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PARTICULAR ABILITY OF CYTOCHROMES P450 3A TO FORM INHIBITORY P450–IRON–METABOLITE COMPLEXES UPON METABOLIC OXIDATION OF AMINODRUGS

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Abstract—The ability of 21 drugs containing an amine function to form inhibitory P450–iron–metabolite complexes absorbing around 455 nm was studied on liver microsomes from rats treated with various P450 inducers. These drugs belong to different chemical and therapeutic series and exhibit very different structures. In the case of eight compounds (diltiazem, lidocaine, imipramine, SKF 525A, fluoxetine, L-α-acetylmethadol, methadol and desmethyltamoxifen) whose oxidation by microsomes from rats treated with several inducers was studied, only dexamethasone (DEX)-treated rat microsomes and, to a lesser extent, phenobarbital (PB)-treated rat microsomes, were able to give significant amounts of 455 nm absorbing complexes. Ten of the 21 compounds studied gave such complexes with DEX-treated rat microsomes, while only three compounds gave complexes (in low amounts) with PB-treated rat microsomes only. For all compounds leading to complexes both with DEX- and PB-treated rat microsomes, much higher amounts of complexes were obtained with DEX-treated rat microsomes. DEX-treated rat microsomes also led to the most intense type I spectral interactions with most of the compounds studied, and very often exhibited the highest N-dealkylation activities towards the tertiary or secondary amine function of the drugs used. A few exceptions aside, there generally exists a qualitative relationship between the ability of P450 3As, induced by DEX, to bind and N-dealkylate amino compounds and their propensity to lead to 455 nm absorbing complexes. This was confirmed by *in vivo* experiments showing that rats treated with diltiazem, tamoxifen or imipramine accumulated large amounts of 455 nm absorbing complexes in their liver only after pretreatment with DEX and, to a lesser extent, with PB. This particular ability of P450 3As to oxidize amino drugs with formation of inhibitory P450-metabolite complexes could be of great importance for the appearance of drug interactions in man.

Key words: drug interactions; nitrosoalkane complexes; N-dealkylation; amines; P450 3A; inhibitors

P450 enzymes constitute a superfamily of heme-thiolate proteins that catalyse the primary oxidation of a wide variety of natural endogenous substrates such as steroids, fatty acids, prostaglandins, leukotrienes and lipid hydroperoxides. They also play an important role in the metabolism of exogenous compounds like drugs, procarcinogens, solvents and environmental pollutants. Their broad substrate specificity is now well understood on the basis of enzyme multiplicity [1]. More than 220 P450s have been sequenced and characterized and classified on the basis of primary amino acid sequence similarity [2]. Changes in the P450-dependent metabolism of a drug very often occur when a co-administered drug acts as an inducer or inhibitor of the same P450 isozyme. This may lead to dramatic changes of the pharmacokinetic parameters of the drug and, from time to time, to the appearance of severe secondary toxic effects. In order to understand and even predict such drug interactions in man, it is important to determine which P450 is mainly involved in the oxidative metabolism of a given drug, and which drugs are able to act as strong inhibitors or inducers of a given P450.

P450s of the 3A subfamily have been shown to be highly conserved in mammal liver [3, 4] and also to be present in many other organs such as intestine, gut and kidney [5, 6]. They are induced by glucocorticoids like dexamethasone [7], imidazoles like clotrimazole [8] and macrolide antibiotics like troleandomycin (TAO) [9–11]. They play a major role in the oxidative metabolism of a surprisingly large number of drugs with very different structures such as nifedipine [12], troleandomycin and erythromycin [13, 14], quinidine [15], cyclosporin A [16, 17], midazolam [18], lidocaine [19], amiodarone [20], dihydroergotamine [21], imipramine [22, 23] and diltiazem [24]. They are also involved in the 6β-

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hydroxylation of testosterone [25], the reduction of glyceryltrinitrate [26] and the oxidation of *Nω*-hydroxyarginine into citrulline and NO [27]. In humans, P450 3A4 enzymes are the most abundant P450s in the liver [4, 5]. They have been shown to be mainly involved in the metabolism of the drugs mentioned above, and it is noteworthy that most of these drugs contain an amine function.

