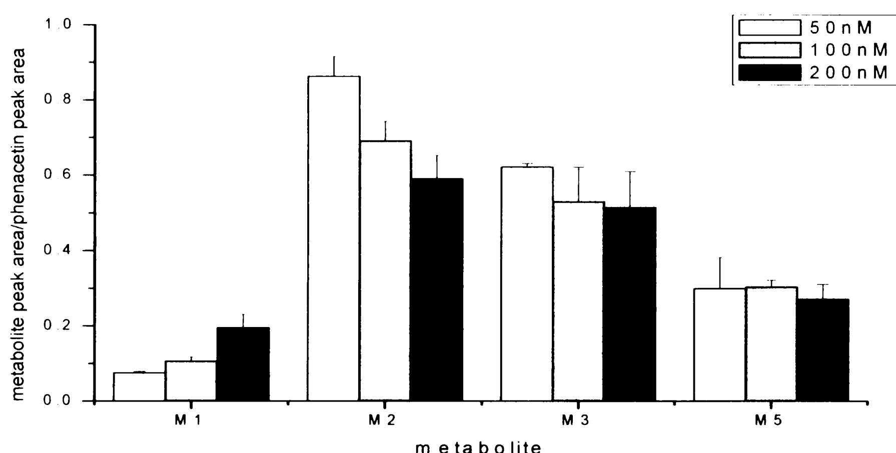

Figure 30. Ellipticine oxidation by rat recombinant CYP2B1. Fifty to 200 nM CYP and 10 μM ellipticine were used in all of the experiments.

Table 7. Percentage levels of individual ellipticine metabolites generated by rat recombinant CYP2B1. Fifty to 200 nM CYP and 10 μM ellipticine were used in all of the experiments.

c CYP	M1	M2	M3	M5
50 nM	4.1	46.4	33.4	16.1
100 nM	6.5	42.4	32.5	18.6
200 nM	12.4	37.6	32.8	17.2

5.1.4 Oxidation of ellipticine by rat recombinant CYP2C

Cytochromes P450 of a 2C subfamily are enzymes expressed constitutively and in large amounts in rat liver. CYP2C6, 2C11, 2C12 and 2C13 represented the CYP2C enzyme subfamily in this study. Although CYP2C11 is the most abundant isoform from the 2C subfamily (rat liver), CYP2C6 was more than three times more efficient to oxidize ellipticine. It predominantly generated 9-hydroxyellipticine (Figure 31, p 53; Table 8, p 53). CYP2C11

also formed predominantly 9-hydroxyellipticine but to a lower extent than CYP2C6 (Figure 32, p 54; Table 9, p 54). Other ellipticine metabolites were formed only in minority by these two enzymes. CYP2C12 generated 12-, 13- and 7-hydroxyellipticine in majority (Figure 33, p 54; Table 10, p 55). The retention times of 13-hydroxyellipticine and 7-hydroxyellipticine are very close – 7.4 and 7.8 minutes, respectively. Hence, the quantification of each ellipticine metabolite separately was impossible and they were quantified together. CYP2C13 was not very efficient enzyme participating in the oxidation of ellipticine (Figure 34, p 55; Table 11, p 55). In the case of CYP2C13, the separate quantification of 13- and 7-hydroxyellipticine was possible.

An increase in CYP2C11 concentration resulted in a decrease in the percentage levels of 12-, 13- and 7-hydroxyellipticine, while an increase in the production of 9-hydroxyellipticine (Table 9, p 54). The percentage levels of 9-hydroxyellipticine increased when the CYP2C12 concentration increased, whereas the portions of ellipticine N^2-oxide decreased (Table 10, p 55).

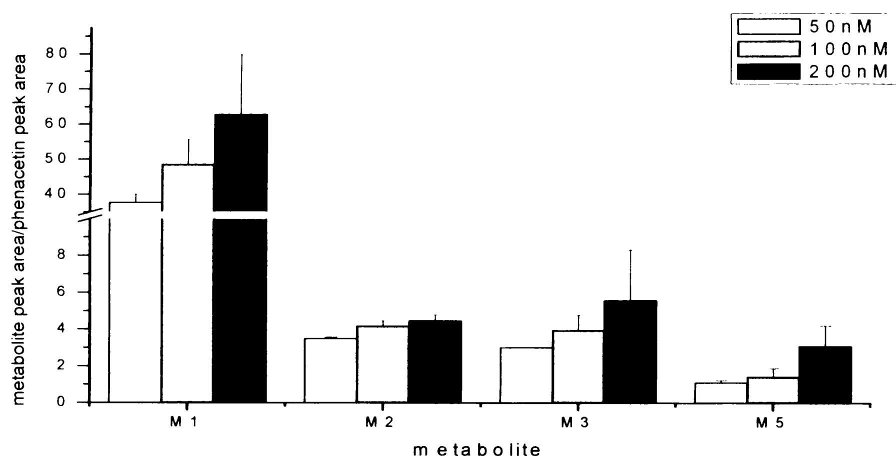

Figure 31. Ellipticine oxidation by rat recombinant CYP2C6. Fifty to 200 nM CYP and 10 μM ellipticine were used in all of the experiments.

Table 8. Percentage levels of individual ellipticine metabolites generated by rat recombinant CYP2C6. Fifty to 200 nM CYP and 10 μM ellipticine were used in all of the experiments.

c CYP	M1	M2	M3	M5
50 nM	83.3	7.7	6.6	2.4
100 nM	83.6	7.2	6.8	2.4
200 nM	82.7	5.9	7.3	4.1

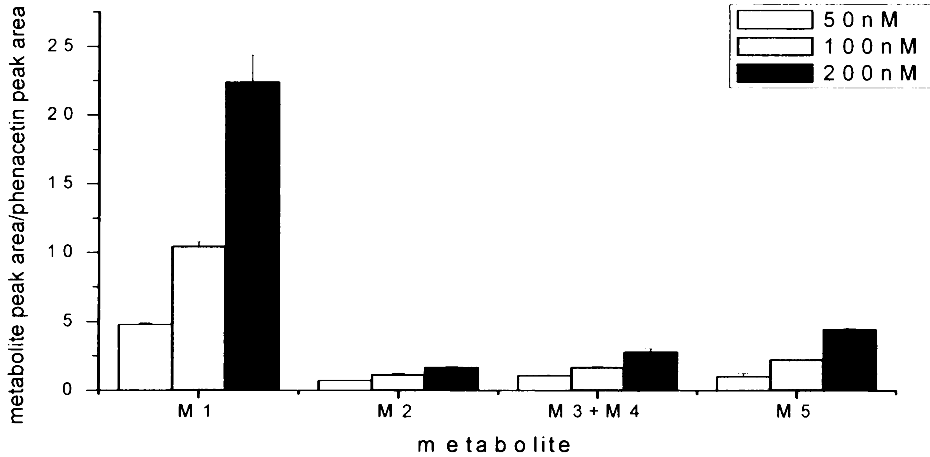

Figure 32. Ellipticine oxidation by rat recombinant CYP2C11. Fifty to 200 nM CYP and 10 μM ellipticine were used in all of the experiments.

Table 9. Percentage levels of individual ellipticine metabolites generated by rat recombinant CYP2C11. Fifty to 200 nM CYP and 10 μM ellipticine were used in all of the experiments.

c CYP	M1	M2	M3+M4	M5
50 nM	62.6	9.7	14.2	13.5
100 nM	67.3	7.5	10.9	14.3
200 nM	71.6	5.4	8.9	14.1

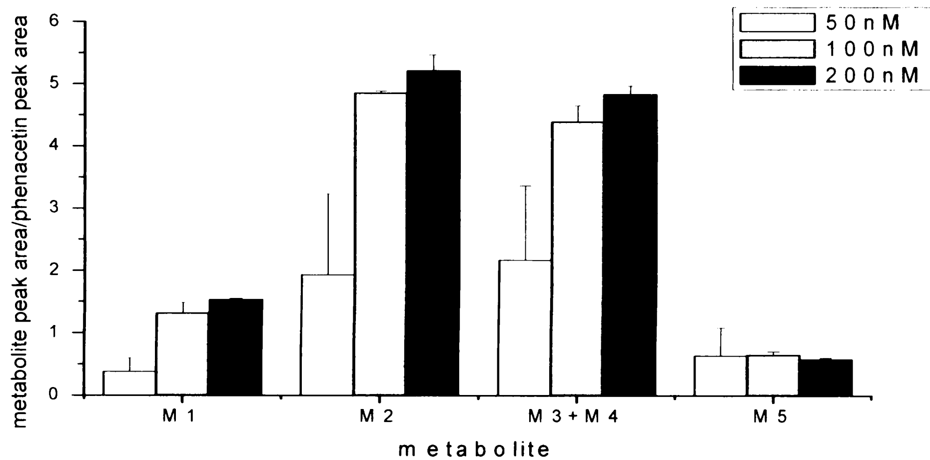

Figure 33. Ellipticine oxidation by rat recombinant CYP2C12. Fifty to 200 nM CYP and 10 μM ellipticine were used in all of the experiments.

Table 10. Percentage levels of individual ellipticine metabolites generated by rat recombinant CYP2C12. Fifty to 200 nM CYP and 10 μM ellipticine were used in all of the experiments.

c CYP	M1	M2	M3+M4	M5
50 nM	7.4	37.8	42.3	12.5
100 nM	11.7	43.4	39.1	5.8
200 nM	12.6	42.9	39.7	4.8

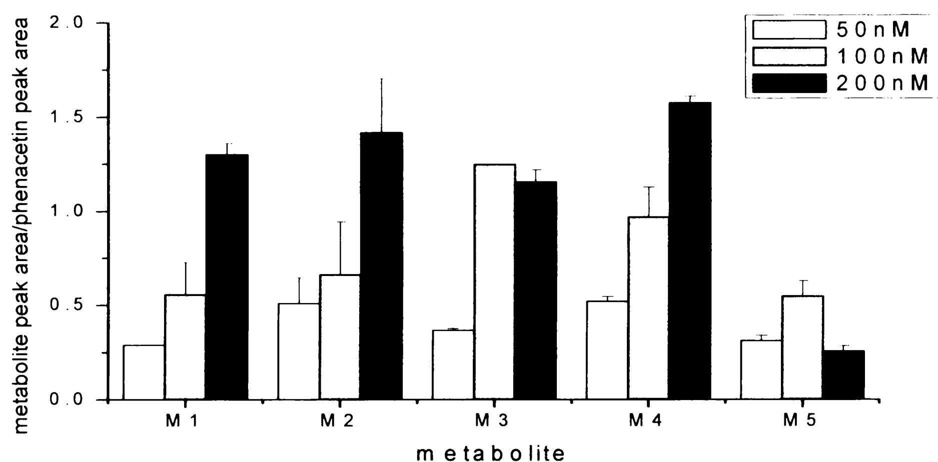

Figure 34. Ellipticine oxidation by rat recombinant CYP2C13. Fifty to 200 nM CYP and 10 μM ellipticine were used in all of the experiments.

