CHARLES UNIVERSITY IN PRAGUE
FACULTY OF SCIENCE, DEPARTMENT OF BIOCHEMISTRY

Modulation of metabolic activation of ellipticine by components of the mixed function monooxygenase system

Summary of PhD Thesis

RNDr. Barbora Mrázová

Supervisor: Prof. RNDr. Marie Stiborová, DrSc.

Praha 2010

INTRODUCTION

Ellipticine (5,11-dimethyl-*6H*-pyrido [4,3-*b*] carbazole, *figure 1*) and its more soluble derivates are alkaloids isolated from *Apocyanaceae* plants, which exhibit significant antitumor activities[15, 21, 24, 31]. Ellipticine was first isolated in 1959 from the leaves of the evergreen tree *Ochrosia elliptica Labil*[6]. Nevertheless, its pharmacological efficiencies (and efficiencies of some of its derivates) was found in 1967, when they were prepared by chemical syntheses[7]. Ellipticine and its more soluble derivates, 9-methoxy-ellipticine and 2-methyl-9-hydroxyellipticine in the form of acetate, have been utilized pharmacologically since 1970s. They are highly efficient against osteolytic breast cancer with metastases, acute myeloblastic leukemia, kidney sarcoma and thyroid carcinoma[1, 17, 18, 19, 23]. Ellipticines exhibit also significant anti-HIV activity because of their ability to inhibit retroviral integrase. This is the reason, why ellipticine is also investigated to be used for AIDS treatment[24, 32].

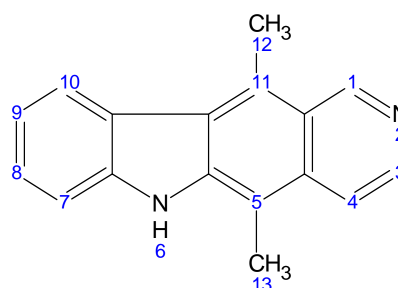

Figure 1 The structure of ellipticine

In organisms, cytochromes P450 oxidize ellipticine up to five metabolites, 9-hydroxyellipticine (M1), 12-hydroxyellipticine (M2), 13-hydroxyellipticine (M3), 7-hydroxyellipticine (M4) and N^2-oxide of ellipticine (M5). These metabolites are formed not only by cytochromes P450 of human liver microsomes, but also by those of microsomes of model organisms (rats and rabbits)[34]. The other enzymes catalyzing the ellipticine oxidation and its activation to more efficient metabolites forming DNA adducts are peroxidases. Two ellipticine metabolites are generated by peroxidases; the major product is the ellipticine dimer, in which the two ellipticine skeletons are connected via

N(6) of the pyrrole ring of one ellipticine molecule and C9 in the second one. The minor metabolite is ellipticine N^2-oxide[27, 29, 30, 37].

The mode of action of ellipticine is considered to be based mainly on DNA intercalation[2, 4, 8, 19] and inhibition of topoisomerase II activity[3, 11, 13, 16, 25]. Ellipticine also cause selective inhibition of p53 protein phosphorylation[26, 41] and oxidative phosphorylation, which leads to the decrease in amounts of ATP in cell and its final damage[33]. It is evident that the explanations of anticancer activity mentioned above are based mainly on nonspecific mechanisms. However, this sharply contrasts with specificity of ellipticines against only several cancer diseases.

A new mechanism for the ellipticine action was described, showing the formation of covalent DNA adducts by ellipticine, after its enzymatic activation with cytochromes P450 and peroxidases. These enzymes generate the pharmacologically more efficient derivates of ellipticine that form these DNA adducts. Deoxyguanosine was determined to be the target deoxynucleoside in DNA, to which the ellipticine reactive metabolites are bound[38]. 13-hydroxyellipticine and 12-hydroxyellipticine (the latter formed also spontaneously from ellipticine N^2 oxide by the Polonowski rearrangement[34, 38]), are responsible for the formation of two major DNA adducts *in vitro*.

The formation of this CYP-mediated covalent DNA adduct by ellipticine was also detected in V-79 Chinese hamster lung fibroblasts transfected with human CYP enzymes[12], in human breast adenocarcinoma MCF-7 cells[5], in human leukemia cells (HL-60 and CCRF-CEM)[30] and in neuroblastoma[28] and glioblastoma[22] cells. Covalent DNA adducts are also generated by ellipticine *in vivo* in some organs (livers, spleen, lungs, kidney, heart, brain) of rats exposed to this anticancer drug[36] and in DNA of rat breast adenocarcinoma[39].