Several compounds, including drugs, which contain an amine function are oxidized by P450 enzymes with the eventual formation of inhibitory P450–iron(II)–nitrosoalkane metabolite complexes, characterized by a Soret peak at 455 nm [13, 28–31]. Some of these compounds, such as macrolide antibiotics of the erythromycin or oleandomycin series [9, 13, 32–34], SKF 525A [30, 35, 36], L-α-acetylmethadol [37] and amiodarone [38], have been found to lead to inhibitory complexes that are stable *in vivo*. This phenomenon is mainly responsible for the severe problems encountered in patients having received drugs in association with erythromycin or troleandomycin [33, 34, 39, 40]. In the case of macrolide antibiotics, P450 3As are mainly affected by this inhibitory effect.

In order to determine which drugs containing an amine function could act as P450 inhibitors via the formation of P450–iron–nitrosoalkane complexes and which P450 isozymes are mainly involved in complex formation, we have studied the interactions of 21 amino drugs with liver microsomal P450s from rats treated by various inducers, as well as the interaction of some of these drugs with rat liver P450s *in vivo*. Figure 1 shows the structure of various drugs or compounds used in this study, all of which contain an amine function. Some of them—i.e. benzphetamine [28] and iodoamphetamine [41], SKF 525A [28, 30, 35–37], L-α-acetylmethadol [37, 42], orphenadrime (also called FAR8328V) [43], amiodarone [38] and desipramine [44, 45]—have been found to form 455 nm absorbing P450–iron–metabolite complexes upon oxidation with liver microsomes. Only a few of them—i.e. SKF 525A [35, 36], L-α-acetylmethanol [37], and amiodarone [38]—have been described as precursors of such P450 complexes *in vivo*. However, the nature of the P450 isozymes primarily involved in the formation of the 455 nm absorbing complexes has rarely been examined. Benzphetamine [28, 37], iodoamphetamine [41], L-α-acetylmethadol [37] and SKF 525A [35, 37, 44] have been found to give higher amounts of complex with microsomes from PB*-treated rats than with microsomes from untreated or 3MC-treated rats. Indeed, the possible involvement of P450 3As in such complex formation has only been studied in the case of erythromycin derivatives (or related macrolide antibiotics) [9, 33, 34], amiodarone [38] and SKF 525A [36]. The results described below clearly show that P450s of the 3A subfamily are particularly important in the formation of inhibitory P450–Fe(II)–nitrosoalkane metabolite complexes upon oxidation of drugs containing a tertiary or secondary amine function.

*Abbreviations: PB, phenobarbital; 3MC, 3-methylcholanthrene; DEX, dexamethasone; CLO, clofibrate; G6P, glucose-6-phosphate; G6PDH, glucose-6-phosphate dehydrogenase.

MATERIALS AND METHODS

Chemicals. NADPH, 3MC, DEX, imipramine, desipramine, diltiazem, amitriptyline, tamoxifen, ranitidine, clomipramine, promethazine and amiodarone were supplied by the Sigma Chemical Co. (Poole, U.K.), and CLO by Fluka (Buchs, Switzerland). Troleandomycin was kindly supplied by Pfizer laboratory (Sandwich, U.K.), fluoxetine by Lilly Research Lab. (Indianapolis, U.S.A.), *N*-desmethyltamoxifen by ICI (Cergy, France), benzphetamine by Upjohn (Paris, France) and erythromycin by Roussel Uclaf. *N*-hydroxy-didesmethylimipramine was synthetized according to a published procedure [46]. FAR 90X2, FAR 8328V, FAR 9058D were kindly provided by Dr A. Bast (Vrije University, Amsterdam, The Netherlands) [43].

