CYTOCHROME P450 3A ACTIVITIES AND THEIR MODULATION BY α-NAPHTHOFLAVONE *in vitro* ARE DICTATED BY THE EFFICIENCIES OF MODEL EXPERIMENTAL SYSTEMS

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The knowledge on efficiencies of different *in vitro* systems containing cytochromes P450 (CYP) of a 3A subfamily is crucial to screen potential substrates of these CYPs. We evaluated and compared efficiencies of several *in vitro* CYP3A enzymatic systems to oxidize the model substrates, α-NF and testosterone, under the standardized experimental conditions. Five CYP3A systems were tested: (i) human hepatic microsomes rich in CYP3A4, (ii) hepatic microsomes of rabbits treated with a CYP3A6 inducer, rifampicine, (iii) microsomes of Baculovirus transfected insect cells containing recombinant human CYP3A4 and NADPH:CYP reductase with or without cytochrome b_5 (Supersomes™), (iv) membranes isolated from *Escherichia coli*, containing recombinant human CYP3A4, NADPH:CYP reductase and cytochrome b_5, and (v) human CYP3A4 or rabbit CYP3A6 reconstituted with NADPH:CYP reductase with or without cytochrome b_5 in liposomes. All systems oxidize testosterone to its 6β-hydroxylated metabolite and α-NF to trans-7,8-dihydrodiol and 5,6-epoxide. The most efficient systems oxidizing both compounds were CYP3A4-Supersomes™ containing cytochrome b_5, followed by human hepatic microsomes. This finding suggests these systems to be suitable for general evaluating a variety of compounds as potential substrates of CYP3A4. The lowest efficiencies to oxidize α-NF and testosterone were found for CYP3A4 expressed in membranes of *E. coli*, and for reconstituted CYP3A4 or CYP3A6. Utilizing the tested enzymatic systems, we also explain here the discrepancies, which showed previously the controversial effects of α-NF on CYP3A-mediated reactions. We demonstrate that inhibition or stimulation of the CYP3A-mediated testosterone hydroxylation by α-NF is dictated by efficiencies of individual enzymatic systems to oxidize the CYP3A substrates.

Keywords: Cytochrome P450 3A; α-Naphthoflavone; Testosterone; Metabolism; Cooperativity; Enzymatic oxidation.

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The cytochrome P450s (CYP)+ are a family of hemoprotein enzymes that play important roles in the metabolism of drugs and carcinogens, as well as endogenous compounds such as prostaglandins, fatty acids and steroids[1,2].

CYP3A is one of the major subfamilies expressed in human livers and is found at high levels in the intestinal tract[3]. This enzyme oxidizes endogenous and exogeneous compounds as well as more than half of the drugs in therapeutic use[3]. The CYP3A subfamily expressed in human livers consists of CYP3A4 [4], CYP3A5 [5,6], and CYP3A7 [5,7]. The CYP3A4 enzyme is the most abundant form of CYP3A (~30% of total CYP) expressed in adult human livers[8]. CYP3A enzymes are induced by rifampicine (RIF) in the human (CYP3A4/5) and rabbit (CYP3A6), but not in rat (CYP3A1/2)[9]. The CYP3A4 enzyme exhibits homotropic cooperativity (non-Michaelis–Menten kinetics) with a number of substrates[10–14]. The enzyme also provides important examples of heterotropic activation characterized by increased oxidation of one substrate in the presence of an effector. One of the most studied modulators/effectors of CYP3A4 is α-naphthoflavone (α-NF) that may, depending on reaction conditions, also serve as a substrate or an inhibitor of this and other CYPs [15–24]. During the past decade understanding of the mechanism of CYP3A4 cooperativity has progressed from a static model with multiple binding sites[13,14,16,20] to a more complex dynamic model suggesting effector-induced conformational rearrangements of the enzyme along with multiple ligand binding[26,27].

The activity of CYP3A4 and its cooperativity is also frequently influenced by the levels of the redox partners, such as cytochrome b_5, relative to the CYP [13,28]. Indeed, the role of cytochrome b_5 in catalytic function of CYP has great importance[29–34]. Several hypotheses trying to explain influence of cytochrome b_5 on CYP reactions have been proposed. The role of this protein as a source of electrons for CYP3A is well known[29–31], but increasing evidence points to allosteric effects of cytochrome b_5, mediated in part by an effect on the CYP spin state[32,33]. Modulatory effects of cytochrome b_5 are further supported by findings that this protein both increases activity of several CYPs and also inhibits their activities in some cases[5,33]. In addition, interactions of CYP with cytochrome b_5 may affect a degree of oligomerization of this enzyme in membranes[34]. The CYP3A4 activity could also be stimulated by divalent cations such as Mg^{2+}, Ca^{2+} or Sr^{2+}. These cations

+ *Abbreviations*: CYP, cytochrome P450; α-NF, α-naphthoflavone; NADPH, reduced form of nicotinamide adenine dinucleotide phosphate; n, Hill coefficient; K_s, dissociation constant; RIF, rifampicine.

could stimulate electron transfer from NADPH:CYP reductase to several acceptors[35].

Because of importance of CYP3A enzymes in metabolism of xenobiotics and endogenous compounds, understanding whether these enzymes are involved in metabolic activation and/or detoxication of additional compounds having pharmacological and/or toxic relevance is important in the assessment of the susceptibility of an individual to these substances. Therefore, investigation which of several *in vitro* experimental models are appropriate to mimic metabolism of xenobiotics in organisms is the major challenge for many laboratories. In addition, because assay conditions and utilization of different *in vitro* systems can substantially alter the catalytic activity of CYP3A, caution should be exerted when results found *in vitro* are extrapolated to situation *in vivo* and when results from different laboratories are compared.

Therefore, the aim of the present work was to evaluate and compare the efficacy of several CYP3A enzymatic systems, which have not been tested for their efficiency to oxidize the model substrates (α-NF and testosterone) under the same (standardized) experimental conditions in one laboratory. Moreover, because there has been some controversy in the literature regarding the effects of α-NF on CYP3A activity[20–25], we have also concentrated our efforts on elucidation of effects of this compound on oxidation of testosterone catalyzed by CYP3A in these enzyme systems.