The most effective enzymes activating ellipticine to form DNA adducts are human and rat cytochromes P450 of the 3A and 1A subfamilies[20, 34, 38, 40].

AIM OF THE STUDY

The aim of this study was to extend our knowledge on metabolism of anticancer drug ellipticine and its mechanism of action.

The aims of the present work are as follows:

.

- To study ellipticine metabolism by cytochromes P450 of a 1A subfamily
- To evaluate ellipticine potential to induce the expression of cytochromes P450 1A1/2 in liver, lung and kidney of rats
- To prepare cytochrome b_5 without its hem cofactor (apo-cytochrome b_5)
- To investigate the effect of cytochrome b_5 on ellipticine metabolism by CYP1A1/2
- To investigate the effect of apo-cytochrome b_5 and other proteins with and without heme in its molecule on oxidation of ellipticine by CYP1A1/2
- To examine the effect of cytochrome b_5, its apo-form and other proteins, on ellipticine oxidation by CYP3A4

RESULTS AND DISCUSSION

The results shown in this study extend our knowledge on pharmacological effects of anticancer drug ellipticine and its mechanism of action. The main results found in this work are summarized as follows:

The different molar ratio of NADPH:CYP reductase and CYP1A and the concentration of CYP1A in the reconstituted system correlate with its efficiency to oxidize ellipticine

We investigated the influence of individual compounds of the mixed function oxidase system on ellipticine oxidation catalyzed by CYPs of a 1A subfamily *in vitro*. The results show that efficiencies of CYP1A1/2 enzymes reconstituted with NADPH:CYP reductase to oxidize ellipticine depends on the CYP:reductase ratios in the reconstitution systems (*figure 2*). Furthermore, an increase in the concentration of CYP1A1 and 1A2 in incubations results in an increase in formation of ellipticine metabolites, predominantly in generation of 9-hydroxyellipticine and 7-hydroxyellipticine (*figure 3*). The results reflect the situation of ellipticine oxidation catalyzed by CYP1A1/2 in human, rat and rabbit hepatic microsomes[20, 35].

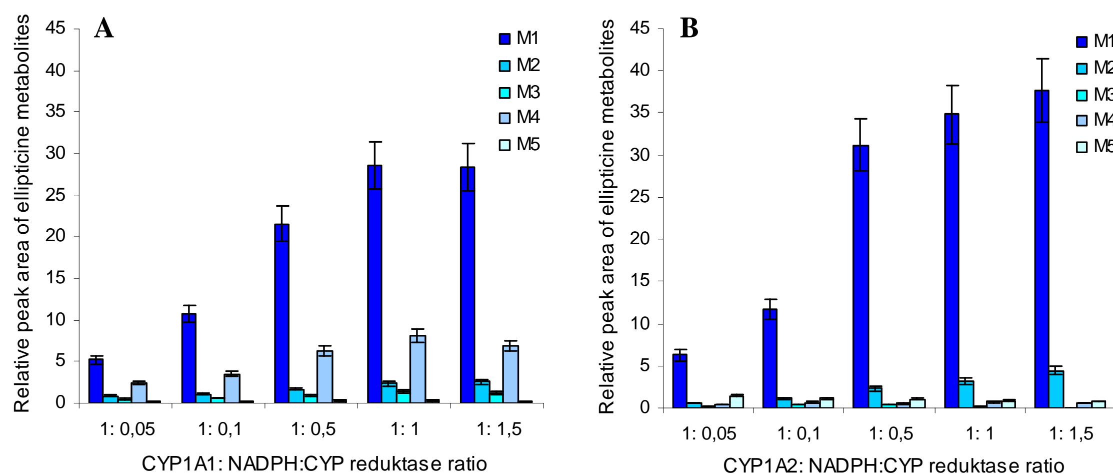

Figure 2 *The effect of NADPH:CYP reduktase on ellipticine oxidation by CYP1A1 (A) and CYP1A2 (B) in reconstituted system. The values are averages and standard deviations of triplicate incubations.*