Preparation of microsomes. Male Sprague–Dawley rats (175–200 g) were treated either for 5 days with PB (0.1% in drinking water) or, for 3 days, with 3MC 20 mg/kg, DEX 100 mg/kg, CLO 500 mg/kg, i.p. in corn oil. Rats were treated with imipramine, diltiazem or tamoxifen (100 mg/kg i.p. in saline) for 3 days. Control rats received saline. Rats were killed 24 hr after the last treatment. For the associated treatments, rats were treated for 3 days with PB, 3MC, DEX or CLO as indicated above and, 36 hr before death, received a single dose of 100 mg/kg (i.p. in saline) of imipramine, diltiazem or tamoxifen. Microsomes were prepared according to the usual techniques [47]. Yeast microsomes containing human expressed cytochromes P450 were prepared as already described for P450 3A4 [48], 1A1 [49], 2C8, 2C9 and 2C18 [50].

Assays. Protein [51], cytochrome P450 [52] and cytochrome P450 metabolite complexes [53] were determined by published procedures.

The amounts of cytochrome P450–metabolite complexes formed *in vivo* were measured by using the difference spectra obtained between microsomal preparation minus microsomal preparation treated by 50 μM potassium ferricyanide for 5 min [28, 53, 54], assuming an extinction coefficient ε (455–490 nm) of 65,000 M^{-1} cm^{-1} for the 455 nm absorbing complexes [44, 54].

Binding of substrates to rat liver cytochrome P450s was studied by difference visible spectroscopy on a Kontron spectrophotometer (Uvikon 820 equipped with a diffusion sphere), after addition of increasing amounts of the compounds dissolved in DMSO or water to hepatic microsomes containing 2 μM P450. 455 nm absorbing cytochrome P450 complex formation was measured by differential spectroscopy with microsomes containing 2 μM cytochrome P450 (0.15 μM in the case of yeast-expressed human P450), 0.1 mM substrate and 0.5 mM NADPH. Difference spectra were recorded from 1 mL cuvettes (1 cm in length) between 400 and 520 nm every 2 min and maximum absorbance of the 455 nm absorbing cytochrome P450 metabolite complex was determined [44, 53, 54].

