NEW SELECTIVE INHIBITORS OF CYTOCHROME P450 2B4 AND AN ACTIVATOR OF CYTOCHROME P450 3A6 IN RABBIT LIVER MICROSOMES

Lucie BOŘEK-DOHALSKÁ[1,*], Petr HODEK[2] and Marie STIBOROVÁ[3]

Department of Biochemistry, Faculty of Science, Charles University, Hlavova 2030, 128 43 Prague 2, Czech Republic; e-mail: [1] dohalska@yahoo.com, [2] hodek@prfdec.natur.cuni.cz, [3] stiborov@prfdec.natur.cuni.cz

Received June 22, 1999
Accepted October 20, 1999

We investigated interactions of adamantane, diamantane and their two substituted derivatives, 2-isopropenyl-2-methyladamantane (2-PMADA, **1**) and 3-isopropenyl-3-methyldiamantane (3-PMDIA, **2**), with various isoforms of rabbit cytochrome P450 (CYP). The data of spectroscopic experiments showed that all the substances are bound to the substrate binding site of rabbit CYP2B4 and CYP3A6. **1** and **2** are compounds having higher affinities to these CYP isoforms than adamantane and diamantane. All compounds inhibit CYP2B4 specific enzyme activity (the 7-pentoxyresorufin *O*-depentylase activity). The 50% inhibition of CYP2B4 was due to 3.82, 0.61, 0.66 and 0.37 μM adamantane, diamantane, **1** and **2**, respectively. The products formed during the CYP2B4-mediated metabolism of studied substances are less effective inhibitors than parent compounds. An opposite effect of **1** on CYP3A6 was determined. The specific enzyme activity of CYP3A6 increased to 138% of control when **1** was used in the presence of 40 μM erythromycin as a substrate. Here, we report the finding of a new activator of CYP3A6 having the structure quite different from that of CYP3A6 activators known to date.

Key words: Cytochrome P450; CYP2B4; CYP3A6; Inhibitors; Activators; Adamantane; Diamantane; 2-Isopropenyl-2-methyladamantane; 3-Isopropenyl-3-methyldiamantane; Oxidations.

Cytochrome P450 (CYP) is a family of hemoproteins that play an important role in the oxidative activation and detoxification of numerous drugs, other foreign compounds, and endogenous substances. These enzymes exhibit broad substrate specificity and are subject to differential regulation[1,2].

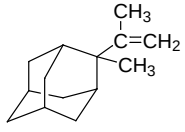
The current view is that the chemistry of the reaction of the activated oxygen atom with the substrate is relatively constant in oxidation reaction catalyzed by CYPs (ref.[3]), and the catalytic specificity of the different CYP enzymes is the result of steric forces imposed by the individual proteins upon the substrates. The knowledge of the forces that govern the catalytic

specificity of CYP enzymes is imperative for an overall understanding of these proteins and, consequently, the many associated biochemical phenomena in pharmacology, toxicology, endocrinology and clinical medicine[4].

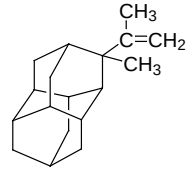
An important goal of current research on CYP is to identify its individual isoforms involved in the metabolism of therapeutically active drugs. One of the approaches for elucidation of the catalytic specificity of CYPs in the microsomal samples is utilization of selective inhibitors. These inhibitors can be classified into four groups: (i) competitive, (ii) noncompetitive, (iii) mechanism-based and (iv) CYP specific inhibitory antibodies[5]. In addition, the studies using specific inhibitors of individual CYPs help to elucidate the mechanism of their action and modulation of their activities *in vivo* and *in vitro*[5]. On the other hand, there is a limited number of compounds which are known to stimulate catalytic activity of some CYPs.

Among the CYPs activated in this way, the enzymes of CYP3A subfamily are probably the most important example[1]. Johnson and co-workers[6] showed that substrates of the rabbit CYP3A6 could act as activators of their own metabolism. Moreover, the importance of CYP3A subfamily enzymes is also underlined by their high efficiency in metabolism of a majority of drugs used in human medicine.