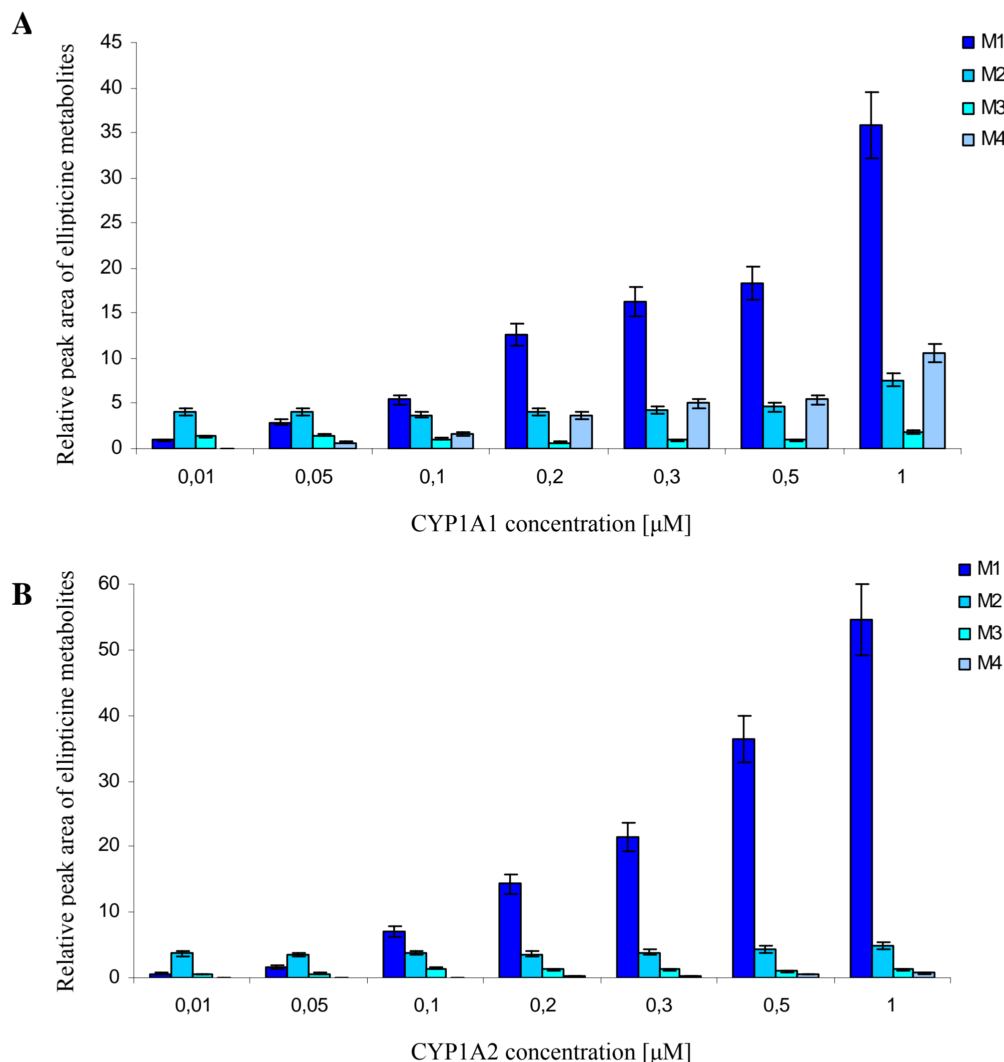

Figure 3 *The effect of the different concentrations of cytochromes P450 1A1 (A) and 1A2 (B) on ellipticine oxidation in reconstituted system. The values are averages and standard deviations of triplicate incubations.*

Ellipticine modulates its own pharmacological and genotoxic potencial by induction of CYP1A1/2

Ellipticine was found to induce expression of CYP1A1 and CYP1A2 enzyme proteins in livers (*figure 4*), lungs and kidneys (*figure 5*) of rats treated i.p. with this drug, thereby increasing their enzymatic activities. The ellipticine-mediated CYP1A induction also increases the DNA adduct formation, which correlates with an increase in production of activation metabolites, responsible for the DNA adduct formation, as found in experiments following ellipticine oxidation by hepatic microsomes isolated from rats treated with 40 mg

ellipticine/kg (*figure 6A*). However, only an increase in oxidation of ellipticine to detoxication products, 9-hydroxy- and 7-hydroxyellipticine, was found in lung and kidney microsomes of rats treated with ellipticine (*figure 6B,C*).