N-dealkylase activities were measured by for-

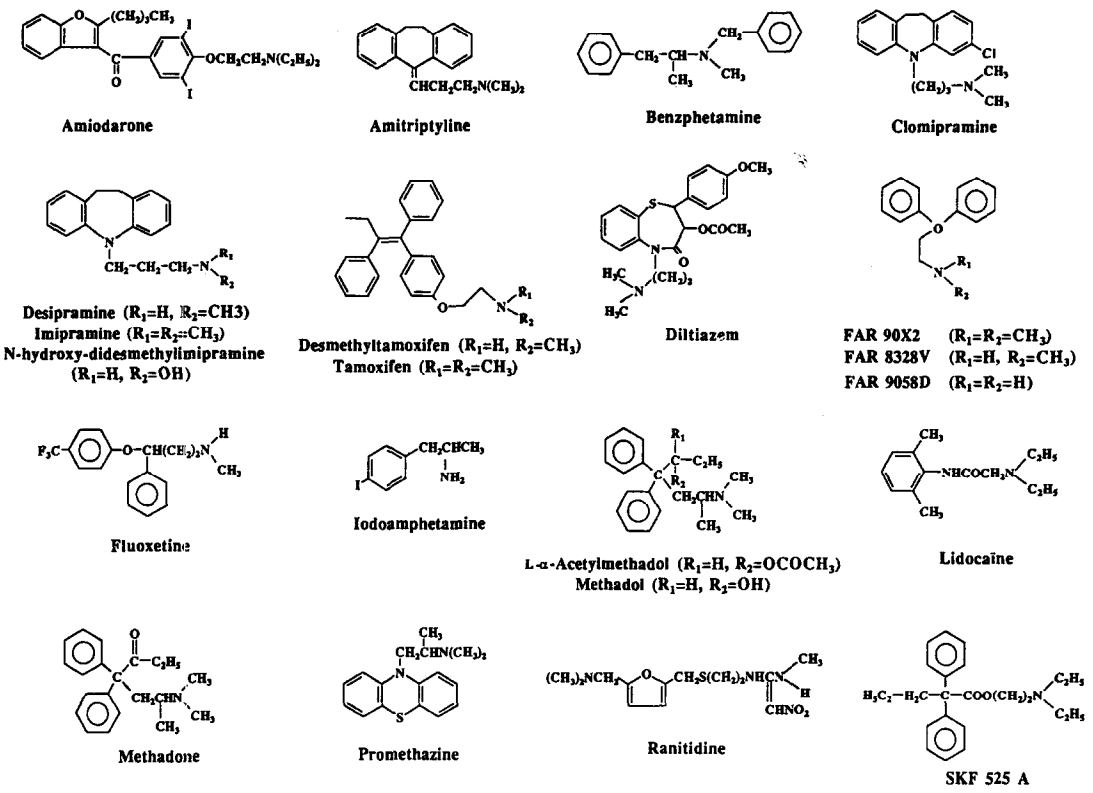

Fig. 1. Chemical structure of the compounds used in this study.

maldehyde or acetaldehyde formation according to a standard technique [55] using 0.1 mM substrate, 1 μM P450 and an NADPH generating system (NADP 1 mM, G6P 10 mM, G6PDH 1 UI) for 10 min at 37°.

RESULTS AND DISCUSSION

Formation of P450–Fe(II)–metabolite complexes upon oxidation of amino compounds by liver microsomes from rats pretreated with various inducers

SKF 525A, a well-known inhibitor of microsomal P450s, has already been shown to give a 455 nm absorbing P450–iron–metabolite complex, both in rats *in vivo* [30, 35, 36] and *in vitro* in liver microsomes from PB-treated rats [28, 44]. The formation of this complex was studied upon metabolic oxidation of SKF 525A by liver microsomes from rats pretreated by standard P450 inducers in the presence of NADPH. DEX, 3MC, PB and CLO were used as inducers of P450s of the 3A, 1A, 2B and 4 family, respectively [7, 10, 56, 57]. As shown in Fig. 2, only microsomes from DEX-treated rats gave an intense formation of 455 nm absorbing complex. This would correspond to 80% of total microsomal P450 engaged in this complex if one admits an ε value of 65,000 $M^{-1}\,cm^{-1}$ for the absorbance, ΔA (455–490 nm), of the 455 nm absorbing complex, as described previously for the amphetamine-derived complex [44, 54, 58]. PB-treated rat microsomes gave a much lower amount of complex (10% of total P450) while the other microsomes failed to give significant complex formation (<5% of total P450) under identical conditions. All the other compounds studied (Fig. 2), namely diltiazem, lidocaine, imipramine, fluoxetine, L-α-acetylmethadol, desmethyltamoxifen and methadol, which belong to different series and exhibit very different structures, were oxidized by DEX-treated rat microsomes with formation of a 455 nm absorbing complex (representing between 10 and 80% of total P450). It is noteworthy that four drugs (desmethyltamoxifen, imipramine, lidocaine and methadol) only formed this complex with DEX-treated rat microsomes. On the contrary, 3MC-treated rat microsomes failed to give significant complex formation with the eight compounds studied, while microsomes from untreated rats or rats treated with CLO gave a P450 iron–metabolite complex only with L-α-acetylmethadol. Finally, Fig. 2 shows that PB-treated rat liver microsomes were able to generate such a complex with four of the tested compounds although its amount (around 10%) was always much lower than that observed with DEX-treated rat microsomes.

Based on these results, the possible formation of 455 nm absorbing complexes with a much larger number of compounds was then studied with liver microsomes from rats treated either with PB or with DEX. Table 1 shows that significant amounts of 455 nm absorbing complex were observed with 10 of