Adamantane (ADA) and diamantane (DIA) are substrates of CYPs having a high affinity to CYP2B1 and/or CYP2B2 (refs[7,8]) while their affinity to CYP1A1/2 isoforms is negligible[7,9]. Thus, they are used as compounds, which specifically inhibit rat liver CYP2B subfamily enzymes[8–10]. However, the knowledge of affinity of these compounds to CYPs of other biological species is rather limited. Therefore, in the present work, we evaluate the affinity of these two substances to microsomal CYPs of another animal species, namely rabbit. Moreover, two substituted derivatives of these



1



2

compounds, 2-isopropenyl-2-methyladamantane (2-PMADA, **1**) and 3-isopropenyl-3-methyldiamantane (3-PMDIA, **2**) are also studied from the point of view of their interaction with CYPs present in rabbit liver microsomes.

EXPERIMENTAL

Chemicals

7-Pentoxy- and 7-ethoxyresorufin were obtained from Fluka Chemie AG (Switzerland). Erythromycin, β-naphthoflavone (2-phenylbenzo[*f*]chroman-4-one), rifampicin, $NADP^+$, NADPH and D-glucose 6-phosphate dehydrogenase were purchased from Sigma Chemical Co. (St. Louis, MO). Phenobarbital was from Kulich Co. (Hradec Králové, Czech Republic) and D-glucose 6-phosphate from Reanal (Budapest, Hungary). All chemicals were reagent grade or better. 2-PMADA and 3-PMDIA were synthesized according to Olah and collaborators[11]. The final products were analyzed by HPLC, mass and NMR spectra.

Animals and Pretreatment

Adult male rabbits (2.5–3.0 kg, VELAZ, Czech Republic) were fed *ad libitum* on pellet chow and water one week before treatment. Then, rabbits were pretreated with β-naphthoflavone (80 mg/kg in olive oil i.p. for 3 days), phenobarbital (0.1% in drinking water for 6 days) and rifampicin (50 mg/kg in 40 mM NaOH i.p. for 3 days) to induce individual liver CYP enzymes. Control microsomes were prepared from untreated rabbits.

Isolation and Characterization of Microsomes

Microsomes were prepared according to Gut and co-workers[12] and stored in aliquots in liquid nitrogen until use. Protein concentrations in samples were determined as described by Wiechelman and collaborators[13] with bovine serum albumin as a standard. Total CYP content was measured based on complex of reduced CYP with CO (ref.[14]). Microsomes were characterized by Western blot according to Gan and co-workers[15] with minor modifications: blots were incubated in 3% blocking solution of skim milk powder with 0.3% Triton X100 instead of 10% blocking solution of skim milk powder without the detergent. The incubation mixtures contained the polyclonal chicken antibodies (10 μg/ml) prepared according to Polson and collaborators[16] against rabbit CYP2B4, CYP2E1 and CYP3A6.

Spectral Measurements

The binding of adamantane, diamantane, 2-PMADA and 3-PMDIA to CYP in microsomes prepared from livers of rabbits treated with different inducers was monitored by difference spectroscopy (Specord M-40, Zeiss, Jena, Germany)[17] using cuvettes of 1 cm optical path. The concentration of microsomal protein was adjusted to 1.5 mg per ml of 0.1 M potassium phosphate buffer, pH 7.4. The tested diamantoid compounds (dissolved in methanol) were directly added to the sample cuvette containing microsomes. The same volume of the solvent was added to the reference cuvette. Absorption spectra were recorded at ambient temperature between 350 and 500 nm. When shifts of minima or maxima of absorbance were observed, ΔA between the actual minima and maxima were determined. Spectral dissociation constants (K_s) were calculated using double-reciprocal plots of the absorbance difference ΔA *versus* substrate concentrations[18].