Table 11. Percentage levels of individual ellipticine metabolites generated by rat recombinant CYP2C13. Fifty to 200 nM CYP and 10 μM ellipticine were used in all of the experiments.

c CYP	M1	M2	M3	M4	M5
50 nM	14.5	25.6	18.3	26.0	15.6
100 nM	14.0	16.6	31.4	24.3	13.7
200 nM	22.8	14.9	20.2	27.6	4.5

5.1.5 Oxidation of ellipticine by rat recombinant CYP2D1/2

There was a significant difference in the efficiency of the two CYP2D enzymes in ellipticine oxidation. CYP2D1 was 17 times more efficient in oxidizing ellipticine than CYP2D2

(Figures 35 and 36, p 56-57; Tables 12 and 13, p 56-57). The difference was even more pronounced in the formation of 9-hydroxyellipticine (60 times) and of 13-hydroxyellipticine (28 times). An increase in CYP2D1/2 concentration correlated with an increase in the formation of all ellipticine metabolites.

An increase in CYP2D2 concentration produced a decrease in the percentage levels of 12- and 13-hydroxyellipticine, while an increase in the production of ellipticine N^2-oxide (Table 13, p 57).

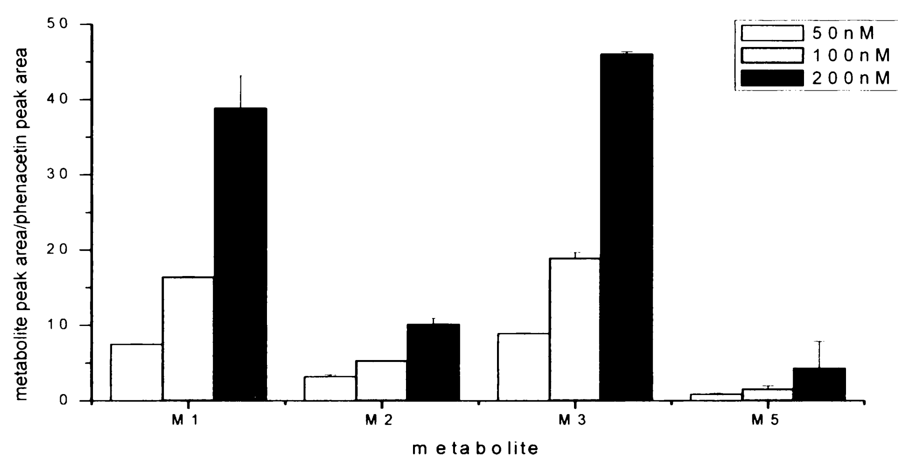

Figure 35. Ellipticine oxidation by rat recombinant CYP2D1. Fifty to 200 nM CYP and 10 μM ellipticine were used in all of the experiments.

Table 12. Percentage levels of individual ellipticine metabolites generated by rat recombinant CYP2D1. Fifty to 200 nM CYP and 10 μM ellipticine were used in all of the experiments.

c CYP	M1	M2	M3	M5
50 nM	36.6	15.6	43.6	4.2
100 nM	38.9	12.6	44.8	3.7
200 nM	39.1	10.2	46.3	4.4

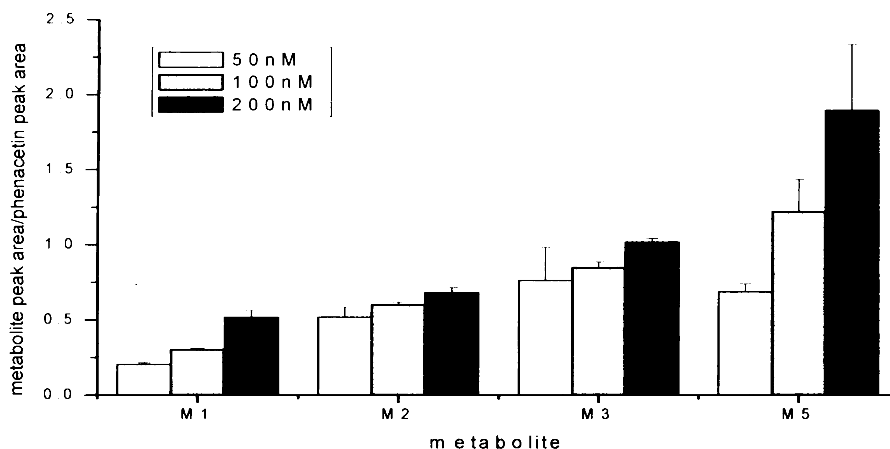

Figure 36. Ellipticine oxidation by rat recombinant CYP2D2. Fifty to 200 nM CYP and 10 µM ellipticine were used in all of the experiments.

Table 13. Percentage levels of individual ellipticine metabolites generated by rat recombinant CYP2D2. Fifty to 200 nM CYP and 10 µM ellipticine were used in all of the experiments.

c CYP	M1	M2	M3	M5
50 nM	9.4	23.9	35.1	31.6
100 nM	10.2	20.2	29.5	41.1
200 nM	12.6	16.6	24.7	46.1

5.1.6 Oxidation of ellipticine by rat recombinant CYP2E1

CYP2E1 exhibited low efficiency to oxidize ellipticine. In majority, 9- and 13-hydroxyellipticine were generated by this enzyme (Figure 37, p 58; Table 14, p 58).

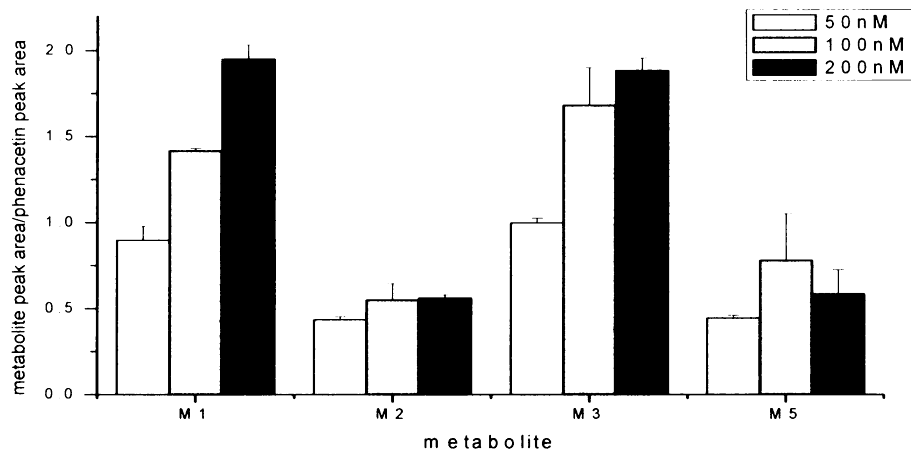

Figure 37. Ellipticine oxidation by rat recombinant CYP2E1. Fifty to 200 nM CYP and 10 μM ellipticine were used in all of the experiments.

Table 14. Percentage levels of individual ellipticine metabolites generated by rat recombinant CYP2E1. Fifty to 200 nM CYP and 10 μM ellipticine were used in all of the experiments.

c CYP	M1	M2	M3	M5
50 nM	32.4	15.7	36.0	16.1
100 nM	32.0	12.4	38.0	17.6
200 nM	39.1	11.3	37.8	11.8

5.1.7 Oxidation of ellipticine by rat recombinant CYP3A1/2

Both CYP3A isoforms generated high amounts of 13-hydroxyellipticine. CYP3A1 was the most effective CYP isoform oxidizing ellipticine. 9-hydroxyellipticine followed by 13-hydroxyellipticine were the predominant ellipticine metabolites generated by CYP3A1 (Figure 38, p 59; Table 15, p 59). CYP3A2 generated ellipticine N^2-oxide in majority (Figure 39, p 59; Table 16, p 60). CYP3A1 was also efficient in catalysing this reaction but to a lower extent.

An increase in CYP3A1 concentration resulted in an increase in the percentage levels of 9-hydroxyellipticine, while the production of 13-hydroxyellipticine decreased (Table 15, p 59). The percentage levels of 9- and 13-hydroxyellipticine increased when the CYP3A2

concentration increased, whereas the portions of ellipticine N^2-oxide decreased (Table 16, p 60).

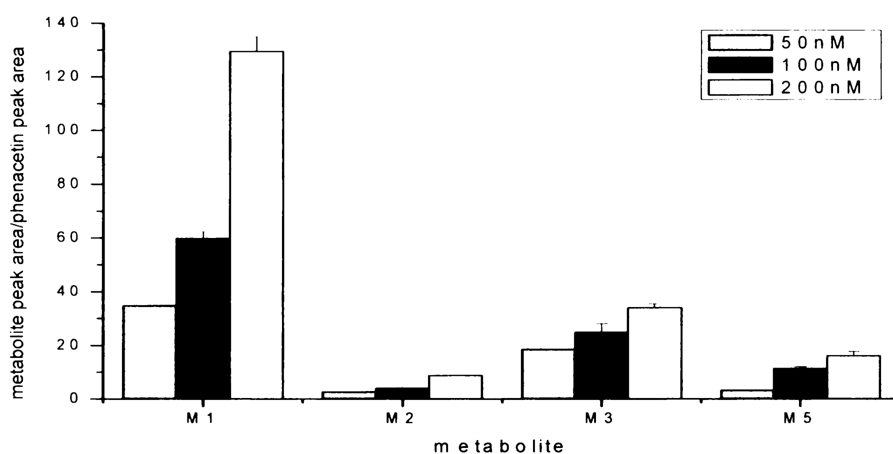

Figure 38. Ellipticine oxidation by rat recombinant CYP3A1. Fifty to 200 nM CYP and 10 μM ellipticine were used in all of the experiments.

Table 15. Percentage levels of individual ellipticine metabolites generated by rat recombinant CYP3A1. Fifty to 200 nM CYP and 10 μM ellipticine were used in all of the experiments.

c CYP	M1	M2	M3	M5
50 nM	58.7	4.5	31.3	5.5
100 nM	59.8	4.0	24.8	11.4
200 nM	68.8	4.6	18.0	8.6

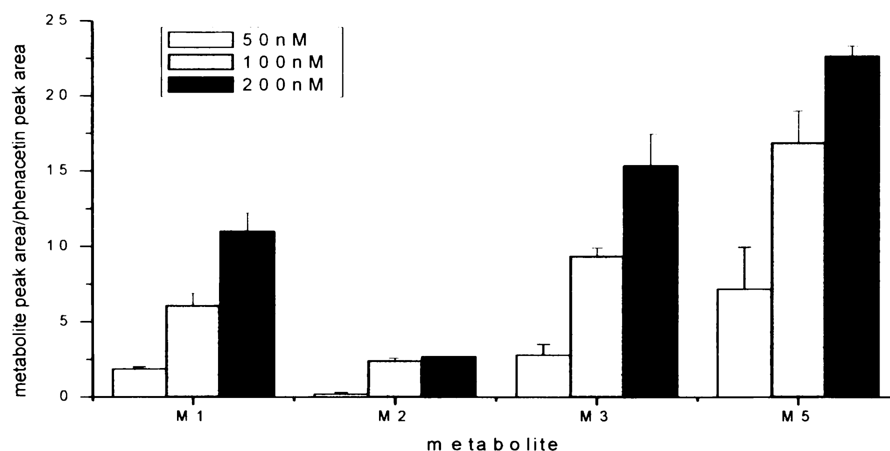

Figure 39. Ellipticine oxidation by rat recombinant CYP3A2. Fifty to 200 nM CYP and 10 μM ellipticine were used in all of the experiments.