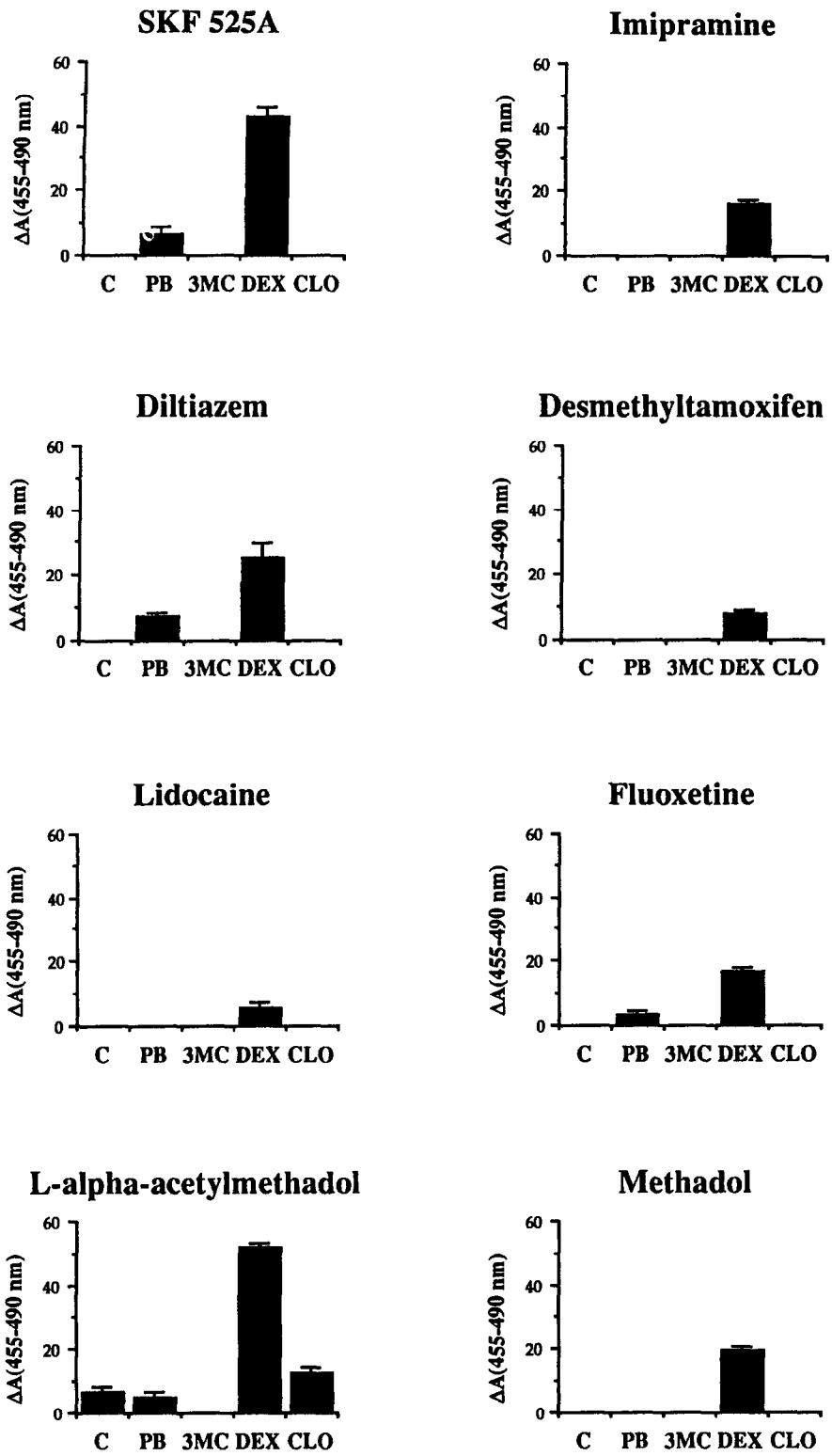

Fig. 2. Amounts of 455 nm absorbing cytochrome P450 metabolite complexes formed upon oxidation of eight amino compounds by liver microsomes from rats treated with various inducers. Substrate (100 μM) was incubated at 37° in the presence of liver microsomes (2 μM P450) from rats either untreated (C) or treated with PB, 3MC, DEX or CLO and 0.5 mM NADPH. Each value is the mean ± SD of three independent determinations of the maximal absorbance at 455 nm [ΔA (455–490 nm). (nmol P450)$^{-1}$.10^3]. Substrate concentration of 100 μM was selected on the basis of preliminary studies performed on the eight compounds by varying their concentration between 10 and 500 μM. Optimal complex formation was always observed for substrate concentrations around 100 μM.