Enzyme Assays

CYP enzyme activities were measured using selective substrates in microsomes from rabbits pretreated with different inducers. 7-Ethoxyresorufin *O*-deethylase (CYP1A1/2 activity) with 1.75 μM substrate and 7-pentoxyresorufin *O*-depentylase (CYP2B4 activity) with 5 μM substrate were assayed with 0.25 mM NADPH and 0.1 μM CYP in 0.1 M potassium phosphate buffer, pH 7.4, for 10 min[19]. Erythromycin *N*-demethylation (CYP3A6 activity) was determined with 40 μM substrate, NADPH-generating system (10 mM $MgCl_2$, 10 mM D-glucose 6-phosphate, 1 mM NADPH, 1 U D-glucose 6-phosphate dehydrogenase per ml) and 2 μM CYP in 0.1 M potassium phosphate buffer, pH 7.4, for 15 min. The reaction was terminated by addition of 0.5 ml of 12.5% trichloroacetic acid. The amount of formaldehyde formed was measured as described by Nash[20]. *O*-Dealkylation of alkoxyresorufins and *N*-demethylation of erythromycin were carried out in total volume of 0.5 ml and 1 ml, at 37 °C in a shaking incubator, with and without addition of diamantoid compounds, respectively. Microsomes were preincubated (10 min at 37 °C) with different concentrations of these compounds in the presence or absence of NADPH and NADPH-generating system.

RESULTS

The interaction of adamantane, diamantane and their substituted derivatives, 2-PMADA and 3-PMDIA, with various isoforms of CYP was investigated using difference spectroscopy.

The data showed that the above-mentioned compounds are typical CYP type I substrates for rabbit liver microsomes of uninduced animals and those of animals induced by PB (inducer of CYP2B4) and RIF (inducer of CYP3A6). Data of spectroscopic experiments with adamantane, diamantane, 2-PMADA and 3-PMDIA are shown in Table I. A comparison of the apparent spectral dissociation constants, K_s, shows that K_s values of 2-PMADA and 3-PMDIA are significantly lower than those for adamantane and diamantane obtained with PB and RIF microsomes. CYPs present in microsomes isolated from livers of uninduced rabbits interact only with 2-PMADA and 3-PMDIA. No interaction except for adamantane (showing high K_s), was obtained with microsomes isolated from rabbit livers treated with β-naphthoflavone (inducer of CYP1A1/2).

In some cases, two values of K_s were determined for 2-PMADA or 3-PMDIA with individual microsomes (Table I). This might be caused by interaction of the substances with two different isoforms of CYP present in microsomes. Indeed, liver microsomes of uninduced rabbits contain several CYP isoforms including, for example: CYP1A1/2, CYP2B4, CYP2C, CYP2E1 and CYP3A6 (ref.[21]). Moreover, although PB microsomes of rabbit livers contain high amounts of CYP2B4, low levels of other CYP isoforms (*i.e.* CYP3A6, CYP2E1) are also present. Likewise, rabbit RIF microsomes contain not only CYP3A6, but other CYP isoforms such as CYP2B4 and CYP2E1,

were also detected in this microsomal system as follows from Western blots analyses (results not shown). Furthermore, other factors which might cause the multiplicity of K_s values (such as binding of compounds to different binding sites of the enzyme protein molecule)[22] cannot be excluded.

The K_s values indicate that 2-PMADA and 3-PMDIA are compounds having higher affinities to CYPs than adamantane and diamantane. Hence, they might be considered strong inhibitors of these CYPs. To evaluate their inhibitory efficiencies with respect to individual CYP isoforms, the effect of these compounds on specific reactions catalyzed by individual CYPs was studied.

Adamantane and diamantane inhibited the CYP2B4 specific enzyme activity (the 7-pentoxyresorufin *O*-depentylase activity) in PB microsomes (Fig. 1a). Diamantane exhibits stronger inhibition than adamantane as revealed by a comparison of IC_{50} values, 3.82 μmol/l for adamantane *versus* 0.61 μmol/l for diamantane, determined under the experimental conditions used (in the presence of 5 μM 7-pentoxyresorufin as a substrate). Moreover, the two derivatives of these inhibitors exerted a more significant effect on the CYP2B4 activity than adamantane and diamantane (Fig. 1); 50% inhibition (IC_{50}) was due to 0.66 μM 2-PMADA and 0.37 μM 3-PMDIA. In contrast to the inhibition of CYP2B4, no inhibitory effect of adamantane, diamantane, 2-PMADA and 3-PMDIA on CYP1A1/2 was found. The specific enzyme activity of CYP1A1/2 (7-ethoxyresorufin *O*-deethylation) was not

TABLE I
Spectral parameters of cytochrome P450 interaction with adamantane, diamantane, 2-PMADA and 3-PMDIA

Compound	microsomes induced by			
	PB	RIF	β-NF	Control
	K_s, μmol/l			
ADA	9.75	7.00	636	[a]
2-PMADA	2.24	2.00 5.60	[a]	2.18 12.20
DIA	0.83	0.84	[a]	[a]
3-PMDIA	0.44 1.31	0.50 1.21	[a]	1.21 15.43

[a] Not detectable.

affected by any of the tested compounds even when a 10-fold molar excess of these substances over the specific substrate was used.