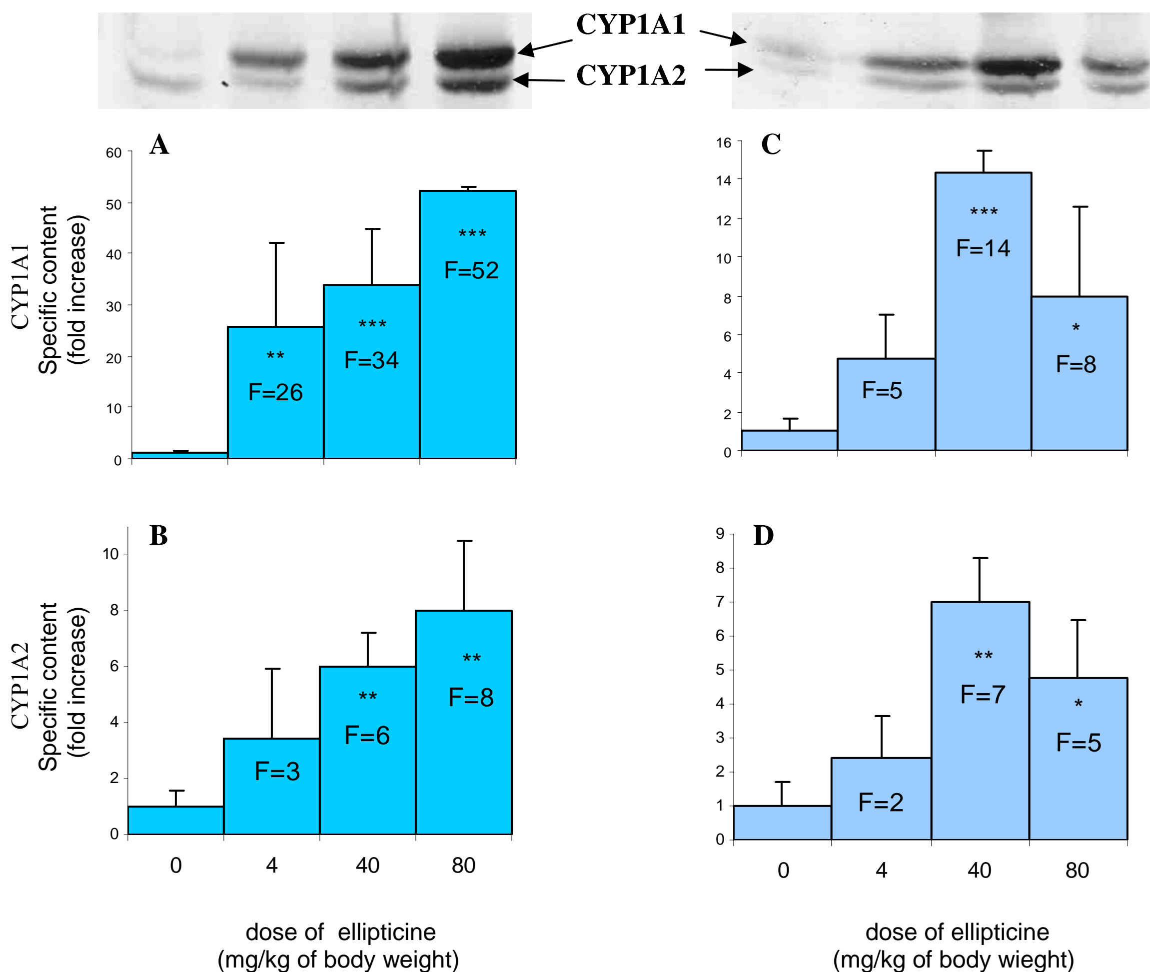

Figure 4 *Induction of CYP1A1 (A, C) and 1A2 (B, D) in livers of male (A, B) and female rats (C, D) untreated and treated with 4, 40 and 80 mg/kg body weight ellipticine.Imunoblots of microsomal CYP1A1/2 are stained with antibody against rat CYP1A1.The values are averages and standard deviations of triplicate incubations, *p<0,05, **p<0,01, ***p<0,001 (Student´s t-test).*

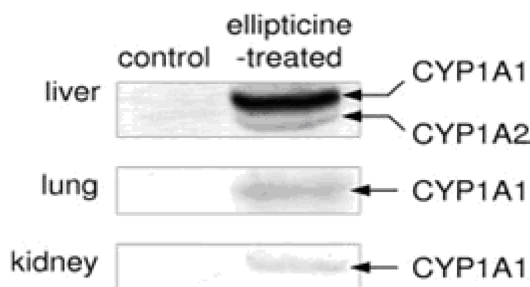

Figure 5 *Imunoblots of microsomal CYP1A1 and 1A2 from livers, lungs and kidneys of untreated and ellipticine-treated (40 mg/kg) male rats stained with antibody against rat CYP1A1.*