EXPERIMENTAL

Chemicals

Glucose-6-phosphate, $NADP^+$, NADPH, α-NF, 3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate (CHAPS), dilauroyl phosphatidylcholine, and dithiothreitol were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Testosterone and 6β-hydroxytestosterone were purchased from Merck (Darmstadt, Germany). Glucose-6-phosphate dehydrogenase was from Serva (Heidelberg, Germany). Bicinchoninic acid was from Pierce (Rockford, IL, USA). All chemicals were of a reagent grade or better.

Animals and Pretreatment

The study was conducted in accordance with the Regulations for the Care and Use of Laboratory Animals (311/1997, Ministry of Agriculture of the Czech Republic), which complies with Declaration of Helsinki. 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 RIF (50 mg/kg in 40 mM NaOH i.p. for 3 consecutive days) and used for isolation of microsomes.

Preparation of Microsomes, Isolation of Enzymes and Assays

Microsomes were isolated from livers of rabbits pretreated with RIF as described previously[37] and stored in 0.5 ml aliquots in liquid nitrogen until use. CYP3A6 was isolated from liver microsomes of rabbit induced by RIF. The procedure was analogous to that described by Haugen and Coon[38] and Yang et al.[39] Rabbit liver NADPH:CYP reductase was purified as described earlier[40]. Rabbit liver cytochrome b_5 was isolated from rabbit liver microsomes by the procedure described by Roos[41]. Protein concentrations were assessed using the bicinchoninic acid protein assay with serum albumin as a standard[42]. Total CYP content was measured based on complex of reduced CYP with carbon monoxide[43]. Hepatic microsomes of rabbits treated with RIF contained 0.9 nmol CYP/mg proteins.

Supersomes™, microsomal samples isolated from insect cells transfected with Baculovirus constructs, containing cDNA of CYP3A4, NADPH:CYP reductase with or without cytochrome b_5, expressing all these enzymes were from Gentest corp. (Woburn, MA, USA). A ratio of CYP3A4 to cytochrome b_5 in Supersomes™, in which this protein was expressed, was 1:4. Membranes isolated from of *E. coli*, containing human CYP3A4 was a gift from Dr. Souček (National Institute of Public Health, Prague, Czech Republic). Purified human CYP3A4 was a kind gift from Dr. Anzenbacher (Palacký University in Olomouc, Czech Republic). Male human hepatic microsomes (pooled samples) were from Gentest corp. (Woburn, MA, USA).

Testosterone 6β-Hydroxylation

The incubation mixtures for measuring the testosterone metabolism contained in a final volume of 0.5 ml: 0.1 M potassium phosphate buffer, pH 7.4, 50 μM testosterone (2 μl of stock methanolic solution per incubation), 10 mM $MgCl_2$, 10 mM D-glucose 6-phosphate, 1 mM $NADP^+$, 1 U/ml D-glucose 6-phosphate dehydrogenase, and one from the used enzyme systems: (i) human hepatic microsomes (0.2 μM CYP), (ii) hepatic microsomes of rabbits treated with a CYP3A6 inducer, RIF (0.2 μM CYP), (iii) microsomes of Baculovirus transfected insect cells containing recombinant human CYP3A4 (0.05 μM) and NADPH:CYP reductase with or without cytochrome b_5 (0.2 μM) (Supersomes™), (iv) membranes isolated from *Escherichia coli*, containing recombinant human CYP3A4 (0.05 μM) and cytochrome b_5 (0.2 μM) reconstituted with NADPH:CYP reductase (0.05 μM), and (v) purified human CYP3A4 (0.05 μM) or rabbit CYP3A6 (0.2 μM) reconstituted with NADPH:CYP reductase (0.05 or 0.2 μM) with or without cytochrome b_5 (0.2 or 0.8 μM) in liposomes. Microsomes and Supersomes™ were diluted on the concentration mentioned above. Bacterial membranes were reconstituted 10 min with NADPH:CYP reductase and cytochrome b_5 for 10 min and diluted with buffer to obtain the CYP concentration of 0.05 μM (see above). Reconstitution of purified CYP3A4 and CYP3A6 with NADPH:CYP reductase was carried out essentially as described earlier[44]. Briefly, CYP3A were reconstituted as follows (0.5 μM CYP3A, 0.5 μM NADPH:CYP reductase, 0.5 μg/μl CHAPS, 0.1 μg/μl vesicles (from D,L-dilauroylphosphatidylcholine), 3 mM reduced glutathione and 50 mM HEPES/KOH, pH 7.4). An aliquot containing 25 pmol of reconstituted CYP3A4 or 100 pmol of reconstituted CYP3A6 was added to incubation mixtures. The mixtures were incubated at 37 °C in a shaking incubator for 15 min. The testosterone 6β-hydroxylation catalyzed by these enzymatic systems was linear up to 30 min of incubation. The reaction was terminated by addition of 0.1 ml of 1 M aqueous Na_2CO_3 containing 2 M NaCl. Phenacetin (5 μl of 1 mM stock solution) was added as an internal standard. The metabolites were extracted with 2 ml of CH_2Cl_2 and the extracts were evaporated to dryness. The residues were dissolved in the mobile phase for HPLC (see below). In order to determine the effect of α-NF

on CYP3A-mediated testosterone oxidation, human and rabbit microsomes, Supersomes™ and purified human CYP3A4 or rabbit CYP3A6 reconstituted with NADPH:CYP reductase and/or cytochrome b_5 in liposomes were pre-incubated (at 37 °C for 10 min) with 150 μM of α-NF in the presence or absence of NADPH (1 mM) together with the NADPH-generating system (10 mM $MgCl_2$, 10 mM D-glucose 6-phosphate, 1 mM $NADP^+$, 1 U/ml D-glucose 6-phosphate dehydrogenase). The K_i values for reversible inhibition of testosterone oxidation by α-NF were determined from Dixon plots as described earlier[45]. Testosterone and its metabolites were separated on Nucleosil (C18) HPLC column (4.6 × 25 mm, 5 μm, Macherey–Nagel, Germany). The flow rates, mobile phases and detection wavelength for assays were 0.6 ml/min, 70:30 CH_3OH/H_2O (v/v), and 254 nm, respectively.