Previous studies showed that conversion of inhibitors, which is catalyzed by CYPs, may influence the inhibitory effect[23,24]. Therefore, in the present study, adamantane, diamantane, 2-PMADA and 3-PMDIA were preincubated with individual microsomal samples, NADPH and O_2 prior to addition of the substrate. The inhibition efficiency of the compounds decreased after their preincubation with the enzyme system (Fig. 1b). The products formed during the CYP2B4-mediated metabolism seem to be less effective

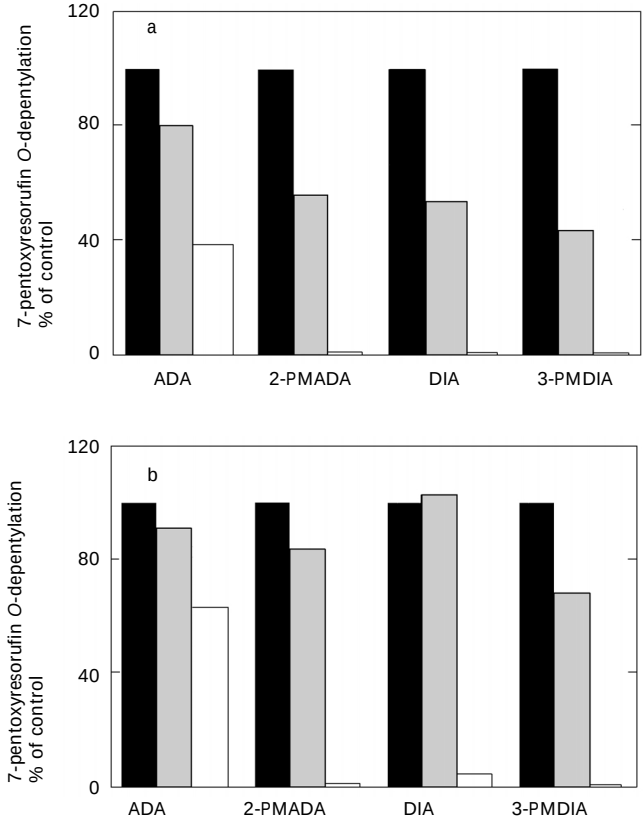


FIG. 1

Effects of adamantane, diamantane, 2-PMADA and 3-PMDIA on 7-pentoxyresorufin (5 μmol/l) *O*-depentylation in PB microsomes preincubated in the absence (a) or presence (b) of NADPH. Control activity in the absence of compounds was 42.5 pmol resorufin/min nmol CYP. The concentration of each chemical was 0 (■), 0.5 (▩) and 5 (❑) μmol/l. Data are averages from duplicate experiments, which differed by less than 15%

inhibitors than the parent substances. In accordance to our previous results, no inhibitory effect of the compounds on the CYP1A1/2 enzyme activities was found after the preincubation of inhibitors with the microsomal system containing CYP1A1/2.

A unique effect of 2-PMADA on CYP3A6 present in RIF microsomes was found. Under the used conditions, the concentration-dependent stimulation of the CYP3A6 enzyme activity was determined (Fig. 2). The activity of CYP3A6 increased to 138% of control when 2-PMADA (140 μmol/l) was