Table 16. Percentage levels of individual ellipticine metabolites generated by rat recombinant CYP3A2. Fifty to 200 nM CYP and 10 μM ellipticine were used in all of the experiments.

c CYP	M1	M2	M3	M5
50 nM	15.6	1.7	23.2	59.5
100 nM	17.5	7.0	26.9	48.6
200 nM	21.3	5.2	29.7	43.8

5.1.8 The effect of cytochrome b_5 on ellipticine oxidation by rat recombinant CYPs

In contrast to most Supersomal samples tested in our study, which contained another microsomal protein cytochrome b5, those expressing rat recombinant CYP1A1/2 and CYP2D1/2 were without cytochrome b_5. To obtain comparable results with all employed CYP isoforms, purified rabbit cytochrome b_5 was added into incubations with these enzymes in the ratio of CYP to cyt b_5 – 1:3, *i.e.* average of ratios of CYP to cyt b_5 in other Supersomes^{TM}. Cytochrome b_5 significantly stimulated ellipticine oxidation by rat CYP1A1/2 (Figure 40, p 61). The retention times of 13-hydroxyellipticine and 7-hydroxyellipticine are very close – 7.4 and 7.8 minutes, respectively. The quantification of each ellipticine metabolite separately was impossible. Therefore, they were quantified together. Nevertheless, with respect to the peak asymmetry it was evident that 13-hydroxyellipticine was formed in higher amounts than 7-hydroxyellipticine when cytochrome b_5 was present. Likewise, formation of 12-hydroxyellipticine and ellipticine N^2-oxide was stimulated by the presence of cytochrome b_5 whereas the formation of 9-hydroxyellipticine was affected only insignificantly (Figure 40, p 61; Table 17, p 62).

Cytochrome b_5 did not influence the pattern and/or amounts of ellipticine metabolites generated by rat recombinant CYP2D1/2 (Figure 41, p 61; Table 18, p 62).

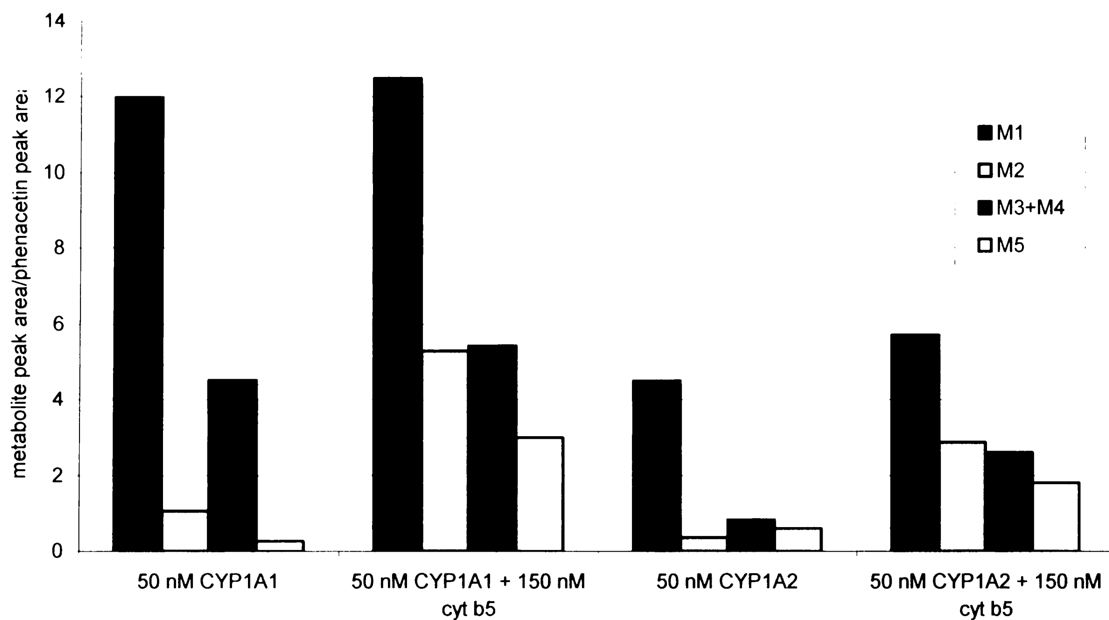

Figure 40. Influence of cytochrome b_5 on ellipticine oxidation by rat recombinant CYP1A. 50 nM CYP, 150 nM cyt b_5 and 10 μM ellipticine were used in the experiments.

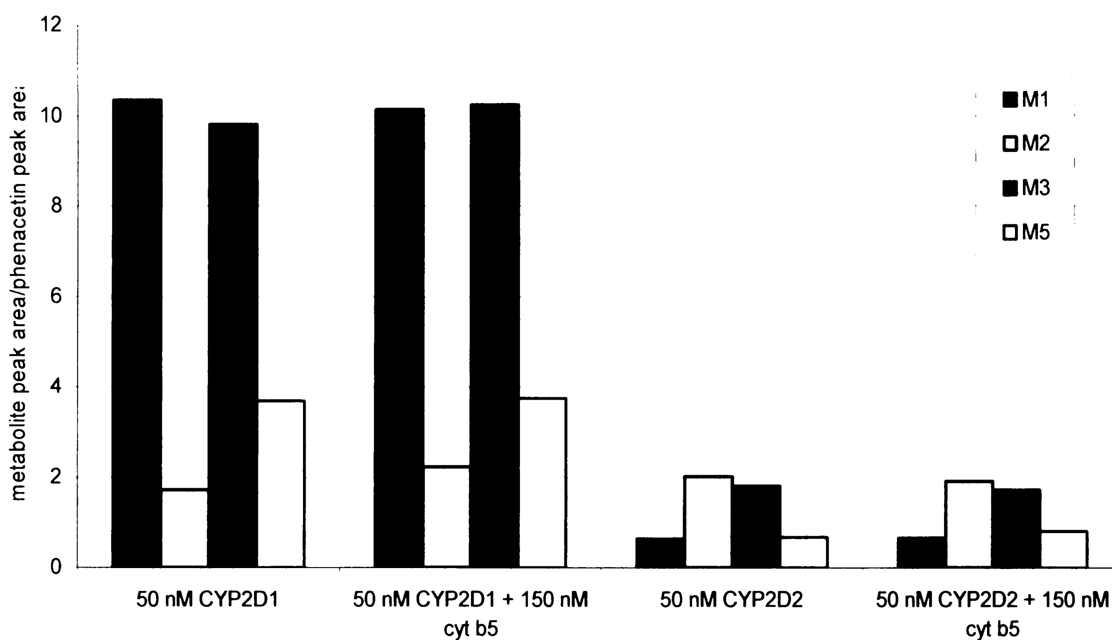

Figure 41. Influence of cytochrome b_5 on ellipticine oxidation by rat recombinant CYP2D. 50 nM CYP, 150 nM cyt b_5 and 10 μM ellipticine were used in the experiments.

Table 17. Percentage levels of individual ellipticine metabolites generated by rat recombinant CYP1A1/2 in the absence and presence of cytochrome b_5 (CYP:cyt b_5 1:3). 50 nM CYP and 10 μM ellipticine were used in all of the experiments.

c CYP1A1 / c cyt b_5	M1	M2	M3+M4	M5
50 nM / 0 nM	67.2	6.0	25.3	1.5
50 nM / 150 nM	47.7	20.2	20.7	11.4
c CYP1A2 / c cyt b_5	M1	M2	M3+M4	M5
50 nM / 0 nM	71.2	5.9	13.3	9.6
50 nM / 150 nM	43.9	22.2	20.0	13.9

Table 18. Percentage levels of individual ellipticine metabolites generated by rat recombinant CYP2D1/2 in the absence and presence of cytochrome b_5 (CYP:cyt b_5 1:3). 50 nM CYP and 10 μM ellipticine were used in all of the experiments.

c CYP2D1 / c cyt b_5	M1	M2	M3+M4	M5
50 nM / 0 nM	40.5	6.7	38.4	14.4
50 nM / 150 nM	38.5	8.5	38.8	14.2
c CYP2D2 / c cyt b_5	M1	M2	M3+M4	M5
50 nM / 0 nM	12.4	39.1	35.3	13.2
50 nM / 150 nM	12.8	37.6	33.6	6.0

5.1.9 Oxidation of ellipticine by rat recombinant CYPs in the presence of cytochrome b5

Figure 42 (p 63) summarizes the formation of ellipticine metabolites by individual rat CYP enzymes in the presence of cytochrome b_5. Cytochrome b_5 was either expressed in Supersomes^{TM} or a purified rabbit cytochrome b_5 was added into the incubation mixture. The concentration of CYP used for this study was 50 nM. The majority of 9-hydroxyellipticine formation regarding other ellipticine metabolites was observed using the rat recombinant CYP1A1/2, CYP2C6/11 and CYP3A1. The highest amount of ellipticine N^2-oxide was formed by CYP3A2. 12-hydroxyellipticine was most efficiently generated by CYP2A1. 13-hydroxyellipticine was formed predominantly by CYP3A1, 2D1, 2A1/2 and 1A1. CYP1A1/2 and CYP2C11/12/13 generated also small amounts of 7-hydroxyellipticine. Despite 13-hydroxyellipticine and 7-hydroxyellipticine were not quantified separately, it was evident from the peak asymmetry that 13-hydroxyellipticine was generated in majority by CYP1A in the presence of cytochrome b_5.