Table 1. Formation of 455 nm absorbing P450–metabolite complexes upon oxidation of various aminodrugs by liver microsomes from rats treated with PB or DEX

Compounds	ΔA (455–490 nm).(nmol P450)$^{-1}$.10^3 (% complexed P450)	
	PB	DEX
Amiodarone	ND	Traces
Amitriptyline	ND	Traces
Benzphetamine	0.5 ± 0.3 (0.8% ± 0.5%)	ND
Clomipramine	ND	ND
Desipramine	ND	31.8 ± 3.2 (49% ± 5%)
Desmethyltamoxifen	ND	7.8 ± 0.6 (12% ± 1%)
Diltiazem	7.1 ± 1.3 (11% ± 2%)	25.3 ± 4.5 (39% ± 7%)
FAR 90 X2	Traces	ND
FAR 8328V	0.5 ± 0.3 (0.8% ± 0.5%)	Traces
FAR 9058D	ND	ND
Fluoxetine	3.2 ± 0.6 (4.9% ± 0.9%)	16.9 ± 1.9 (26% ± 3%)
Imipramine	ND	16.2 ± 1.3 (25% ± 2%)
Iodoamphetamine	2.6 ± 0.6 (4% ± 0.9%)	ND
L-α-Acetylmethadol	5.2 ± 1.3 (8% ± 2%)	52.0 ± 1.0 (80% ± 2%)
Lidocaine	ND	5.8 ± 1.3 (9% ± 2%)
Methadol	ND	19.5 ± 1.0 (30% ± 1%)
Methadone	Traces	ND
Promethazine	ND	Traces
Ranitidine	ND	ND
SKF 525A	6.5 ± 2.6 (10% ± 4%)	42.9 ± 3.2 (66% ± 5%)
Tamoxifen	ND	3.2 ± 0.6 (5% ± 1%)

Conditions used for complex formation: liver microsomes from rats treated either with PB (2.5 nmol P450/mg protein) or with DEX (2 nmol P450/mg protein) containing 2 μM P450, 100 μM substrate and 500 μM NADPH. The amount of P450 existing in the form of a 455 nm absorbing P450 complex was calculated by assuming an ε (455–490 nm) = 65,000 M^{-1} cm^{-1} [44, 54]. Data are means ± SD of two to five independent measurements; ND: not detected; traces mean the appearance of a very weak spectrum with $\Delta A \approx 0.3 \times 10^{-3}$.(nmol P450)$^{-1}$.

the 21 compounds, in DEX-treated rat microsomes (i.e. the eight compounds depicted in Fig. 2 along with desipramine and tamoxifen). Moreover, the amount of complex formed was generally high (between 10 and 80% of total P450) with the exception of tamoxifen. On the contrary, in the case of PB-treated rat microsomes, complex formation was observed with only seven of the 21 compounds studied and never exceeded 10% of total P450. In fact, it was close to the detection limit for two compounds. However, it is noteworthy that the two amphetamine derivatives, iodoamphetamine and benzphetamine, as well as FAR 8328V, gave very low but significant amounts of complex with PB-treated rat microsomes whereas they did not form any complex with DEX-treated rat microsomes.

Thus, of the 21 compounds studied, eight (amiodarone, amitriptyline, clomipramine, FAR 90X2, FAR 9058D, methadone, ranitidine and promethazine) did not form significant amounts of 455 nm absorbing complexes with DEX- and PB-treated rat microsomes under the conditions employed (100 μM substrate, 2 μM P450 and 20 min reaction in the presence of 0.5 mM NADPH). With the exception of three compounds (iodoamphetamine, benzphetamine and FAR 8328V) which gave complexes only with PB-treated rat microsomes, all the others gave complexes with DEX-treated rat microsomes in much higher amounts than with PB-treated rat microsomes.

These first results suggested a major contribution of P450 3As in the formation of 455 nm absorbing complexes upon oxidation of amino drugs exhibiting very different structures. This conclusion was supported by the qualitative relationship existing between the amounts of complexes and of P450 3As (relative to total P450) in the various microsomes. Both amounts are very high in DEX-treated rat microsomes, low in PB-treated rat microsomes and very low in the other microsomes (see 10, 56, 59 for the amounts of P450 3As in rat liver microsomes, and Fig. 2 and Table 1 for the amounts of complexes).