Oxidation of α-NF

Incubation mixtures contained in a final volume of 0.5 ml: 0.1 M potassium phosphate buffer, pH 7.4, 150 μM α-NF (2 μl of stock methanol solution per incubation), 50 μl of NADPH-generating system (see above) and the same enzyme systems as in the case of testosterone oxidation (see above). The mixtures were incubated at 37 °C for 30 min in a shaking incubator. The oxidation of α-NF to 5,6-epoxide and 7,8-dihydrodiol catalyzed by these enzymatic systems was linear up to 40 and 80 min of incubation, respectively[24]. The reaction was terminated by addition of 0.1 ml of 1 M aqueous Na_2CO_3 containing 2 M NaCl. The α-NF metabolites were extracted with 2 ml of CH_2Cl_2 and the extracts were evaporated to dryness. The residues were dissolved in the mobile phase for HPLC. Samples were analyzed by HPLC as described elsewhere[3,46] to identify α-NF oxidation products. Two metabolites with retention times of 13.0 and 21.0 min (Fig. 2), which were previously assigned as the trans-7,8-dihydrodiol and 5,6-epoxide, were formed[3,16,24,46]. Mass spectroscopy (MALDI-TOF using α-cyano-4-hydroxycinnamic acid as a matrix) of the metabolite with a retention time of 13.0 min gave molecular ions at *m/z* 307 $(M + H)^+$ and *m/z* 329 $(M + Na)^+$, suggesting a dihydrodiol derivative. The metabolite with a retention time of 21.0 min gave molecular ions at *m/z* 289 $(M + H)^+$ and *m/z* 311 $(M + Na)^+$ and peaks at *m/z* 273 $(M + H)^+$ and at *m/z* 295 $(M + Na)^+$, that is an indicative of an epoxide metabolite. The results are consistent with previous studies on the metabolism of α-NF by rat microsomes pretreated with 3-methylcholanthere[46] and by purified reconstituted CYP3A4 [16], in which these two metabolites were identified as trans-7,8-dihydrodiol (retention time 13.0 min) and 5,6-epoxide (retention time 21.0 min). Another minor metabolite formed by RIF-microsomes eluted with retention time of 9.6 min has not been identified yet (Fig. 2). Two peaks eluted with retention times of 26.6 and 28.2 min seemed not to be the products of α-NF oxidation, because they are also present in the control reaction samples. These samples containing all reaction components were immediately (without incubation) applied on a HPLC column to be analyzed.

Spectral Measurements

The binding of α-NF to CYP was monitored by difference spectroscopy (Specord M-42, Carl Zeiss, Jena, Germany)[47] using cuvettes of 1 cm optical path. The concentration of CYP3A4 and/or CYP3A6 was adjusted to 1.0 mg per ml of 0.1 M potassium phosphate buffer, pH 7.4. α-NF (0.5–10 μl of 0.1 or 1 mM methanolic stock solution) or testosterone (0.5–10 μl of 1 or 10 mM methanolic stock solution) was directly added to the sample cuvette containing purified CYP3A. The same volume of the solvent was added to the reference cuvette. Absorption spectra were recorded at ambient temperature between 350 and 500 nm. The difference in

absorbance between the wavelength maximum and minimum was plotted versus the α-NF or testosterone concentration, which was analyzed by nonlinear regression methods with Origin 6.0 software. Two equations were compared statistically to determine the best fit: $\Delta A = B_{max}S/(K_s + S)$ and $\Delta A = B_{max}S^n/(K_s^n + S^n)$, where S represents substrate concentration, K_s is the spectral dissociation constant, B_{max} is the maximal binding, and n is a measure of the cooperativity[3].

Inactivation Experiments

Primary incubation mixtures contained human microsomes (4 μM CYP) or Supersomes™ containing human recombinant CYP3A4 (1 μM), NADPH:CYP reductase and cytochrome b_5, appropriate α-NF concentrations and 0.1 M potassium phosphate buffer, pH 7.4. After preincubation of the reaction mixture at 37 °C for 5 min, the reactions were initiated by adding NADPH (final concentration of 1 mM). At the indicated times, 20 μl samples of the primary reaction mixture were removed and mixed with 980 μl of a secondary reaction mixture containing 50 μM testosterone, 1 mM NADPH in 0.1 M potassium phosphate buffer, pH 7.4, and incubated at 37 °C for 15 min. The oxidation of testosterone was analyzed as described above.

RESULTS

α-*NF and Testosterone Binding to CYP3A Enzymes*

In order to investigate the binding affinities of α-NF and testosterone to purified human CYP3A4 and rabbit CYP3A6, difference spectroscopy was used. The binding titration of α-NF and testosterone, respectively, typically gave a type I spectrum with both enzymes that is an indicative of the removal of H_2O as the sixth ligand of the heme iron, resulting in a spin shift of the heme iron from low to high spin[47]. For this type of a binding spectrum, an increase in absorbance at 390 nm and a decrease in absorbance at 420 nm are typical. The magnitude of the difference of absorbance maximum and minimum for α-NF and testosterone is assumed to represent the extent of binding to the catalytic site. The values of the spectral dissociation constants are shown in Table I. Both compounds are bound with higher affinities to purified CYP3A6 than to CYP3A4. α-NF exhibits a higher affinity to bind to both these CYP3A than testosterone. The binding produced a rather sigmoidal binding curve (see $n > 1$ in Table I) suggesting that the binding of both compounds to the purified enzymes is cooperative. A type I binding spectrum indicates that testosterone and α-NF should be substrates of the CYP3A enzymes. Indeed, both these enzymes oxidize testosterone and α-NF (Figs 1–4).