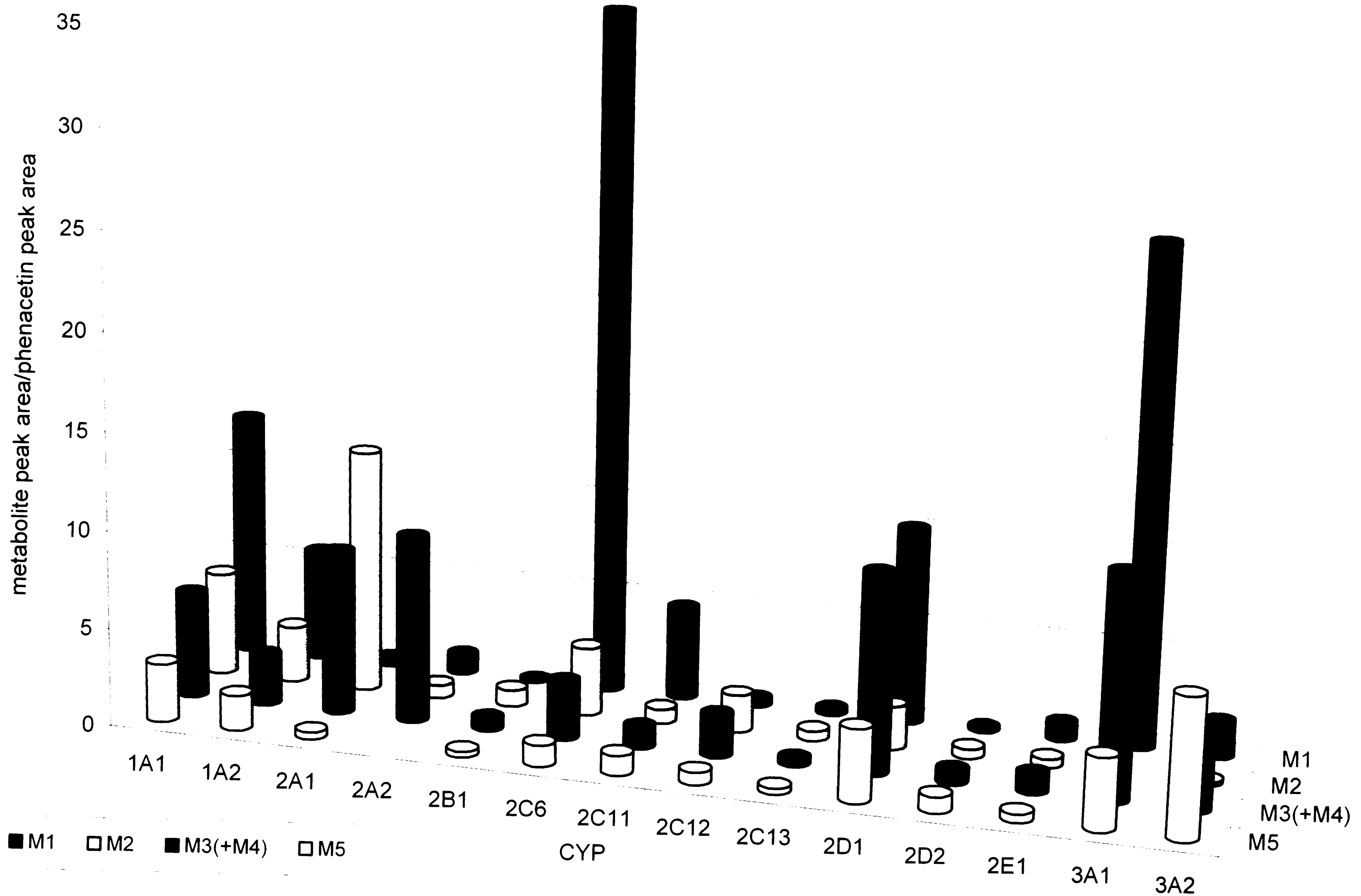

Figure 42. Ellipticine oxidation by rat recombinant CYP enzymes in the presence of cytochrome b_5. 50 nM CYP and 10μM ellipticine were used in all of the experiments. Cytochrome b_5 was added to Supersomes expressing CYP1A1/2 and CYP2D1/2 (CYP:cyt b_5 – 1:3).

5.2 Oxidation of ellipticine by human recombinant CYPs and the effect of cytochrome b_5 on this process

Oxidation of ellipticine by human cytochromes P450 was also investigated in this study. Microsomes from *Baculovirus* transfected insect cells (Supersomes™) expressing human recombinant CYP enzymes were used in the experiments. Up to five ellipticine metabolites were separated by HPLC (Figure 25, p 48) after the incubation of ellipticine with human CYPs in Supersomes™. The metabolites were the same as those generated by rat recombinant CYP enzymes: 9-hydroxyellipticine (M1), 12-hydroxyellipticine (M2), 13-hydroxyellipticine (M3), 7-hydroxyellipticine (M4) and ellipticine N^2-oxide (M5). The structures of all five ellipticine metabolites were identified on the basis of co-chromatography with standards (for the structures see Figure 10, p 16).

To investigate the effect of cytochrome b_5 on ellipticine oxidation by human recombinant CYPs, detailed studies with CYPs both in absence and in presence of cytochrome b_5 were performed. There were no Supersomes™ expressing human CYP enzymes with cytochrome b_5 commercially available except those expressing CYP2B6, CYP2E1 and CYP3A4. Therefore, purified rabbit cytochrome b_5 (CYP:cyt b_5 1:3) was added.

In the case of CYPs of the 1A and 1B subfamilies three different concentrations of the enzymes were used in the experiments. In the case of other human CYPs (CYP2A6, 2B6, 2C subfamily, 2D6*1, 2E1 and 3A4) ellipticine oxidation was analysed using only one CYP concentration – 50 nM.

5.2.1 Oxidation of ellipticine by human recombinant CYP1A1/2

Human recombinant CYP1A1/2 oxidized ellipticine mainly to 9-hydroxyellipticine while other ellipticine metabolites were formed only in minority (Figures 43 and 44, p 65-66; Tables 19 and 20, p 65-66). Three different CYP concentrations (10, 20, 50 nM) were used to perform this study. An increase in CYP concentrations resulted in an increase in formation of ellipticine metabolites. The peak of 12-hydroxyellipticine was not detected in this system.

Nevertheless, its formation could not be excluded because it could have been covered by the great peak area of 9-hydroxyellipticine.

Significant stimulation of ellipticine oxidation by both employed CYP enzymes of the 1A subfamily by cytochrome b_5 was observed (Figures 43 and 44, p 65-66; Tables 19 and 20, p 65-66). Even though the formation of 13-hydroxyellipticine and 7-hydroxyellipticine was not quantified separately (they were eluted with similar retention times showing only shoulders in one broad peak), in the presence of cytochrome b_5 the proportion of generated 13-hydroxyellipticine and 7-hydroxyellipticine remarkably changed. 13-hydroxyellipticine was formed preferentially.

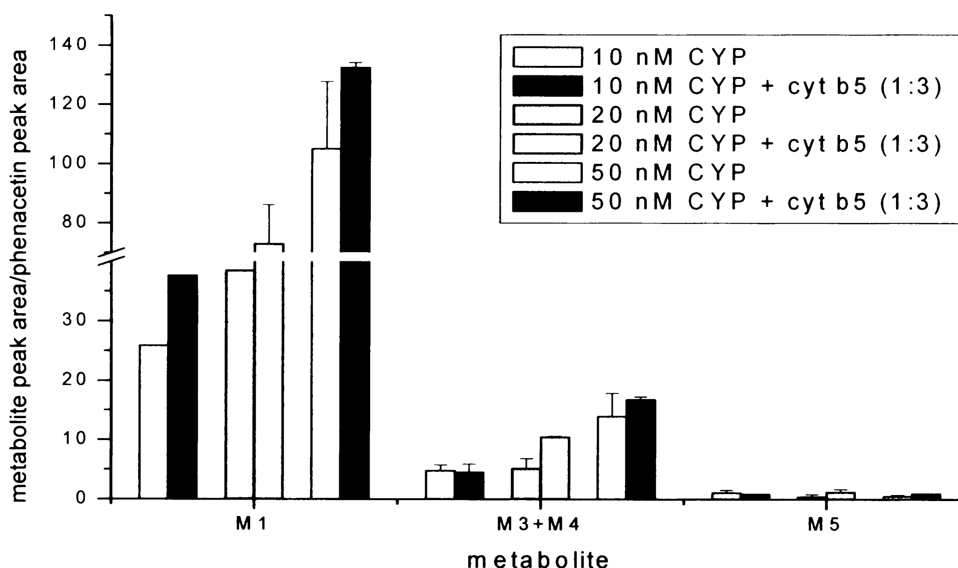

Figure 43. Ellipticine oxidation by human recombinant CYP1A1 in the absence and presence of cytochrome b_5 (CYP:cyt b_5 1:3). Ten to 50 nM CYP and 10 μM ellipticine were used in all of the experiments.

Table 19. Percentage levels of individual ellipticine metabolites generated by human recombinant CYP1A1 in the absence and presence of cytochrome b_5. Ten to 50 nM CYP and 10 μM ellipticine were used in al of the experiments. Purified rabbit cytochrome b_5 was added in a ratio of CYP to cyt b_5 1:3.

c CYP / c cyt b_5	M1	M3+M4	M5
10 nM / 0 nM	81.4	15.2	3.4
10 nM / 30 nM	87.4	10.6	2.0
20 nM / 0 nM	87.4	11.7	0.9
20 nM / 60 nM	86.2	12.4	1.4
50 nM / 0 nM	87.9	11.7	0.4
50 nM / 150 nM	88.2	11.2	0.6

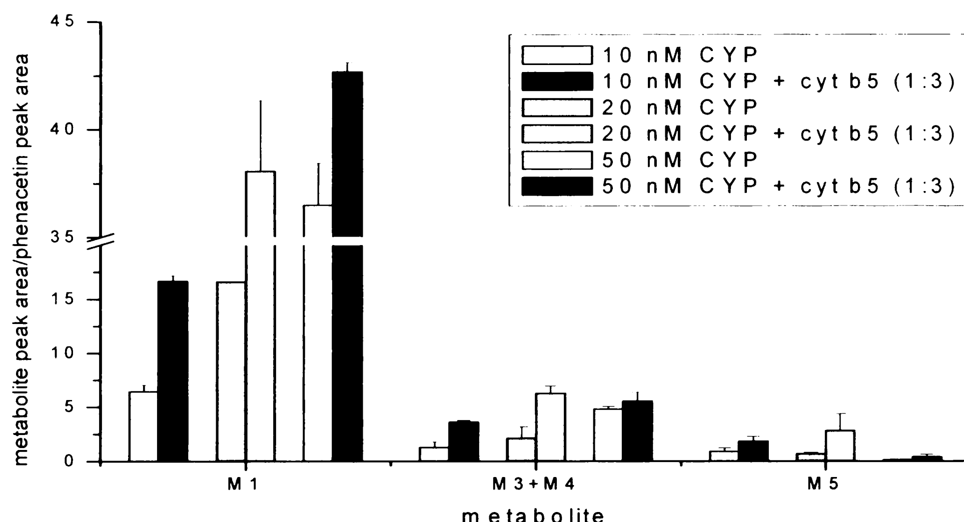

Figure 44. Ellipticine oxidation by human recombinant CYP1A2 in the absence and presence of cytochrome b_5 (CYP:cyt b_5 1:3). Ten to 50 nM CYP and 10 µM ellipticine were used in all of the experiments.

Table 20. Percentage levels of individual ellipticine metabolites generated by human recombinant CYP1A2 in the absence and presence of cytochrome b_5. Ten to 50 nM CYP and 10 µM ellipticine were used in al of the experiments. Purified rabbit cytochrome b_5 was added in a ratio of CYP to cyt b_5 1:3.

c CYP / c cyt b_5	[illegible]	[illegible]	[illegible]
10 nM / 0 nM	74.6	14.9	10.5
10 nM / 30 nM	75.2	16.4	8.4
20 nM / 0 nM	85.3	11.0	3.7
20 nM / 60 nM	80.7	13.3	6.0
50 nM / 0 nM	88.1	11.6	0.3
50 nM / 150 nM	87.8	11.4	0.8

5.2.2 Oxidation of ellipticine by human recombinant CYP1B1

Three different CYP1B1 concentrations (10, 20, 50 nM) were used to investigate ellipticine oxidation by human recombinant CYP1B1. 9-hydroxyellipticine was the major metabolite formed by CYP1B1 (Figure 45, p 67; Table 21, p 67). Its formation slightly increased with an increase in CYP concentration and was stimulated by the presence of cytochrome b_5 (CYP:cyt b_5 1:3). Other ellipticine metabolites were formed only in minority, similar to the case of CYP enzymes from the 1A subfamily. At the highest CYP concentration (50 nM), the

formation of 13-hydroxyellipticine and 7-hydroxyellipticine was significantly inhibited by the presence of cyt b_5. Formation of 12-hydroxyellipticine was not quantified.