Most of the drugs studied involved a tertiary amine function (Fig. 1). The metabolic pathway leading from a tertiary amine to a P450–Fe(II)–nitrosoalkane complex, which has been reported previously [29, 31], is indicated in Fig. 3. It involves a series of reactions including four oxidative steps (steps 1, 2, 3 and 5). In order to understand the origin of this propensity of P450 3As to form 455 nm absorbing complexes, we then compared the capacity of the various microsomes to perform some steps involved in Fig. 3.

Static interaction of amino compounds with microsomal P450s from rats treated with PB and DEX

Interaction of the amino compounds with PB- and DEX-treated rat microsomes was studied by difference visible spectroscopy (Table 2). Most produced a typical "type I" spectrum characterized

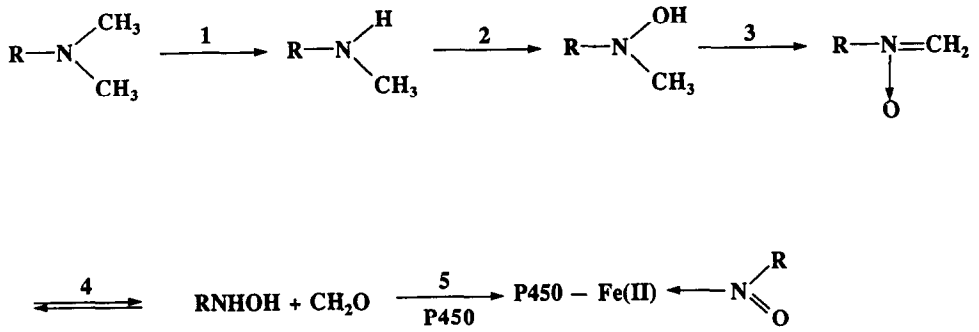

Fig. 3. Metabolic pathway involved in the formation of 455 nm absorbing P450–Fe(II)–nitrosoalkane metabolite complexes upon oxidation of compounds containing an $N(CH_3)_2$ group [29, 31].

Table 2. Spectral interaction of aminocompounds with cytochrome P450 from DEX- or PB-treated rat liver microsomes

Treatment of rats		PB		DEX	
		Difference spectra			
Compounds	Type	$\Delta A_{max.}$ (nmol P450)$^{-1}.10^3$	$K_s(\mu M)$	$\Delta A_{max.}$ (nmol P450)$^{-1}.10^3$	$K_s(\mu M)$
Amiodarone	—	<5	—	<5	—
Amitriptyline	I	<5	—	25	—
Benzphetamine	I	30	5	40	17
Clomipramine	I	<5	—	10	—
Desipramine	I	<5	—	11	10
Desmethyltamoxifen	I	10	5	25	—
Diltiazem	I	9	2	20	6
FAR 8328 V	I	10	—	<5	—
FAR 90 X2	I	10	—	6	—
FAR 9058 D	II	—	—	<5	—
Fluoxetine	I	<5	1	7	2
Imipramine	I	6	1	15	1
Iodoamphetamine	I	10	—	<5	—
L-α-acetylmethadol	I	<5	—	20	16
Lidocaine	I	14	6	14	9
Methadol	I	7	—	17	3
Methadone	I	9	8	12	1
Promethazine	I	5	—	15	—
Ranitidine	—	<5	—	<5	—
SKF 525A	I	13	3	40	3
Tamoxifen	I	8	—	<5	—

Difference spectra were obtained by addition of increasing amounts (0.1–100 μM) of substrate to microsomal suspensions containing 2 μM P450 from rats treated either with PB or DEX. They contained 2.5 and 2 nmol P450/mg protein, respectively. Most gave type I difference spectra (λ_{max} = 390 nm, λ_{min} = 420 nm). Maximum absorbances at saturating concentrations of the compounds are given below. Apparent spectral dissociation constants, K_s, were determined from double-reciprocal plots of ΔA (390–420 nm) versus [substrate]. The reported ΔA and K_s values were means from four measurements (SD ≈ 15%).