Oxidation of α-NF and Testosterone Catalyzed by CYP3A Enzymes

In order to evaluate efficiencies of different enzymatic systems containing CYP3A to oxidize both compounds *in vitro*, the following five model systems were utilized: (i) human hepatic microsomes rich in CYP3A4, (ii) hepatic microsomes of rabbit treated with a CYP3A6 inducer, RIF, (iii) microsomes of Baculovirus transfected insect cells containing recombinant human CYP3A4 and NADPH:CYP reductase with or without cytochrome b_5 (Supersomes™), (iv) membranes isolated from *E. coli*, containing human CYP3A4, NADPH:CYP reductase with or without cytochrome b_5, and (v) purified human CYP3A4 or rabbit CYP3A6 reconstituted with NADPH:CYP reductase with or without cytochrome b_5 in liposomes.

α-NF is oxidized by all enzymatic systems into two metabolites, tentatively identified as trans-7,8-dihydrodiol (retention time 13.0 min) and 5,6-epoxide (retention time 21.0 min) by mass spectra and previously reported data[16,24,46] (see Fig. 1 for human hepatic microsomes and human recombinant CYP3A4). Under the conditions used in these experiments, 5,6-epoxide was the major product of α-NF oxidation (Figs 1 and 2).

Testosterone is oxidized by CYP3A to three hydroxylation metabolites, 6β-hydroxytestosterone being the predominant one (see Fig. 3 for human hepatic microsomes and human recombinant CYP3A4). Therefore, in order to examine efficiencies of all CYP3A enzymatic systems tested in this study, formation of this metabolite was utilized.

Among the systems tested in this study, Supersomes™ containing human recombinant CYP3A4, NADPH:CYP reductase and cytochrome b_5 were the most efficient system oxidizing α-NF and testosterone into their metabo-

TABLE I
Dissociations constants K_s and Hill coefficients n for interactions of testosterone and α-NF with rabbit CYP3A6 and human CYP3A4

Compound/Type of spectra	K_s, μM/n	
	CYP3A6	CYP3A4
Testosterone/Type I	2.14/1.46	43.2/1.24
α-NF/Type I	0.78/1.24	10.8/1.30

Experimental conditions are described in Experimental. The values in the table are means of two parallel experiments

lites (Figs 2 and 4), followed by human hepatic microsomes and liver microsomes of rabbits pretreated with RIF. When cytochrome b_5 was not present in the system of Supersomes™, 6β-hydroxylation of testosterone catalyzed by this system was almost 8-fold lower than by that with this protein. Nevertheless, under these conditions, a rate of oxidation of this substrate was still higher than that catalyzed by human or rabbit microsomes.

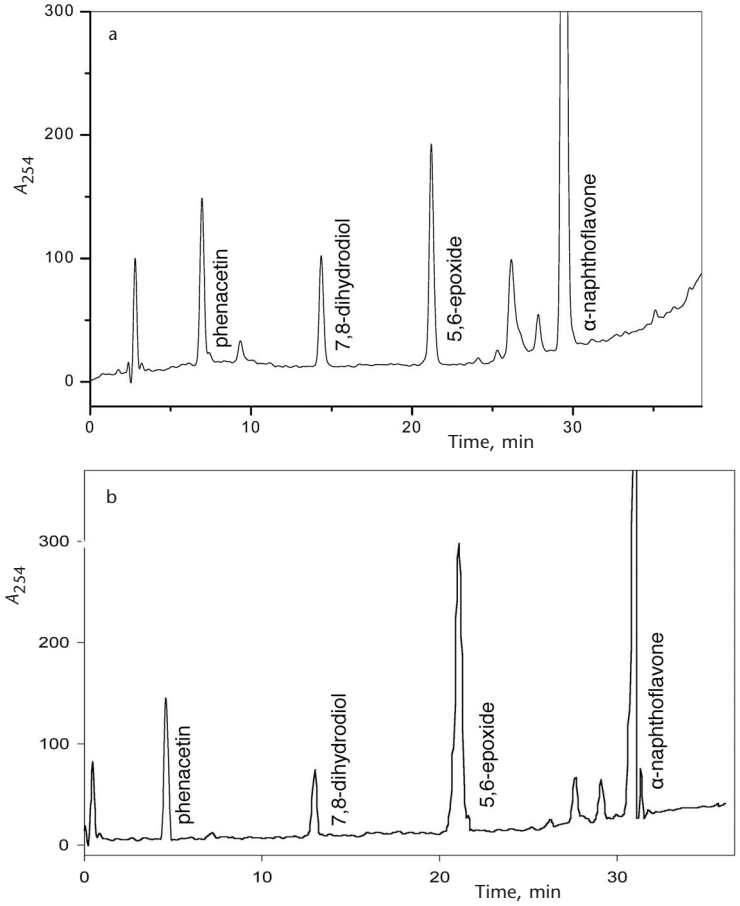

FIG. 1

HPLC separation of α-NF metabolites formed in incubations with 150 μM α-NF and human microsomes (100 pmol CYP) (a) or Supersomes™ containing human CYP3A4 (25 pmol) and cytochrome b_5 (b). For experimental conditions, see Experimental. Two metabolites are formed: 7,8-dihydrodiol (13 min) and 5,6-epoxide (21 min). Peaks in 26.6. and 28.2 min are also present in control incubations. Phenacetin (6 min) was used as an internal standard

Oxidation of α-NF by Supersomes™ containing human recombinant CYP3A4, NADPH:CYP reductase and cytochrome b_5 was 17.3-fold higher than without cytochrome b_5 (Fig. 2). Therefore, cytochrome b_5 seems to be essential for efficiencies of CYP3A4 to oxidize α-NF.

Of all tested CYP3A systems, the lowest efficacy to oxidize α-NF and testosterone was found for CYP3A4 expressed in membranes of *E. coli* and for the purified CYP3A4 or CYP3A6 reconstituted with NADPH:CYP reductase in liposomes (Figs 2 and 4). When cytochrome b_5 was present in these reconstituted systems, up to a 8.5-fold increase in oxidation of α-NF and testosterone was found (Figs 2 and 4). This protein had, however, no effect on oxidation of these substrates by the system of recombinant CYP3A4 expressed in membranes of *E. coli*.