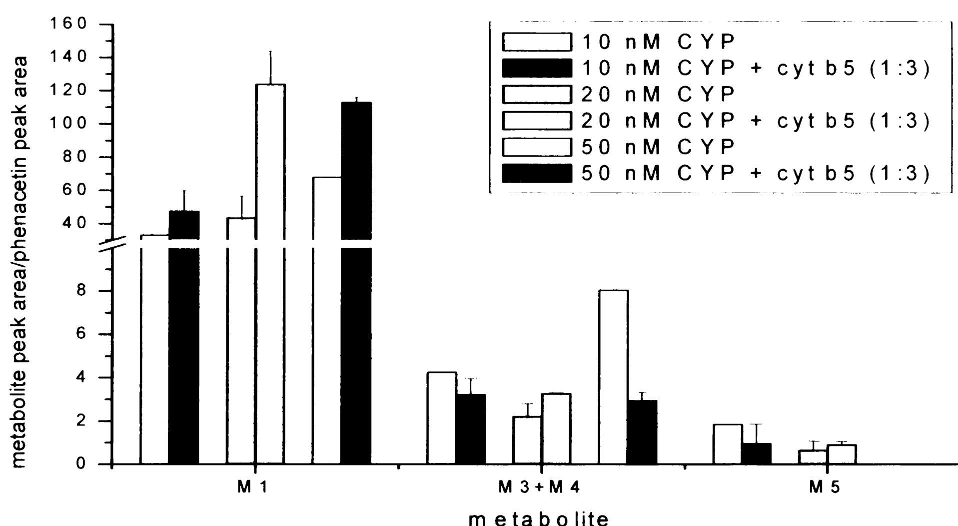

Figure 45. Ellipticine oxidation by human recombinant CYP1B1 in the absence and presence of cytochrome b_5 (CYP:cyt b_5 1:3). Ten to 50 nM CYP and 10 μM ellipticine were used in all of the experiments.

Table 21. Percentage levels of individual ellipticine metabolites generated by human recombinant CYP1B1 in the absence and presence of cytochrome b_5. Ten to 50 nM CYP and 10 μM ellipticine were used in al of the experiments. Purified rabbit cytochrome b_5 was added in a ratio of CYP to cyt b_5 1:3.

c CYP / c cyt b_5	M1	M3+M4	M5
10 nM / 0 nM	84.4	10.9	4.7
10 nM / 30 nM	91.9	6.2	1.9
20 nM / 0 nM	93.8	4.8	1.4
20 nM / 60 nM	96.7	2.6	0.7
50 nM / 0 nM	89.4	10.6	0.0
50 nM / 150 nM	97.5	2.5	0.0

5.2.3 Oxidation of ellipticine by human recombinant CYP2A6

Human recombinant CYP2A6 generated four ellipticine metabolites, but only in low levels (Figure 46, p 68; Table 22, p 68). The formation of 9-, 12- and 13-hydroxyellipticine was stimulated by the presence of cytochrome b_5. In contrast to this finding, formation of ellipticine N^2-oxide slightly decreased in the presence of cytochrome b_5. In the presence of

cytochrome b_5, 12-hydroxyellipticine was found to be the prominent ellipticine metabolite generated by CYP2A6.

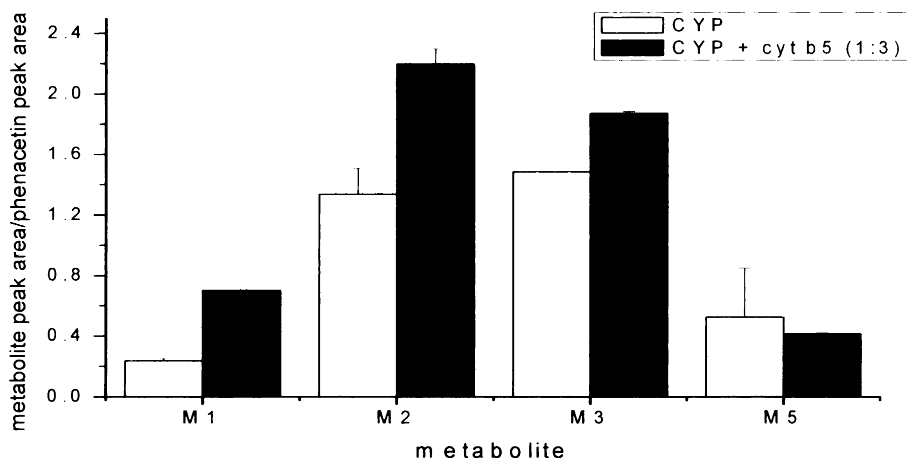

Figure 46. Ellipticine oxidation by human recombinant CYP2A6 in the absence and presence of cytochrome b_5 (CYP:cyt b_5 1:3). 50 nM CYP and 10 μM ellipticine were used in all of the experiments.

Table 22. Percentage levels of individual ellipticine metabolites generated by human recombinant CYP2A6 in the absence and presence of cytochrome b_5. 50 nM CYP and 10 μM ellipticine were used in al of the experiments. Purified rabbit cytochrome b_5 was added in a ratio of CYP to cyt b_5 1:3.

c CYP / c cyt b_5	[illegible]	[illegible]	[illegible]	[illegible]
50 nM / 0 nM	6.6	37.4	41.4	14.6
50 nM / 150 nM	13.5	42.4	36.1	8.0

5.2.4 Oxidation of ellipticine by human recombinant CYP2B6

Ellipticine was a poor substrate of human recombinant CYP2B6. Slight stimulation of its oxidation by this human CYP isoform by cytochrome b_5 was observed (Figure 47, p 69; Table 23, p 69).

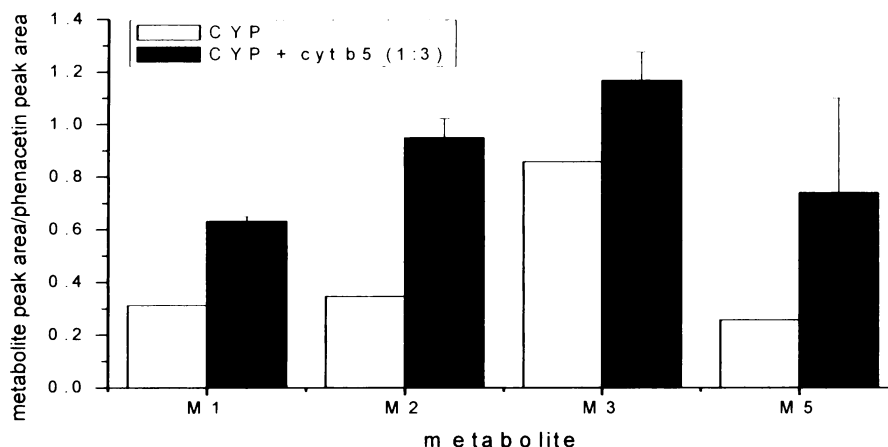

Figure 47. Ellipticine oxidation by human recombinant CYP2B6 in the absence and presence of cytochrome b_5 (CYP:cyt b_5 1:3). 50 nM CYP and 10 μM ellipticine were used in all of the experiments.

Table 23. Percentage levels of individual ellipticine metabolites generated by human recombinant CYP2B6 in the absence and presence of cytochrome b_5. 50 nM CYP and 10 μM ellipticine were used in al of the experiments. Purified rabbit cytochrome b_5 was added in a ratio of CYP to cyt b_5 1:3.

c CYP / c cyt b_5	M1	M2	M3	M5
50 nM / 0 nM	17.6	19.5	48.4	14.5
50 nM / 150 nM	18.1	27.2	33.5	21.2

5.2.5 Oxidation of ellipticine by human recombinant CYP2C

CYP2C19 was the most effective isoform from CYP2C subfamily to oxidize ellipticine (Figure 50, p 71; Table 26, p 71). CYP2C9*1 (CYP2C9 with site mutation at [144]Arg) was 2.5 times (Figure 49, p 70; Table 25, p 71), and CYP2C8 was 7.2 times less effective in this process (Figure 48, p 70; Table 24, p 70) than CYP2C19. All the employed CYP2C forms generated 9-hydroxyellitícine as the major metabolite. Formation of all the ellipticine metabolites was stimulated by cytochrome b_5. In the absence of cytochrome b_5, no ellipticine N^2-oxide was generated by CYP2C9*1 in detectable amounts.

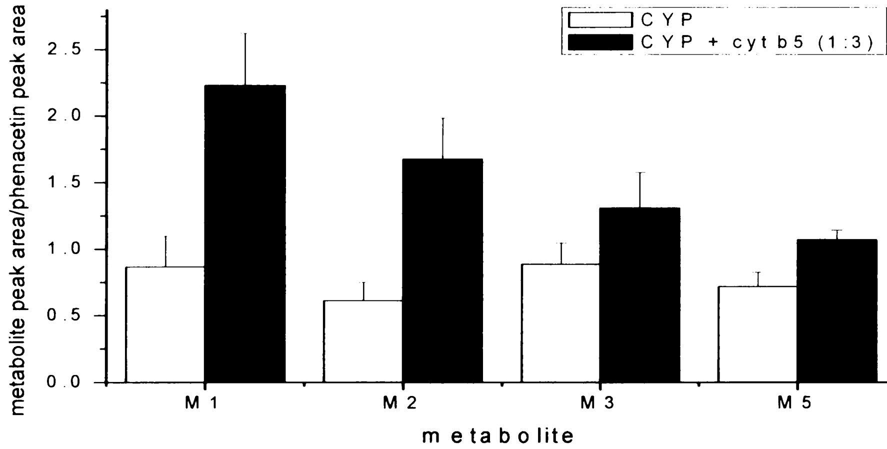

Figure 48. Ellipticine oxidation by human recombinant CYP2C8 in the absence and presence of cytochrome b_5 (CYP:cyt b_5 1:3). 50 nM CYP and 10 μM ellipticine were used in all of the experiments.

Table 24. Percentage levels of individual ellipticine metabolites generated by human recombinant CYP2C8 in the absence or presence of cytochrome b_5. 50 nM CYP and 10 μM ellipticine were used in al of the experiments. Purified rabbit cytochrome b_5 was added in a ratio of CYP to cyt b_5 1:3.

c CYP / c cyt b_5	[illegible]	[illegible]	[illegible]	[illegible]
50 nM / 0 nM	28.1	19.9	28.7	23.3
50 nM / 150 nM	35.5	26.7	20.8	17.0

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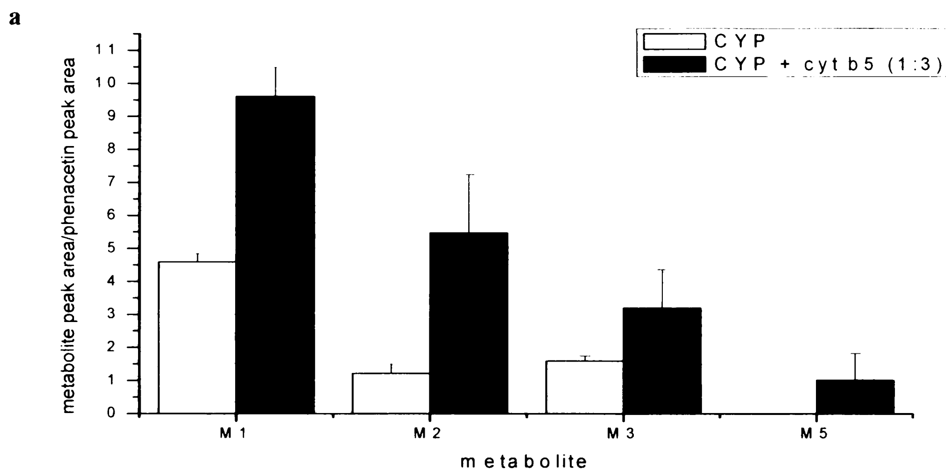

Figure 49. Ellipticine oxidation by human recombinant CYP2C9*1 in the absence and presence of cytochrome b_5 (CYP:cyt b_5 1:3). 50 nM CYP and 10 μM ellipticine were used in all of the experiments.