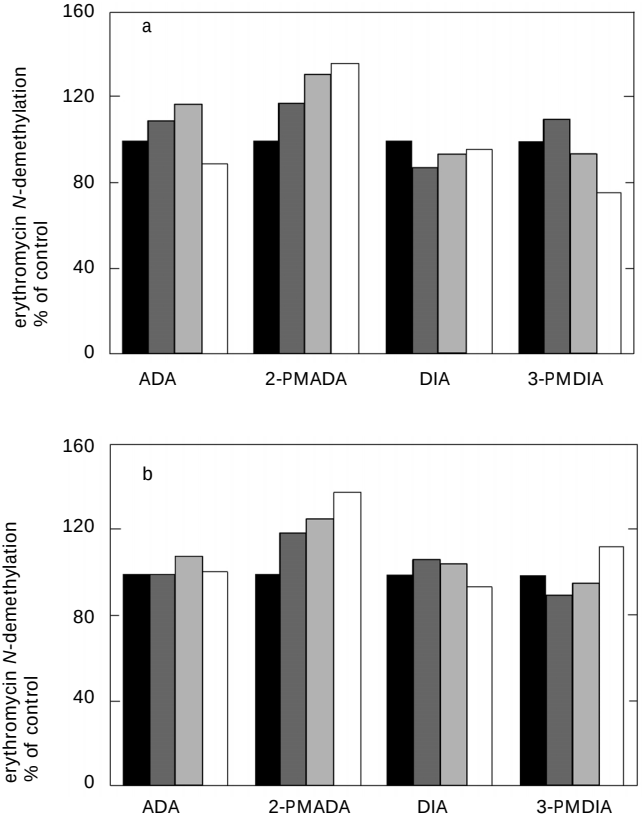


FIG. 2

Effects of adamantane, diamantane, 2-PMADA and 3-PMDIA on erythromycin (40 μmol/l) *N*-demethylation in RIF microsomes preincubated in the absence (a) or presence (b) of NADPH-generating system. Control activity in the absence of compounds was 1.03 nmol HCHO/min nmol CYP. The concentration of adamantane, diamantane, 2-PMADA and 3-PMDIA was 0 (■), 4 (▩), 40 (▨) and 140 (❑) μmol/l. Data are averages from duplicate experiments, which differed by less than 15%

used in the presence of 40 μM erythromycin as the substrate. Adamantane, diamantane and 3-PMDIA did not affect the CYP3A6 erythromycin *N*-demethylase activity significantly (Fig. 2a). No influence on stimulation of the CYP3A6 activity was observed when the diamantoid substances were preincubated with RIF microsomes, NADPH and O_2 prior to addition of erythromycin (Fig. 2b).

DISCUSSION

The present work clearly shows that adamantane and diamantane have high affinities not only to rat liver CYP2B1/2, but also to the rabbit orthologues CYP2B4. Diamantane exhibits a stronger interaction with CYP2B4 than adamantane, similarly to the rat CYP2B1/2 (refs[8,9]). Therefore, these diamantoid compounds might be successfully used as selective inhibitors also for rabbit CYP2B4. Interestingly, the derivatives of adamantane and diamantane, containing the isopropenyl group in their molecules, have higher affinities to CYP2B4 than the parent compounds. This conclusion was confirmed by testing their inhibitory effect on the specific CYP2B4 enzyme activity.

Vinylic and acetylenic analogues of CYP substrates have long been recognized as mechanism-based inhibitors of CYPs (refs[1,25–30]). No data are, however, available about the inhibitory effects of isopropenylic analogues of the substrates on CYPs. Thus, we studied, whether the reactive isopropenylic group present in diamantoid compounds, might exhibit such effects on CYPs as certain vinylic and acetylenic compounds. No evidence for mechanism-based inactivation of CYP2B4 was seen with the isopropenylmethyl derivatives of adamantane and diamantane. On the contrary, preincubation of these compounds with microsomes, NADPH and O_2 prior to addition of the enzyme substrate (7-pentoxyresorufin) led to a decrease in the inhibitory effect. This probably results from the fact that products of the inhibitor conversion are less efficient CYP2B4 inhibitors.

Recently, Strobel *et al.*[31] described the effect of another derivative of adamantane on the CYP2B4 and CYP2B5 enzymes. Similarly to our results, the 1-adamantyl propargyl ether also acts as a strong reversible inhibitor of CYP2B4, binding tightly in the active center. It was not reported as a mechanism-based inhibitor of CYP2B4. Therefore, even propargyl derivative of adamantane does not behave as a suicide substrate of the CYP2B4 isoform. However, this compound causes mechanism-based inactivation of CYP2B5, which differs from CYP2B4 by only 12 amino acid residues[31]. The molecu-

lar basis for the susceptibility of both CYPs to inactivation was assessed in the work cited above[31].