Effect of α-NF on Testosterone 6β-Hydroxylation Catalyzed by CYP3A

In further part of our work we studied the effect of α-NF on testosterone 6β-hydroxylation catalyzed by the CYP3A systems found in this study to exhibit different efficiencies to oxidize testosterone. Namely, human and

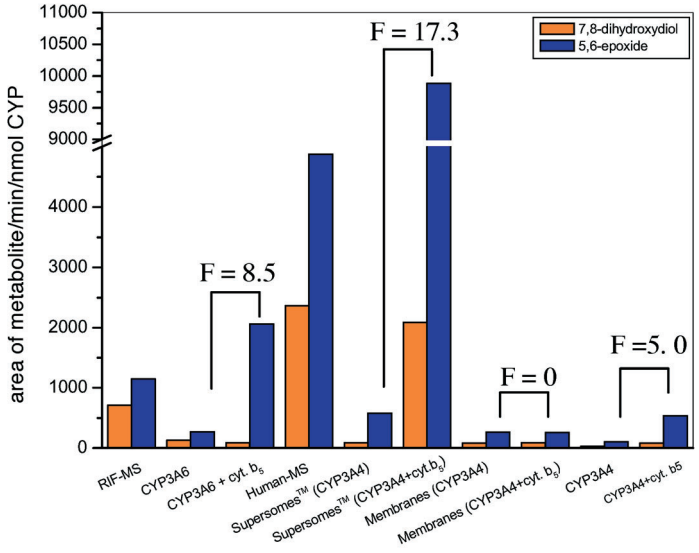

FIG. 2
α-NF oxidation catalyzed by enzymatic systems containing CYP3A4 or CYP3A6. The values in the figure are means of two parallel experiments. F – fold higher levels of α-NF metabolites in the system with cytochrome b_5 than without this protein

rabbit microsomes, Supersomes™ and purified human CYP3A4 or rabbit CYP3A6 reconstituted with NADPH:CYP reductase and cytochrome b_5 in liposomes were employed in these experiments. As shown in Fig. 5, α-NF can either inhibit, or stimulate or have no effects on testosterone oxidation catalyzed by the CYP3A systems. Effects of α-NF on testosterone 6β-hydroxylation in the studied enzyme systems are summarized in Table II.

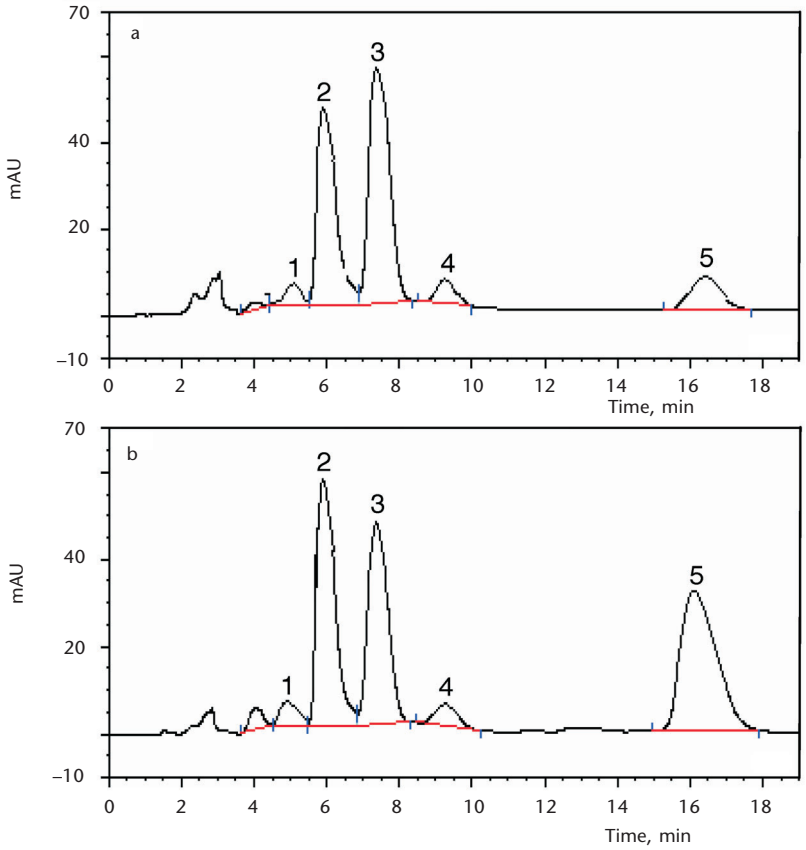

FIG. 3

HPLC separation of testosterone metabolites formed in incubations containing 50 μM testosterone and Supersomes™ containing human CYP3A4 (25 pmol) and cytochrome b_5 (a) and human microsomes (100 pmol CYP) (b). For experimental conditions, see Experimental. Three metabolites are formed: X-hydroxytestosterone (4.9 min, peak 1), 6β-hydroxytestosterone (7.3 min, peak 3) and Y-hydroxytestosterone (9.1 min, peak 4). Phenacetin (5.9 min, peak 2) was used as an internal standard. Peak 5 is of testosterone

The influence of α-NF on testosterone oxidation was studied using two different protocols, which could distinguish reversible and irreversible inhibition effect of this compound. In the first one, the reaction mixtures containing CYP3A and α-NF were incubated 10 min prior an addition of testosterone and NADPH (protocol A). In the other protocol, α-NF was pre-incubated with the CYP3A systems for the same time interval (10 min) in the presence of NADPH prior adding testosterone (protocol B) (Fig. 5). The control incubations contained the enzymatic system pre-incubated 10 min with NADPH before adding testosterone (Fig. 5).