Table 25. Percentage levels of individual ellipticine metabolites generated by human recombinant CYP2C9*1 in the absence and presence of cytochrome b_5. 50 nM CYP and 10 µM ellipticine were used in al of the experiments. Purified rabbit cytochrome b_5 was added in a ratio of CYP to cyt b_5 1:3.

c CYP / c cyt b_5	M1	M2	M3	M5
50 nM / 0 nM	62.0	16.5	21.5	0.0
50 nM / 150 nM	49.8	28.4	16.6	5.2

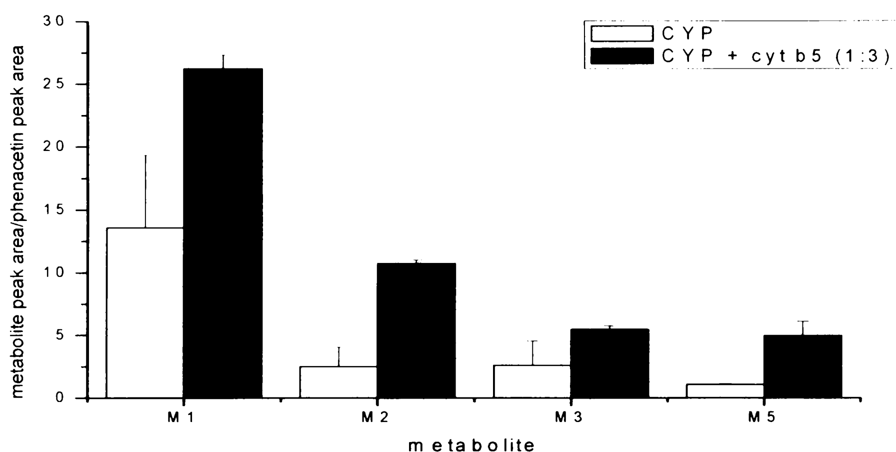

Figure 50. Ellipticine oxidation by human recombinant CYP2C19 in the absence and presence of cytochrome b_5 (CYP:cyt b_5 1:3). 50 nM CYP and 10 µM ellipticine were used in all of the experiments.

Table 26. Percentage levels of individual ellipticine metabolites generated by human recombinant CYP2C19 in the absence and presence of cytochrome b_5. 50 nM CYP and 10 µM ellipticine were used in al of the experiments. Purified rabbit cytochrome b_5 was added in a ratio of CYP to cyt b_5 1:3.

c CYP / c cyt b_5	M1	M2	M3	M5
50 nM / 0 nM	68.7	12.6	13.2	5.6
50 nM / 150 nM	53.3	22.7	11.5	10.5

5.2.6 Oxidation of ellipticine by human recombinant CYP2D6*1

Human recombinant CYP2D6*1 (CYP2D6 with site mutation at [374]Val) oxidizes ellipticine mainly to ellipticine N^2-oxide (Figure 51, p 72; Table 27, p 72). The reaction was strongly stimulated by the presence of cytochrome b_5. 9-hydroxyellipticine was also formed

by CYP2D6*1, but to a lower extent than ellipticine N^2-oxide. Other ellipticine metabolites (12-hydroxyellipticine and 13-hydroxyellipticine) were formed in minority.

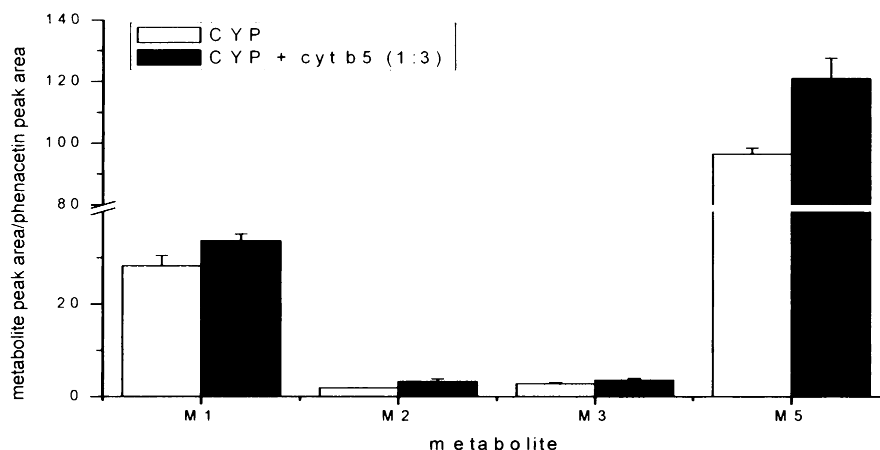

Figure 51. Ellipticine oxidation by human recombinant CYP2D6*1 in the absence and presence of cytochrome b_5 (CYP:cyt b_5 1:3). 50 nM CYP and 10 μM ellipticine were used in all of the experiments.

Table 27. Percentage levels of individual ellipticine metabolites generated by human recombinant CYPD6*1 in the absence and presence of cytochrome b_5. 50 nM CYP and 10 μM ellipticine were used in al of the experiments. Purified rabbit cytochrome b_5 was added in a ratio of CYP to cyt b_5 1:3.

c CYP / c cyt b5	M1	M2	M3	M5
50 nM / 0 nM	21.8	1.5	2.2	74.5
50 nM / 150 nM	20.8	2.0	2.2	75.0

5.2.7 Oxidation of ellipticine by human recombinant CYP2E1

In the absence of cytochrome b_5, human recombinant CYP2E1 oxidized ellipticine predominantly to 13-hydroxyellipticine (Figure 52, p 73). In the presence of cytochrome b_5, the percentage levels of individual ellipticine metabolites significantly changed (Table 28, p 73): 9-hydroxyellipticine was the metabolite formed as the major one; its formation was the most significantly stimulated by cytochrome b_5. Likewise, formation of other ellipticine metabolites was stimulated by cytochrome b_5, but to a lower extent (Figure 52, p 73).

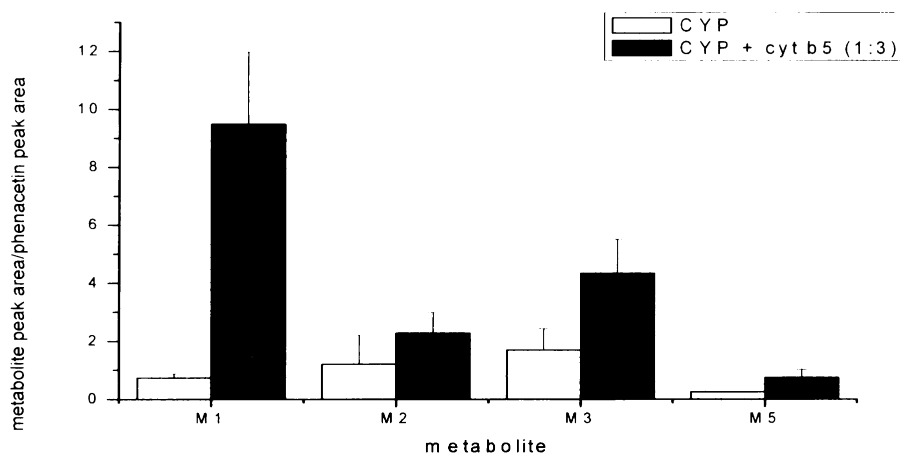

Figure 52. Ellipticine oxidation by human recombinant CYP2E1 in the absence and presence of cytochrome b_5 (CYP:cyt b_5 1:3). 50 nM CYP and 10 µM ellipticine were used in all of the experiments.

Table 28. Percentage levels of individual ellipticine metabolites generated by human recombinant CYP2E1 in the absence and presence of cytochrome b_5. 50 nM CYP and 10 µM ellipticine were used in al of the experiments. Purified rabbit cytochrome b_5 was added in a ratio of CYP to cyt b_5 1:3.

c CYP / c cyt b_5	M1	M2	M3	M5
50 nM / 0 nM	19.0	30.9	43.5	6.6
50 nM / 150 nM	56.3	13.5	25.7	4.5

5.2.8 Oxidation of ellipticine by human recombinant CYP3A4

CYP3A4 is the most abundant CYP isoform in human liver[87]. Human recombinant CYP3A4 generated four metabolites from ellipticine: ellipticine N^2-oxide, 9-hydroxyellipticine, 13-hydroxyellipticine and 12-hydroxyellipticine (Figure 53, p 74; Table 29, p 74). Formation of all ellipticine metabolites was significantly stimulated when cytochrome b_5 was present in the reaction mixture.

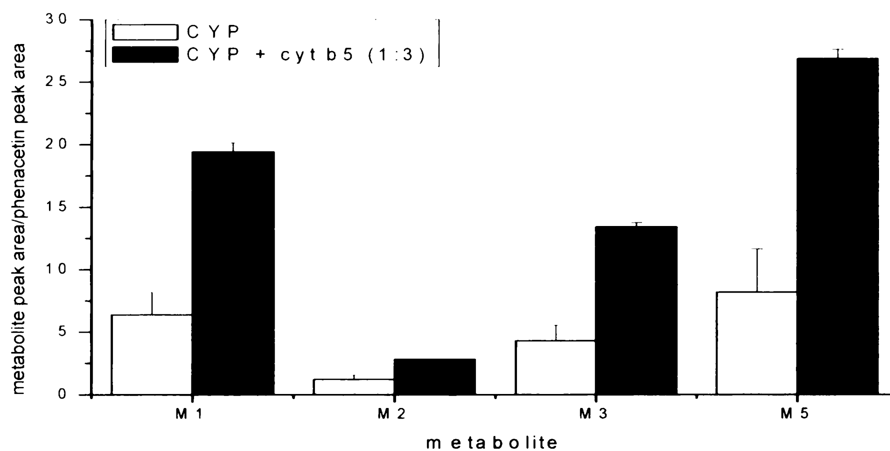

Figure 53. Ellipticine oxidation by human recombinant CYP3A4 in the absence and presence of cytochrome b_5 (CYP:cyt b_5 1:3). 50 nM CYP and 10 μM ellipticine were used in all of the experiments.

Table 29. Percentage levels of individual ellipticine metabolites generated by human recombinant CYP3A4 in the absence and presence of cytochrome b_5. 50 nM CYP and 10 μM ellipticine were used in al of the experiments. Purified rabbit cytochrome b_5 was added in a ratio of CYP to cyt b_5 1:3.

c CYP / c cyt b_5	M1	M2	M3	M5
50 nM / 0 nM	31.8	6.0	21.5	40.7
50 nM / 150 nM	31.1	4.5	21.5	42.9

5.2.9 Comparison of ellipticine oxidation by human recombinant CYPs

Figures 54 and 55 (p 75-76) show the comparison of efficiency of individual human CYP isoforms in oxidizing ellipticine in the absence and presence of cytochrome b_5. CYP2A6, 2B6, 2C8, 2C9*1 and 2E1 are represented in a separate graph from the other CYPs (CYP1A1/2, 1B1, 2C19, 2D6*1 and 3A4) because they were much less efficient to oxidize ellipticine. As can be seen in Figure 54, CYP2D6*1 generated the highest amounts of ellipticine N^2-oxide. 9-hydroxyellipticine was formed predominantly by CYP1A1 and 1B1. CYP2C19 was the most efficient to oxidize ellipticine to 12-hydroxyellipticine, especially in the presence of cytochrome b_5. The largest amounts of 13-hydroxyellipticine were generated by CYP1A1 (CYPs of the 1A subfamily generated also 7-hydroxyellipticne) and CYP3A4.