As already mentioned, the characteristic of CYP3A subfamily enzymes is their ability to be activated by certain compounds. α-Naphthoflavone is the well known specific effector of human CYP3A4 and rabbit CYP3A6 which stimulates some reactions but not others[1,4,32,33]. Several substances with partial or modified structures of α-napthoflavone also activate CYP3A4 (ref.[33]). The mechanism of activation by α-naphthoflavone and other flavones is not yet fully understood. One explanation for the stimulation by flavones is the enhanced affinity of CYP3A4 for NADPH: CYP reductase[34]. Another mechanism proposed to explain both cooperativity and flavone stimulation is increased substrate affinity due to the presence of the effector at a distinct site[32,33] and involves at least some aspects of an allosteric model. Binding of an activator affects the conformation of the active site and thus stimulates the catalytic activity. Ueng *et al.*[35] have shown the stimulation of 8,9-epoxidation and inhibition of 3α-hydroxylation of a CYP3A4 substrate, aflatoxin B_1. Their results support a model in which an allosteric site is involved, although the proximity of this putative site to the catalytic site cannot be ascertained yet. On the other hand, Shou *et al.*[33] proposed that the stimulation of CYP3A4-catalyzed benzo(*a*)pyrene oxidation by α-naphthoflavone is related to co-occupancy of a single, large binding site by the substrate and α-naphthoflavone, as opposed to an allosteric mechanism. More recently, Korzekwa and co-workers[22] supported this mechanism. They demonstrated that CYP3A4 (and even other CYP isoforms, namely, CYP2B6, 2C8, 2C9 and 3A5) may exhibit atypical enzyme kinetics depending on the substrate(s) employed and that these results may be explained by a model which includes simultaneous binding of two substrate molecules in the active center. As the CYP3A4 enzyme possesses a rather flexible protein molecule and hence the conformation of its active center might be dramatically changed upon binding of ligands (substrates, inhibitors or activators)[36,37], the latter explanation of stimulation mechanism is more likely. However, to confirm such assumption, additional activators having structure different from that of α-naphthoflavone and its derivatives should be examined.

Here, we show for the first time the CYP3A6 activator, 2-PMADA, which is structurally quite different from α-naphthoflavone. A detailed study of the mechanism of CYP3A6 (and/or CYP3A4) activation by this compound is under way.

The study was supported by grants of the Ministry of Education of the Czech Republic (VS96141), the Grant Agency of the Czech Republic (203/99/1628 and 303/99/0893) and the Grant Agency of the Charles University (245/1999).