Using the protocol A, testosterone 6β-hydroxylation catalyzed by CYP3A of rabbit hepatic microsomes and human recombinant CYP3A4 in Supersomes™ (with or without cytochrome b_5) was inhibited by α-NF, by ~50%, but this reaction catalyzed by human hepatic microsomes was not influenced by this compound (Fig. 5). A degree of inhibition of testosterone oxidation was up to 2.5-fold increased by the pre-incubation of microsomes or Supersomes™ with α-NF and NADPH (protocol B). Under these conditions, α-NF also inhibited testosterone oxidation catalyzed by

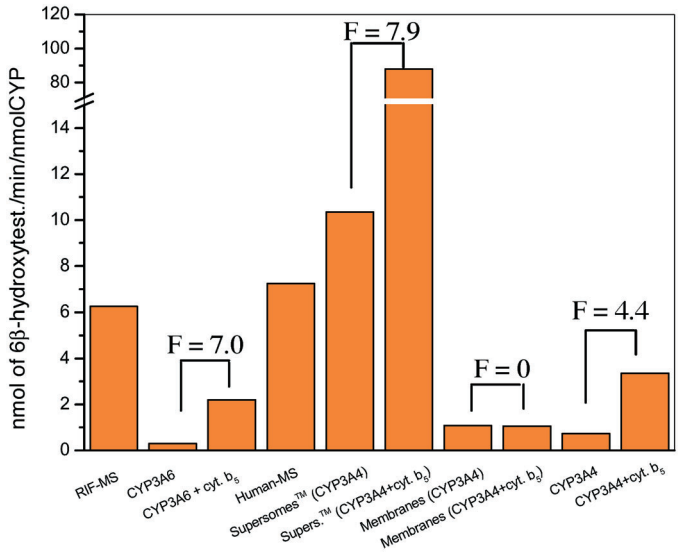

FIG. 4
6β-Hydroxylation of testosterone catalyzed by enzymatic systems containing CYP3A4 or CYP3A6. The values in the figure are means of two parallel experiments. F – fold higher levels of 6β-hydroxytestosterone in the system with cytochrome b_5 than without this protein

human hepatic microsomes (Fig. 5). These findings suggest that α-NF seems to act as an irreversible inhibitor of testosterone 6β-hydroxylation. On the contrary, only a low and nonsignificant decrease in inhibition of testosterone oxidation by α-NF was detectable using this pre-incubation protocol (protocol B versus protocol A) in the system of Supersomes™ expressing CYP3A4 without cytochrome b_5 (Fig. 5).

Recently, we have found that α-NF acts as a mechanism-based inhibitor of CYP3A6 reconstituted with NADPH:CYP reductase and cytochrome b_5 and CYP3A6 present in rabbit hepatic RIF-microsomes[24]. Here, we increased our study by investigating α-NF-mediated inhibition of another CYP3A enzyme, human CYP3A4. Experiments were conducted at 37 °C so that aliquots of incubations (containing α-NF, human microsomes and/or CYP3A4 with NADPH:CYP reductase and cytochrome b_5 in Supersomes™ and

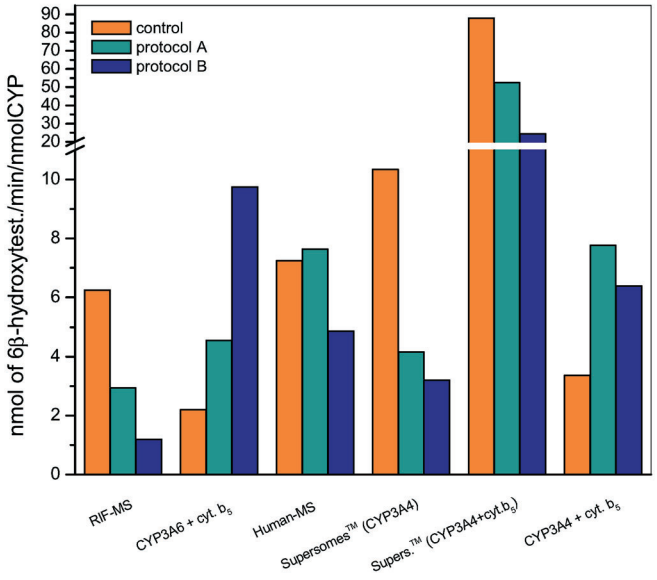

FIG. 5

Effect of α-NF on testosterone 6β-hydroxylation by the enzymatic systems containing CYP3A4 and CYP3A6. The control incubations contained the enzymatic system pre-incubated 10 min with NADPH before adding testosterone. Protocol A: the reaction mixtures containing CYP3A and α-NF were incubated 10 min prior an addition of testosterone and NADPH. Protocol B: α-NF was pre-incubated with the CYP3A systems for the same time interval (10 min) in the presence of NADPH prior adding testosterone. The values in the figure are means of two parallel experiments

NADPH) could be taken at the indicated time intervals and transferred to the secondary incubations containing testosterone and NADPH. Incubations containing microsomes and α-NF without NADPH or those without α-NF (control incubations) did not lead to a substantial loss in the CYP3A-mediated oxidation of testosterone (Fig. 6a). However, a loss of the CYP3A4 enzymatic activity in Supersomes™ was detected under the same conditions (without α-NF) after 10 min of incubation. This finding indicates that CYP3A4 is rather instable in Supersomes™. Therefore, this CYP3A4 system should be handled with care. The irreversible inactivation of 6β-hydroxylation of testosterone was found as a consequence of α-NF incubation in the presence of NADPH (Fig. 6). α-NF was found to cause a decrease in the extent of 6β-hydroxylation of testosterone in a time- and concentration-dependent manner (Fig. 6). This inhibition was NADPH- and α-NF-dependent (Fig. 6). Since there was a 50-fold dilution in enzyme concentration in the transfer of an aliquot from the primary to the secondary reaction mixture, the competitive effects of α-NF within the secondary reaction were minimal.