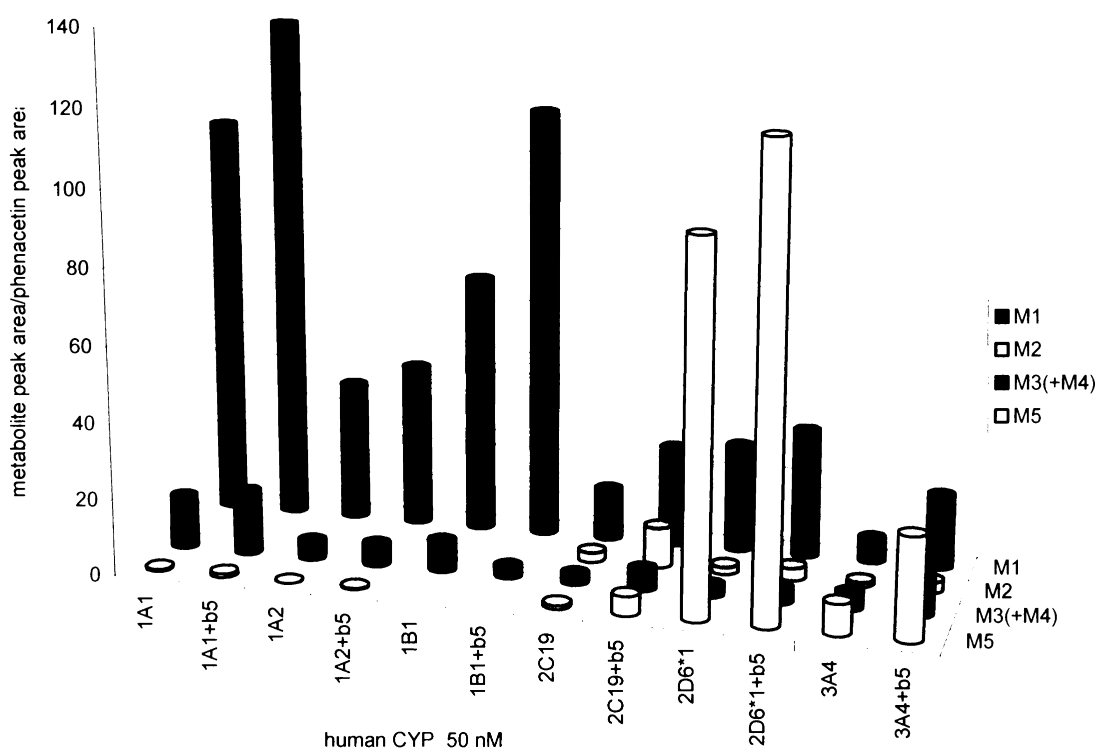

Figure 54. Comparison of the efficiency of individual human CYP isoforms (CYP1A1/2, 1B1, 2C19, 2D6*1 and 3A4) in ellipticine oxidation in the absence and presence of cytochrome b_5. 50 nM CYP and 10 μM ellipticine were used in all of the experiments. The ratio of CYP to cyt b_5 was 1:3.

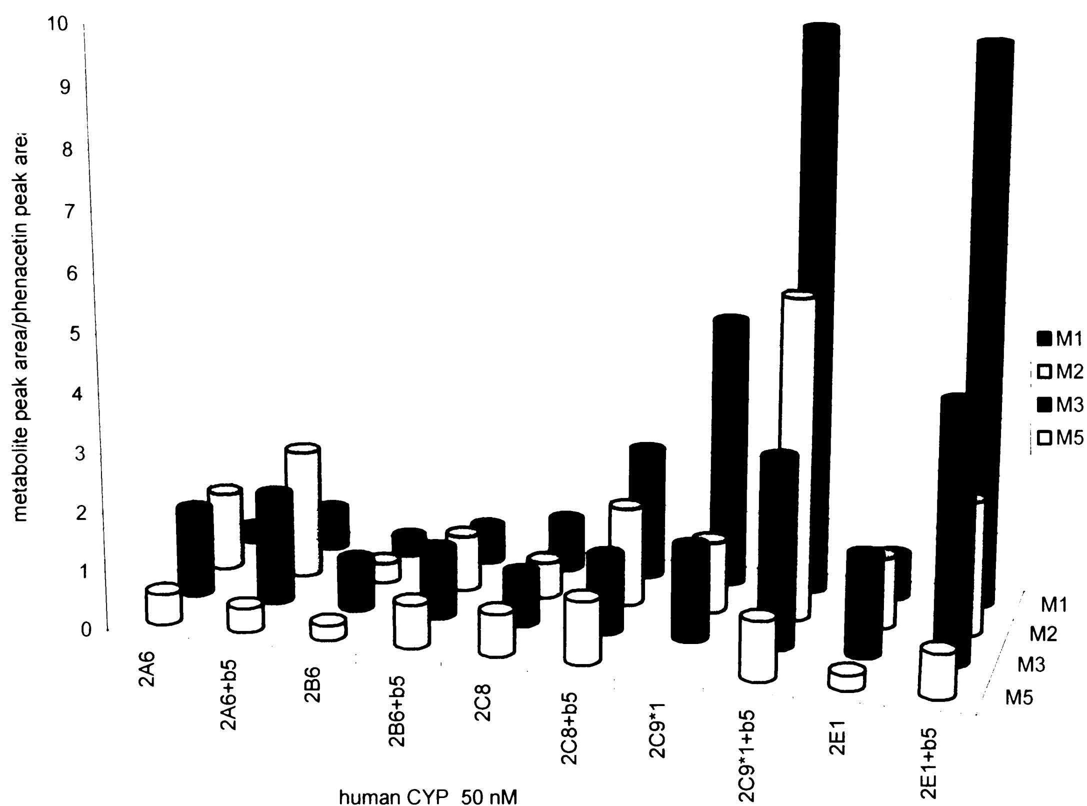

Figure 55. Comparison of the efficiency of individual human CYP isoforms (CYP2A6, 2B6, 2C8, 2C9*1 and 2E1) in ellipticine oxidation in the absence and presence of cytochrome b_5. 50 nM CYP and 10 μM ellipticine were used in all of the experiments. The ratio of CYP to cyt b_5 was 1:3.

5.3 Oxidation of ellipticine by human recombinant flavin-containing monooxygenase 3 (FMO3)

Ellipticine metabolism by human recombinant flavin-containing monooxygenase isozyme (FMO3) in Supersomes™ was also investigated. No ellipticine metabolite formation was detectable by this enzyme.

5.4 Preparation of the standard of 13-hydroxyellipticine

The procedure of preparation of the standard of 13-hydroxyellipticine was optimized as described in Chapter 4.7, p 44.

6 Discussion

Most drugs are biotransformated when enter the human organism. The biotransformation pathways can be either detoxifying, or bioactivating. Likewise, ellipticine is also oxidized by various biotransformation enzymes (*e.g.* cytochromes P450[57], peroxidases[77]) to form more or less pharmacologically efficient metabolites.

The present thesis is focused on ellipticine oxidation by rat and human recombinant cytochromes P450. It is known that some mechanisms of ellipticine's anticancer action are partially specific against tumoral tissues. For example, covalent DNA adduct formation by ellipticine is dependent on its metabolic activation[95]. Therefore, knowledge of ellipticine oxidation by individual biotransformation enzymes is of enormous importance. Although there are considerable inter-individual differences in expression levels of the enzymatic system biotransforming drugs among the human population, an evaluation of the ellipticine's fate in humans can be partially evolved from the studies performed with model organisms. Rats have been found to be suitable animal models for mimicking the ellipticine oxidation in humans[104]. In former studies, liver microsomes with various inducers or inhibitors of individual CYP forms/subfamilies were used to study ellipticine metabolism *in vitro*[104]. CYPs are present in a very complex mixture in such microsomal systems, thus it is difficult to determine contribution of individual CYP forms to the formation of individual ellipticine metabolites.

In the present study, use of Supersomes™ (microsomes isolated from *Baculovirus* transfected insect cells) expressing recombinant CYPs enabled us to study ellipticine oxidation by rat and human individual CYP forms and to compare the orthologous CYP enzymes (Figure 56, p 78, Table 30, p 79) in both organisms. In general, rat CYPs were found to be less efficient in oxidizing ellipticine than the human ones.