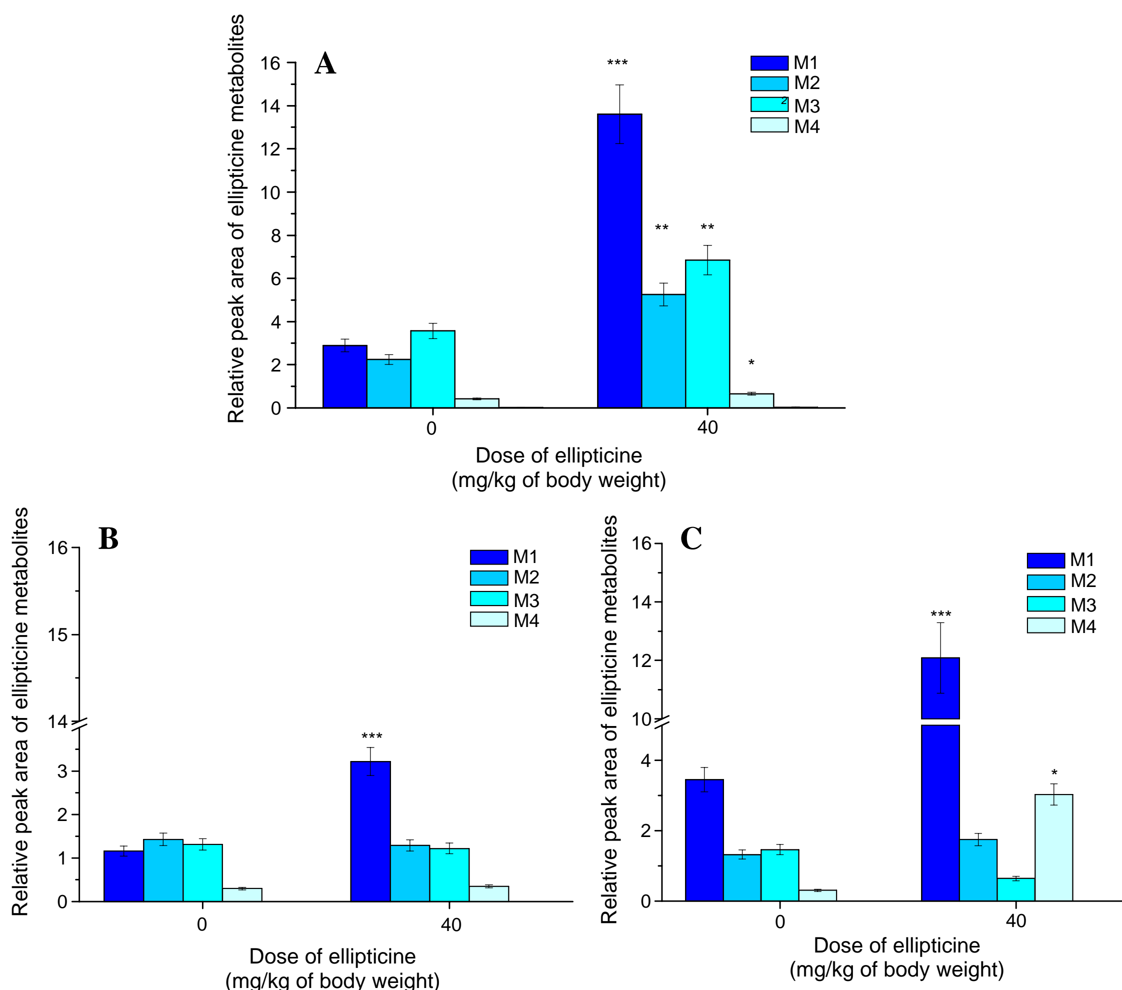

Figure 6 *Ellipticine metabolism in rat liver (A), lung (B) and kidney microsomes (C) of control animals and those treated with 40 mg/kg ellipticine. The values are averages and standard deviations of triplicate incubations, *p<0,05, **p<0,01, ***p<0,001 (Student´s t-test).*

The rabbit apo-cytochrome b_5 (cytochrome b_5 without its heme cofactor) was prepared utilizing bovine myoglobin

A biologically active apo-cytochrome b_5 (without any damages in its protein function) was prepared using transfer of heme from purified rabbit cytochrome b_5 to horse heart apo-myoglobin. This apo-cytochrome b_5, after its reconstitution with hemin, reveals the properties identical to those of the native cytochrome b_5.