The reaction of α-NF with the enzyme protein is probably a bimolecular reaction. It is evident; however, from the linear nature of relationships in Fig. 6 where CYP3A4-mediated testosterone 6β-hydroxylation is plotted against time in a semi-log plot, that inactivation proceeds as a pseudo-

TABLE II
Effect of α-NF on testosterone 6β-hydroxylation in different enzymatic CYP3A systems

Enzyme system	Effect of α-NF
Oxidation of testosterone with higher efficiency	
Supersomes™ containing CYP3A4, cytochrome b_5 and NADPH:CYP reductase	irreversible inhibition
Supersomes™ containing CYP3A4 and NADPH:CYP reductase	reversible inhibition
Human hepatic microsomes	irreversible inhibition
Rabbit hepatic RIF-microsomes	irreversible inhibition
Oxidation of testosterone with lower efficiency	
CYP3A4 reconstituted with NADPH:CYP reductase and cytochrome b_5	stimulation
CYP3A6 reconstituted with NADPH:CYP reductase and cytochrome b_5	stimulation

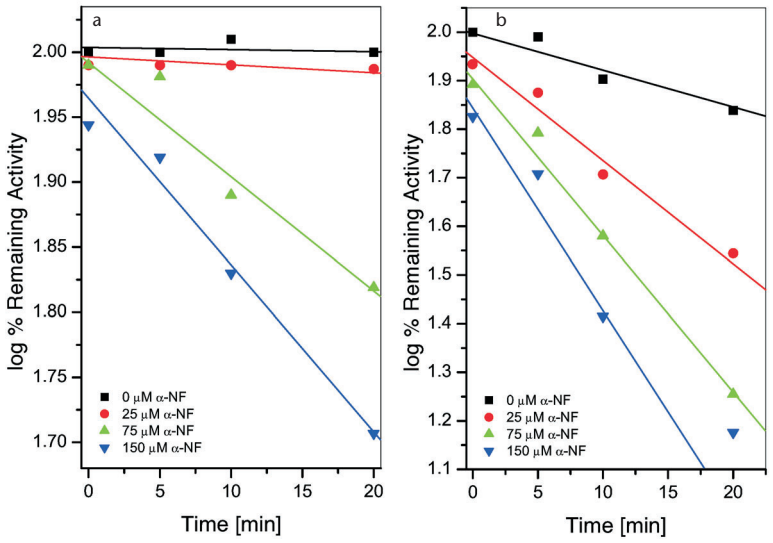

FIG. 6
Inactivation of CYP3A4 of human microsomes (a) and Supersomes™ with NADPH:CYP reductase and cytochrome b_5 (b) by α-NF. The values in the figure are means of two parallel experiments

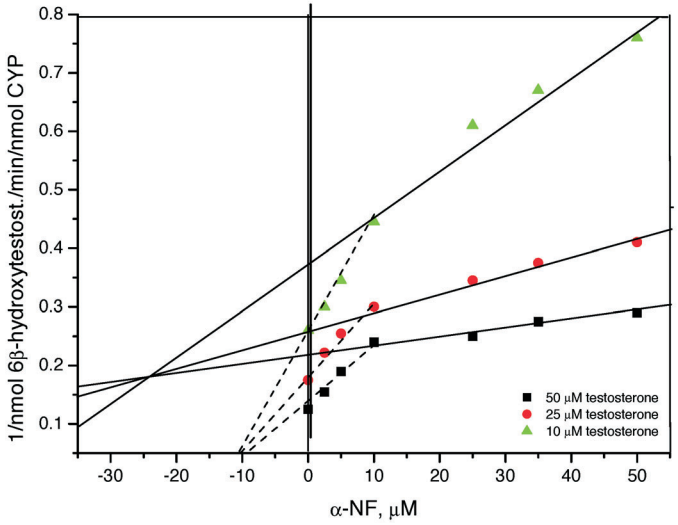

FIG. 7
Inhibition of CYP3A4 (Supersomes™) measured as testosterone 6β-hydroxylation by α-NF (Dixon plots). The values in the figure are means of two parallel experiments

monomolecular reaction. Pseudo-first-order inactivation kinetics was observed with α-NF concentrations from 25 to 150 μM. This fact may be explained by the distinct excess of α-NF as compared with the amount of CYP (concentration 6–38-fold). Therefore, the reaction can be described by the first-order equations. A double-reciprocal plot of the first-order rate constant for inactivation (k_i) and α-NF concentration gave a straight line, indicative a saturable inhibition (data not shown). The concentrations required for half-maximum inactivation (K_i) are 125.4 and 41.9 μM, and the times required for half of the enzyme to become inactivated are 29.6 and 12.9 min for CYP3A of human microsomes and Supersomes™ with cytochrome b_5, respectively.

In the case of human CYP3A4 in Supersomes™ containing NADPH:CYP reductase without cytochrome b_5, α-NF acts as reversible inhibitor of testosterone oxidation (Fig. 7). Kinetic studies showed non-linear Dixon plots. At the low concentration of α-NF, the inhibiton seems to be non-competitive with respect to testosterone, having K_i value of 10 μM. At higher concentration of α-NF, however, this compound acts as a competitive inhibitor with respect to testosterone. The K_i value for such a competitive inhibition is 24 μM.

In contrast to the inhibition effect of α-NF on testosterone oxidation by the above mentioned CYP3A enzymatic systems, up to a 4.4-fold increase in oxidation of testosterone was caused by α-NF in the systems exhibiting low CYP3A enzymatic activity composed from purified CYP3A6 reconstituted with NADPH:CYP reductase with cytochrome b_5 in liposomes (Fig. 5). Likewise, the CYP3A4-catalyzed testosterone oxidation in another CYP3A reconstituted system (containing CYP3A4) was stimulated by α-NF (Fig. 5).

DISCUSSION

In this report we compared several enzymatic systems to find *in vitro* experimental models that might mimic the CYP3A-mediated enzymatic activity and its modulation by effectors in organisms.

The results found in this work demonstrate large differences in efficacy of individual CYP3A systems to oxidize their substrates. These findings indicate that data shown in a variety of former studies employing different enzymatic systems to evaluate a degree of participation of CYP3A enzymes in xenobiotic metabolism should be carefully interpreted. The results clearly demonstrate that microsomes of eukaryotic organisms containing recombinant human CYP3A4, NADPH:CYP reductase and cytochrome b_5 (Supersomes™ in the case of our study), followed by hepatic microsomal

systems of human and rabbit, were the best system oxidizing the tested substrates, α-NF and testosterone. This finding suggests these systems to be suitable for general evaluating a variety of compounds as potential substrates of the CYP3A enzymes.