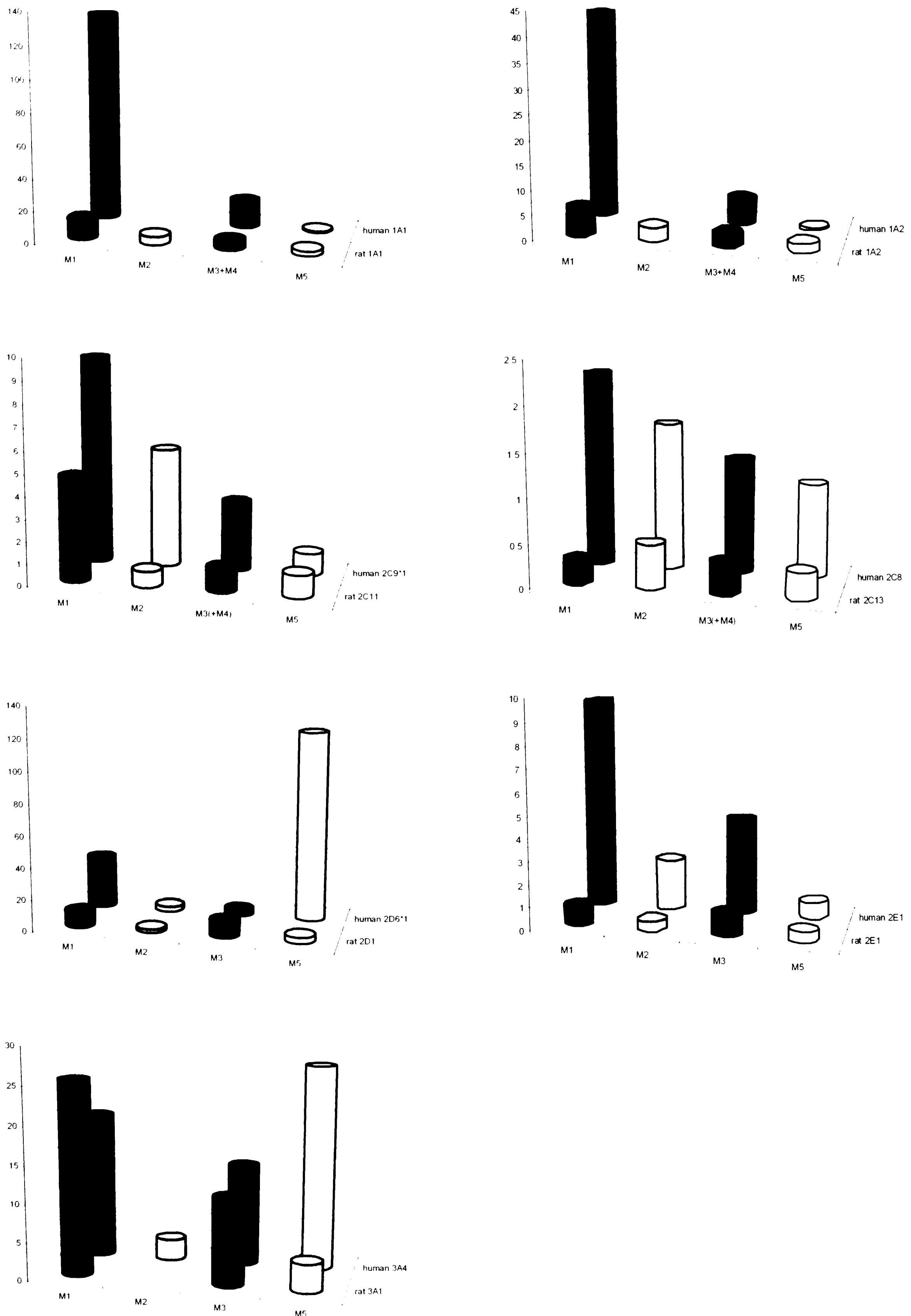

Figure 56. Ellipticine oxidation by orthologous rat and human CYPs. 50 nM CYP, 10 μM ellipticine and cytochrome b_5 in the average ratio of CYP: cyt b_5 1:3 were used in all of the experiments.

Table 30. Sequence homology of selected CYP forms in rats and humans. Modified from [92].

rat	human	amino acid sequence homology [%]	rat	human	amino acid sequence homology [%]
1A1	1A1	78	2C11	2C9	77
1A2	1A2	70	2C13	2C8	68
-	1B1	-	2C12	-	-
2A1	-	-	-	2C19	-
2A2	-	-	2D1	2D6	71
2A3	2A6	-	2D2	-	-
2B1	2B7/11	76/74	2E1	2E1	78
-	2B6	-	3A1	3A3/4	78/73
2C6	2C10	75	3A2	3A5/7	71/-

Cytochromes P450 of a 3A subfamily generated ellipticine metabolites responsible for covalent DNA adduct formation (12-, 13-hydroxyellipticine and ellipticine N^2-oxide) in majority. With respect to the highest abundance of CYP3A4 in human liver (30%)[87], CYP3A4 seems to be the most important CYP form concerning ellipticine genotoxic bioactivation *in vitro*[34, 95, 104]. Indeed, the predominant ellipticine metabolite formed by human hepatic microsomes is 13-hydroxyellipticine followed by ellipticine N^2-oxide[103].

These data are in accordance with the inhibition studies performed with rat and human microsomal systems. In these studies, the specific inhibition of CYP3A enzymes (ketoconazole, troleandomycin) correlated with a very strong decrease in DNA adducts formation in both rats and humans[104].

CYP enzymes of a 1A subfamily generated predominantly the detoxification ellipticine metabolite, 9-hydroxyellipticine. The amount of 9-hydroxyellipticine formed by human CYP1A1/2 was particularly high; it represented more than 80% of all ellipticine metabolites. Moreover, ellipticine oxidation by these human CYP forms to its other metabolites was also very effective. The amounts of especially 13-hydroxyellipticine generated by human CYP1A were also considerably high. Since the abundance of CYP1A2 in human liver is about 13%[87], its contribution to ellipticine-DNA adduct formation should also be considered. However, CYP1A were found to be responsible predominantly for the formation of

9-hydroxyellipticine, and in lower amounts also of 7-hydroxyellipticine, in human liver microsomes[103].

The 2C subfamily of CYPs is expressed in high levels in human liver (20%)[87]. This subfamily consists of 15 CYP forms in humans and 9 CYP forms in rats[130]. Only 3 human and 4 rat enzymes from this CYP subfamily were employed in this study. Rat CYP2C6 catalysed ellipticine oxidation to 9-hydroxyellipticine effectively, followed by human CYP2C19 and rat 2C11. Rat CYP2C13 was inefficient in oxidizing ellipticine as well as its orthologous form – human 2C8, but in both cases activating ellipticine biotransformation pathways (formation of metabolites forming the DNA adducts, *i.e.* 12-, 13-hydroxyellipticine and ellipticine N^2-oxide) predominated. Likewise, rat CYP2C12 generated the detoxification metabolite 9-hydroxyellipticine only in minority.

There were significant differences observed concerning ellipticine oxidation by rat CYP2D1 and its orthologous form, human CYP2D6*1. However, the extreme efficiency of CYP2D6*1 in catalysing ellipticine oxidation to its N^2-oxide might have been caused by the site mutation at [374]Val.

Human CYP2E1 (7% in human liver)[87] was more effective to oxidize ellipticine than its orthologous rat form, especially in catalysing ellipticine oxidation to 9-hydroxyellipticine, which was the major metabolite generated by human CYP2E1.

Human CYP1B1 catalysed the formation of 9-hydroxyellipticine with a similar efficiency as human CYP1A subfamily did.

Although, recombinant enzymes are not absolutely ideal models of catalytic properties of enzymes (because of their expression in a different organism), our study using individual enzyme forms provides a basis for interpreting ellipticine oxidation in complex biological systems *in vivo*.

Effect of cytochrome b_5

Cytochrome b_5 is a protein present in endoplasmic reticulum membranes, which is known to have an ability to influence (stimulate/inhibit) enzymatic activities of some CYP forms. The effect of cytochrome b_5 on ellipticine oxidation by rat CYP1A and 2D subfamilies was

studied. Purified rabbit cytochrome b_5 was added into the reaction mixture. The ellipticine oxidation by rat recombinant CYP1A1/2 was considerably stimulated by the presence of cytochrome b_5. Moreover, the percentage levels of individual ellipticine metabolites significantly changed: ellipticine bioactivation (*i.e.* formation of 12-hydroxyellipticine, 13-hydroxyellipticine and ellipticine N^2-oxide) was stimulated at the expense of detoxification reactions (*i.e.* formation of 9-hydroxyellipticine and 7-hydroxyellipticine). On the contrary, ellipticine biotransformation by rat recombinant CYP enzymes from 2D subfamily was not influenced by the presence of cytochrome b_5.

Yamazaki and co-workers[118, 119] studied the effect of cytochrome b_5 on drug metabolism of human recombinant CYPs expressed in Supersomes™. The drug oxidation activities of some CYP forms increased in the presence of cytochrome b_5: CYP2A6, 2B6, 2C8, 2C19, 2E1, 3A4 and 3A5[118, 119]. On the other hand, some CYPs have not shown effects of cytochrome b_5 concerning oxidation of selected drugs: 1A1 (ethoxyresorufin, 7-ethoxycoumarin), 1A2 (phenacetin, 7-ethoxycoumarin), 1B1 (ethoxyresorufin, 7-ethoxycoumarin) and 2D6 (bufuralol, dextromethorpan)[118, 119].

However, ellipticine oxidation by all human recombinant CYP enzymes employed in the present study was stimulated by the presence of cytochrome b_5. The percentage levels of individual ellipticine metabolites changed in the case of human CYP2E1 where higher portion of 9-hydroxyellipticine formed. On the contrary, human CYP2C9*1 generated larger parts of the ellipticine metabolites responsible for the DNA adduct formation, *i.e.* 12-, 13-hydroxyellipticine and ellipticine N^2-oxide. Proportions of ellipticine metabolites generated by other human recombinant CYPs were affected only slightly or insignificantly.

Mechanism of the effect of cytochrome b_5 on the reactions catalysed by the MFO system has not been fully elucidated yet. There are two hypotheses[118]:

1. Cytochrome b_5 affects the electron transfer between cytochrome P450 and NADPH:CYP reductase; and
2. Cytochrome b_5 interacts with cytochrome P450 and causes conformational changes in the protein molecule that result in a change in its catalytic properties.

Within the present study, there have been observed changes not only in the levels of the ellipticine metabolites, but also in the apportionment of individual metabolites. Hence, this finding supports the second hypothesis.

7 Conclusion

Ellipticine oxidation by individual rat and human recombinant cytochromes P450 in Supersomes™ was investigated in the present study. Reverse-phase high-performance liquid chromatography was used for the separation of ellipticine metabolites. All the CYP enzymes employed in the study (see Table 31) exhibited a potency to oxidize ellipticine to up to five metabolites: 9-, 12-, 13-, 7-hydroxyellipticine and ellipticine N^2-oxide.

Table 31. Rat and human CYP forms employed in the present study

Rat	1A1, 1A2, 2A1, 2A2, 2B1, 2C6, 2C11, 2C12, 2C13, 2D1, 2D2, 2E1, 3A1, 3A2
Human	1A1, 1A2, 1B1, 2A6, 2B6, 2C8, 2C9*1, 2C19, 2D6*1, 2E1, 3A4

Rat recombinant cytochromes P450

The results obtained in the experiments using rat recombinant cytochromes P450 for the ellipticine oxidation could be summarized as follows: 9-hydroxyellipticine was the major metabolite formed by rat recombinant CYPs. Rat CYP2C6, followed by 3A1, 1A1 and 2D1, was the most efficient CYP enzyme generating this ellipticine metabolite. 12-hydroxyellipticine formed predominantly by CYP2A1. Ellipticine oxidation to 13-hydroxyellipticine was most efficiently catalysed by CYP3A1 followed by 2D1 and 2A1/2. The only CYP subfamilies generating low amounts of 7-hydroxyellipticine were CYP1A and 2C. Rat CYP3A2, 3A1, 2D1 and 1A1/2 exhibited the highest potency to oxidize ellipticine to its N^2-oxide.

Cytochrome b_5 stimulated ellipticine oxidation by CYP1A1/2. The percentage levels of individual metabolites changed. The formation of 9-hydroxyellipticine was stimulated only insignificantly, whereas 12-hydroxyelipticine and ellipticine N^2-oxide were generated in much higher amounts in the presence of cytochrome b_5. The portion of 13- and 7-hydroxyellipticine changed on behalf of 13-hydroxyellipticine when cytochrome b_5 was present. CYP2D subfamily was not influenced by the addition of cytochrome b_5 concerning ellipticine oxidation.

Human recombinant cytochromes P450

In the case of ellipticine oxidation by human recombinant cytochromes P450, CYP1A1 and 1B1 exhibited an extreme efficiency to catalyse ellipticine oxidation to 9-hydroxyellipticine. CYP2D6*1 generated very efficiently ellipticine N^2-oxide. 12-hydroxyellipticine was formed in highest amounts by CYP2C19 and 2C9*1. The only CYP subfamilies generating 7-hydroxyellipticine were CYP1A and 2C. CYP3A4 and 1A1 generated large amounts of 13-hydroxyellipticine when cytochrome b_5 was present.

Cytochrome b_5 stimulated ellipticine oxidation by all the human CYP forms employed in the study.

Human FMO3

Human recombinant flavin-containing monooxygenase 3 expressed in Supersomes™ did not generate detectable amounts of ellipticine metabolites.

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