Cytochrome b_5 affects ellipticine oxidation by CYP1A1/2 and kinetics of this reaction

Cytochrom b_5 changed not only a simple enhancement of formation of all ellipticine metabolites in ellipticine oxidation by CYP1A1 and 1A2, but a different pattern and amounts of these metabolites were produced. Namely, cytochrome b_5 stimulated generation of activation metabolites of ellipticine (12-hydroxy- and 13-hydroxyellipticine), while detoxication products of its oxidation were decreased (*figure 7*). Moreover, interaction between CYP1A1 and cytochrome b_5 changed the kinetics of ellipticine oxidation from hyperbolic to sigmoidal. On the contrary, no cooperativity was observed using the CYP1A2 reconstitued system.

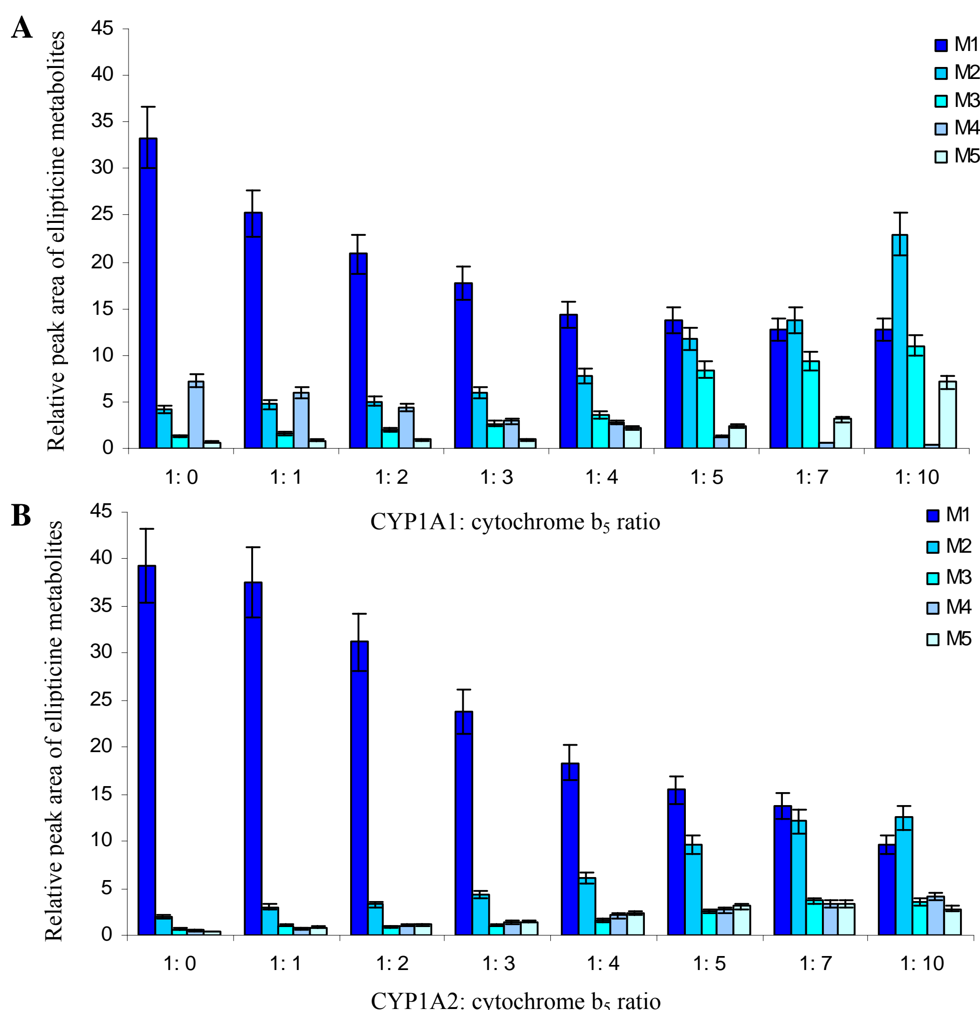

Figure 7 *The effect of cytochrome b_5 on ellipticine oxidation by CYP1A1 (A) and 1A2 (B). CYP1A1/2 and NADPH:CYP reduktase was used in molar ratio 1:1. The values are averages and standard deviations of triplicate incubations.*