REFERENCES

1. Guengerich F. P.: *Chem. Res. Toxicol.* **1991**, *4*, 391.
2. Gonzales F. J., Gelboin H. V.: *Environ. Health Perspect.* **1992**, *98*, 85.
3. Guengerich F. P., MacDonald T. L.: *Acc. Chem. Res.* **1984**, *17*, 9.
4. Brian W. R., Sari M. A., Iwasaki M., Shimada T., Kaminsky L. S., Guengerich F. P.: *Biochemistry* **1990**, *29*, 11280.
5. Guengerich F. P.: *Cancer Res.* **1988**, *48*, 2946.
6. Johnson E. F., Schwab G. E., Vickery L. E.: *J. Biol. Chem.* **1988**, *263*, 17672.
7. Hodek P., Janščák P., Anzenbacher P., Burkhard J., Janků J., Vodička L.: *Xenobiotica* **1988**, *18*, 1109.
8. Stiborová M., Hansíková H., Frei E.: *Gen. Physiol. Biophys.* **1996**, *15*, 211.
9. Hodek P., Burkhard J., Janků J.: *Gen. Physiol. Biophys.* **1995**, *14*, 225.
10. Stiborová M., Schmeiser H. H., Wiessler M., Frei E.: *Cancer Lett.* **1999**, *138*, 61.
11. Olah G. A., Wu A., Farooq O., Prakash G. K. S.: *J. Org. Chem.* **1990**, *55*, 1792.
12. Gut I., Nedelcheva V., Souček P., Stopka V., Gelboin H. V., Ingelman-Sundberg M.: *Arch. Toxicol.* **1996**, *71*, 45.
13. Wiechelman K. J., Braun R. D., Fitzpatrick J. D.: *Anal. Biochem.* **1988**, *175*, 231.
14. Omura T., Sato R.: *J. Biol. Chem.* **1964**, *239*, 2370.
15. Gan Z., Marquardt R. R., Abramson D., Clear R. M.: *J. Food Microbiol.* **1997**, *38*, 191.
16. Polson A., von Wechmar B. H., van Regenmortel M. H. V.: *Immunol. Commun.* **1980**, *9*, 475.
17. Jefcoate C. R.: *Methods Enzymol.* **1978**, *52*, 258.
18. Schenkman J. B., Remmer H., Estabrook R. W.: *Mol. Pharmacol.* **1967**, *3*, 113.
19. Lubet R. A., Mayer R. T., Cameron J. W., Nims R. W., Burke M. D., Wolff T., Guengerich F. P.: *Arch. Biochem. Biophys.* **1985**, *238*, 43.
20. Nash T.: *J. Biochem.* **1953**, *55*, 416.
21. Lu A. Y. H., West S. B.: *Pharmacol. Rev.* **1980**, *31*, 277.
22. Korzekwa K. R., Krishnamachary N., Shou M., Ogai A., Parise R. A., Rettie A. E., Gonzales F. J., Tracy T. S.: *Biochemistry* **1998**, *37*, 4137.
23. Shimada T., Yamazaki H., Foroozesh M., Hopkins N. E., Alworth W. L., Guengerich F. P.: *Chem. Res. Toxicol.* **1998**, *11*, 1048.
24. Kent U. M., Roberts E. S., Chun J., Hodge K., Juncaj J., Hollenberg P. F.: *Chem. Res. Toxicol.* **1998**, *11*, 1154.
25. Ortiz de Montellano P. R., Kunze K. L.: *J. Biol. Chem.* **1980**, *255*, 5578.
26. Gan L. S. L., Acebo A. L., Alworth W. L.: *Biochemistry* **1984**, *23*, 3827.
27. Komives E. A., Ortiz de Montellano P. R.: *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *76*, 746.
28. CaJacob C. A., Chan. W. K., Shephard E., Ortiz de Montellano P. R.: *J. Biol. Chem.* **1988**, *263*, 18640.
29. Hopkins N. E., Foroozesh M. K., Alworth W. L.: *Biochem. Pharmacol.* **1992**, *44*, 787.
30. Roberts E. S., Ballou D. P., Hopkins N. E., Alworth W. L., Hollenberg P. F.: *Arch. Biochem. Biophys.* **1995**, *323*, 303.

31. Strobel S. M., Szklarz G. D., Qun He Y., Foroozesh M., Alworth W. L., Roberts E. S., Hollenberg P. F., Halpert J. R.: *J. Pharmacol. Exp. Therapeut.* **1999**, *290*, 445.
32. Schwab G. E., Raucy J. L., Johnson E. F.: *Mol. Pharmacol.* **1988**, *33*, 493.
33. Shou M., Grogan J., Mancewicz J. A., Krausz K. W., Gonzalez F. J., Gelboin H. V., Korzekwa K. R.: *Biochemistry* **1994**, *33*, 6450.
34. Huang M. T., Johnson E. F., Muller-Eberhard U., Koop D. R., Coon M. J., Connez A. H.: *J. Biol. Chem.* **1981**, *256*, 10897.
35. Ueng Y.-F., Kuwabara T., Chun Y.-J., Guengerich F. P.: *Biochemistry* **1997**, *36*, 370.
36. Lewis D. F., Eddershaw P. J., Goldfarb P. S., Tarbit M. H.: *Xenobiotica* **1996**, *10*, 1067.
37. Anzenbacher P.: *Abstr. 20th Xenobiochemistry Symposium, Smolenice, Slovakia, 20–21 May 1999*, p. 10. Vydavateľstvo STU, Bratislava 1999.