Interestingly, whereas a rate of 6β-hydroxylation of testosterone catalyzed by CYP3A4 in Supersomes™, even without cytochrome b_5, was still higher than testosterone hydroxylation mediated by human hepatic microsomes, oxidation of another tested substrate, α-NF, by this system without cytochrome b_5 was 9- and 17.3-fold lower than by human hepatic microsomes and CYP3A4 in Supersomes™ with cytochrome b_5, respectively. Therefore, cytochrome b_5 seems to be essential for the effective conversion of α-NF by CYP3A4.

The enzymatic activity of CYP3A reconstituted with NADPH:CYP reductase in liposome vesicles was much lower than that of the microsomal systems. Surprisingly, analogously low efficiencies to oxidize α-NF and testosterone were detected using CYP3A4 expressed in membrane of *E. coli* reconstituted with NADPH:CYP reductase with or without cytochrome b_5. Because purified CYP3A4 or 3A6 and CYP3A4 in *E. coli* membranes were reconstituted with NADPH:CYP reductase under the conditions described previously to be appropriate for the reconstitution process[2,21,35], the reconstituted systems should be in the optimal state for their catalytic activity. Therefore, now we can only speculate to explain their low enzymatic activities. One of the reasons might be absence of other components of the mixed-function oxidase system such as cytochrome b_5, stimulating α-NF and testosterone oxidation by CYP3A4 in Supersomes™, and/or additional components of the endoplasmic reticulum membrane. Indeed, the presence of cytochrome b_5 in the systems of CYP3A4/6 reconstituted with reductase increased their enzymatic activity. However, even in the presence of this protein in a ratio to CYPs of 4:1, shown previously to be appropriate for the CYP3A4 enzymatic activity in the reconstitution system[48–51], efficiencies of this system to oxidize α-NF and testosterone was still much lower than the CYP3A4-mediated activity of Supersomes™ or human and rabbit hepatic microsomes. The presence of cytochrome b_5 in the system, which was prepared by reconstitution of CYP3A4 in *E. coli* membranes, was even without any stimulation effect on its enzymatic activity. These findings suggest that other, still unknown, components of hepatic microsomes are necessary to reconstitute full enzymatic activity of CYP3A. Furthermore, in the case of CYP3A4 in membranes of *E. coli*, another, still unsettled, question might arise whether the CYP3A4 protein is expressed and incorporated into the

cell membrane in conformation appropriate for its enzymatic activity needed for α-NF and testosterone oxidation.

Even though we showed here clearly that several microsomal systems of eukaryotes are the most efficient systems oxidizing two CYP3A substrates we investigated in this study, caution should be still exerted when these enzymatic systems would be used generally. Hence, in order to properly evaluate a role of CYP3A in metabolism of various chemicals, we recommend employing a combination of various *in vitro* approaches. Such a procedure seems also to be reasonable mainly for studies concerning the modulation of CYP3A enzyme activities by their effectors. Here we have shown that one of such effectors, α-NF, can modulate CYP3A activity in a different manner, depending on efficacy of the individual enzymatic system. α-NF can act as an irreversible inhibitor of CYP3A in systems, which efficiently oxidize α-NF itself and a second model substrate, testosterone. Namely, CYP3A4 in the presence of cytochrome b_5 in Supersomes™, and/or human or rabbit hepatic microsomes, form apparently large amounts of α-NF reactive intermediates, which are finally capable of inactivating CYP3A, by their covalent binding to the enzyme active site[15,24,46]. This finding corresponds to the results of our previous work, which showed that a-NF can act as a mechanism-based inhibitor of two CYP3A6-catalyzed reactions, 6β-hydroxylation of progesterone and testosterone[24]. On the contrary, in the systems exhibiting lower efficiencies to oxidize α-NF, such as CYP3A4 in Supersomes™ without cytochrome b_5, α-NF acts as a reversible inhibitor competing with binding of testosterone to the active site of the enzyme. The enzymatic activity of CYP3A4 in this system seems not to be sufficient to oxidize α-NF to reactive species in such amounts, which would inactivate the enzyme.

In the case of the CYP3A systems exhibiting the lowest efficacy to oxidize α-NF and testosterone, namely the CYP3A4 or 3A6 reconstituted with reductase and cytochrome b_5, stimulation effects of α-NF was found on testosterone oxidation. Hence, an increase in efficiency of these systems was produced by α-NF. One of the explanations of the enhancement of CYP3A activities might be allosteric interactions. The results found previously show that at least two binding sites of CYP3A4 for α-NF are present in CYP3A4 [3,24]. The presence of more than one binding site of α-NF in CYP3A4 is also supported by the results demonstrating that α-NF binding to CYP3A4 showed sigmoidicity. It was found that one binding and oxidation site of CYP3A4 for α-NF present in the active center of the enzyme is distinct from a testosterone site of interaction[3,24]. Binding of α-NF to the binding site different from a testosterone site of interaction can change a

CYP3A4 protein conformation that exhibits higher efficiency to oxidize testosterone. Nevertheless, this suggestion needs to be evaluated by further investigations. Likewise, the question whether this feature is of general importance, valid also for other CYP3A substrates awaits further examination.

In conclusion, among the enzymatic systems containing CYP3A4 and 3A6 enzymes tested in our study, we have found the system of human recombinant CYP3A4 expressed in eukaryotic endoplasmic reticulum membranes (microsomes assigned as Supersomes™) together with NADPH:CYP reductase and cytochrome b_5, followed by human hepatic microsomes, the most suitable *in vitro* experimental models exhibiting the highest activity to oxidize α-NF and testosterone. Therefore, these systems themselves or in a combination with inhibiting the CYP3A4 activity with its selective inhibitor, ketoconazole[52], might be generally used to investigate potential substrates of CYP3A4. Nevertheless, employing a combination of other *in vitro* systems is strongly recommended for this purpose, and mainly for studies concerning the modulation of CYP3A4 activities by its effectors.

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