Cytochrome b_5, but not its apo-form, changes the ellipticine oxidation by CYP1A1/2

The heme cofactor of cytochrome b_5 is essential for modulation of ellipticine oxidation by CYP1A1/2, which suggests that cytochrome b_5 supports oxidation reactions due to an electron transfer among the enzymes of the CYP-dependent enzymatic system (*figure 8*). No effect of apo-cytochrome b_5 and/or other proteins with or without heme on ellipticine oxidation of CYP1A1/2 was found (*figure 8*). However, because not only a simple enhancement of formation of all ellipticine metabolites, but differences in patterns and amounts of these metabolites, were produced, the effect of cytochrome b_5 on reactions catalyzed by CYP1A/2 probably results also from alteration of the protein conformation of both enzymes.

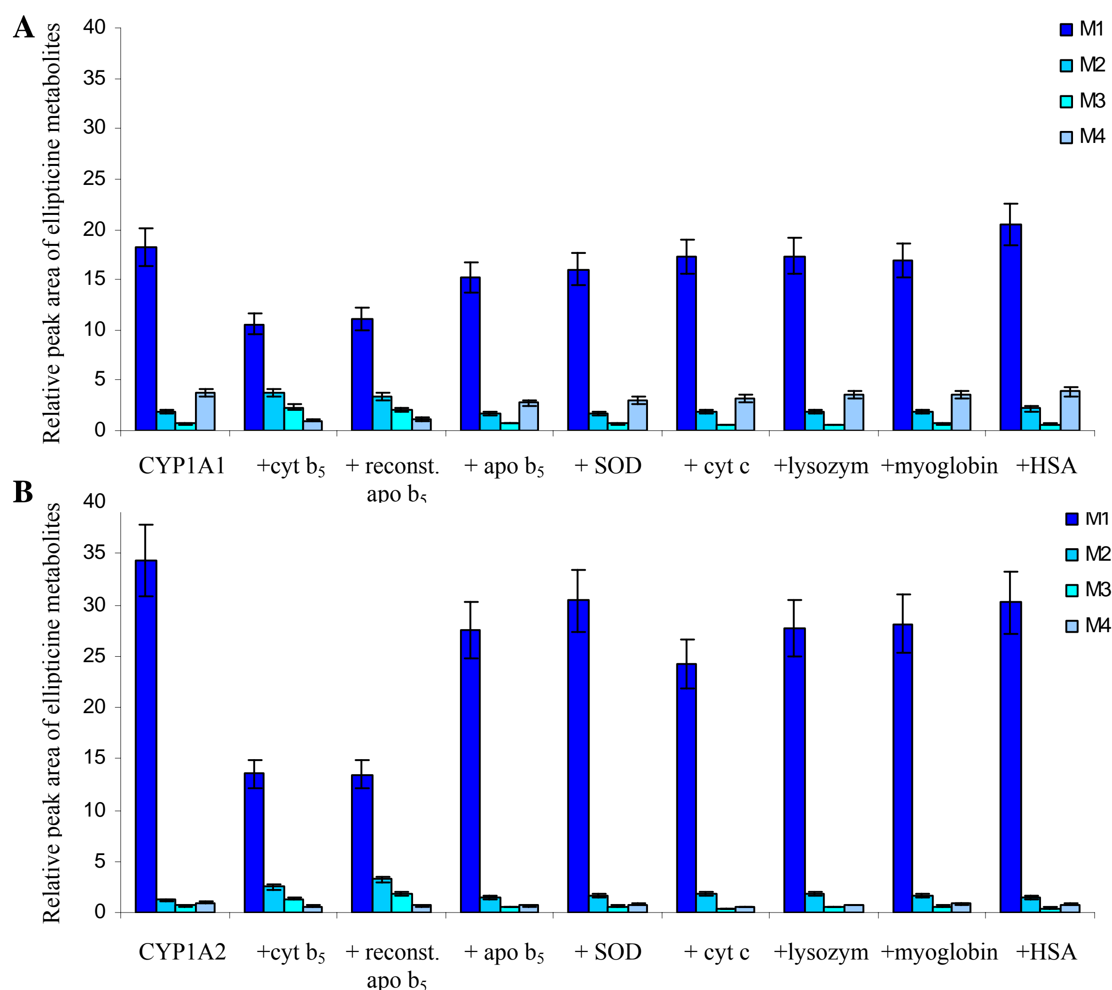

Figure 8 *The effect of cytochrome b_5 ,its apo-form and other compounds on ellipticine oxidation by CYP1A1 (A) and 1A2 (B). The values are averages and standard deviations of triplicate incubations.*

Cytochrome b_5 potentiates production of activation metabolites during ellipticine oxidation by CYP3A4

Human and purified rabbit hepatic cytochrome b_5 are also capable of stimulating oxidation of ellipticine by CYP3A4. Whereas the formation of detoxication product of ellipticine oxidation (9-hydroxyellipticine) is practically not changed by cytochrome b_5, generation of 13-hydroxy- and 12-hydroxyellipticine, the metabolites responsible for formation of DNA adducts, is increased significantly by the cytochrome b_5 protein (*figure 9*). These results correlate with an increase in the ellipticine-DNA adduct formation, mainly with generation of the major adduct 1, which is produced by 13-hydroxyellipticine. Interaction between CYP3A4 and cytochrome b_5 causes an increase in the values of maximal velocities of ellipticine oxidation to all metabolites by CYP3A4, but does not change hyperbolic kinetics of ellipticine oxidation (as it was found in the CYP1A1/2 system). In addition, these findings correspond with binding of ellipticine to the active site of a CYP3A4 model, with its most populated binding mode. The results indicate that the presence of heme in cytochrome b_5 seems to be essential to stimulate CYP3A4-mediated oxidation of ellipticine (*figure 9*), but the allosteric modulation effect of this protein cannot be excluded.

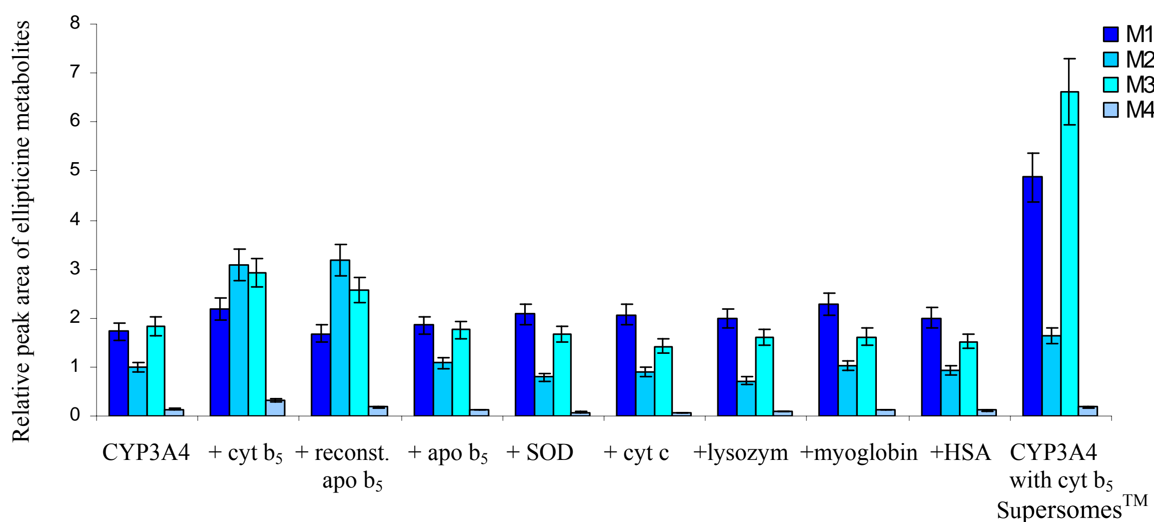

Figure 9 *The effect of cytochrome b_5, its apo-form and other compounds on ellipticine oxidation by human CYP3A4. The values are averages and standard deviations of triplicate incubations.*

The part of results of this PhD. thesis has already been published in scientific journals (see papers 1-4 of List of publications). Two other papers are in preparation (see papers 5 and 6 in List of publications).

The work was supported by the Grant Agency of the Czech Republic (grants 203/06/0329, 305/09/H008 a P301/10/0356), Ministry of Education of the Czech Republic (grants MSM0021620808, 1M0505 a RP MSMT 14/63) and Grant Agency of Charles University (grants 258188 a 1272080).