by a peak around 390 nm and a trough around 420 nm, which corresponds to a transition of P450s from their low-spin Fe(III) to high-spin Fe(III) state upon binding of the substrate on a protein site close to the heme [60]. Only two compounds, amiodarone and ranitidine, did not produce such a type I spectrum with both kinds of microsomes and it is interesting to note that they also failed to lead to significant amounts of 455 nm absorbing complexes (Table 1). Thirteen amino compounds interacted better with DEX-treated rat microsomes than with PB-treated rat microsomes (higher maximum absorbances and/or lower apparent spectral dissociation constants in most cases). Among them, desipramine, desmethyltamoxifen, diltiazem, fluoxetine, imipramine, L-α-acetylmethadol, methadol and SKF 525A were also found to act as much better substrates for 455 nm absorbing complex formation with DEX-treated rat microsomes (Table 1). Five compounds interacted better with PB-treated rat microsomes; of those, benzphetamine, iodoamphetamine and FAR 8328V were also found to act as better substrates for 455 nm absorbing complexes with PB-treated rat microsomes.

Table 3. Oxidative N-dealkylation activities of rat liver microsomes towards various amino compounds

Compounds	PB	DEX
	Activity nmol RCHO.(nmol P450)$^{-1}$.(10 min)$^{-1}$	
Amiodarone	1 ± 1	<0.5
Amitriptyline	11 ± 1	60 ± 2
Benzphetamine	100 ± 22	51 ± 8
Clomipramine	17 ± 1	51 ± 2
Desipramine	12 ± 4	29 ± 1
Desmethyltamoxifen	11 ± 7	25 ± 2
Diltiazem	56 ± 9	100 ± 9
FAR 90 X2	27 ± 1	28 ± 2
Fluoxetine	8 ± 1	13 ± 3
Imipramine	27 ± 3	43 ± 3
L-α-Acetylmethadol	23 ± 3	64 ± 5
Lidocaine	3 ± 1	9 ± 2
Methadol	15 ± 1	47 ± 9
Methadone	17 ± 4	6 ± 2
Promethazine	11 ± 1	19 ± 1
Ranitidine	<0.5	4 ± 1
SKF 525A	5 ± 1	7 ± 1
Tamoxifen	3 ± 1	15 ± 5

Conditions of the assay were described in Materials and Methods using the same microsomes as in Table 2 but with 1 μM P450. Data are means ± SD from three experiments. Similar experiments using 250 μM substrate instead of 100 μM gave very similar results.

This would be in global agreement with a qualitative relationship between the formation of P450–substrate complexes and P450–iron–metabolite complexes for the amino compounds studied. However, this relationship is only qualitative and suffers from some exceptions. For instance, amitriptyline, clomipramine, FAR 90X2, methadone and promethazine interacted well with DEX- and (or) PB-treated rat microsomes, but were found to be unable to form 455 nm absorbing complexes. Moreover, desmethyltamoxifen and methadol gave weak but significant interactions with PB-treated rat microsomes but were unable to form iron–metabolite complexes with these microsomes. Finally, lidocaine gave similar type I interactions with both kinds of microsomes, although it gave significant amounts of 455 nm absorbing complex only with DEX-treated rat microsomes, and tamoxifen gave type I spectra only with PB-treated rat microsomes, although it gave an iron–metabolite complex only with DEX-treated rat microsomes (compare Tables 1 and 2).

N-dealkylation of the amino compounds by DEX- and PB-treated rat liver microsomes

The amino compounds containing a secondary or tertiary amine function with an N-CH_3 or N-C_2H_5 moiety were submitted to oxidation by DEX- and PB-treated rat microsomes in the presence of NADPH. The possible formation of formaldehyde or acetaldehyde was detected by using the Nash reagent [55]. As shown in Table 3 most of these compounds underwent an oxidative dealkylation. This activity was higher with DEX-treated rat microsomes for 15 of the 18 compounds tested, whereas PB-treated rat microsomes were more active only for amiodarone, benzphetamine and methadone. However, there was no quantitative relationship between the N-dealkylase activity of microsomes and their ability to form a 455 nm absorbing complex. For instance, PB-treated rat microsomes exhibited a great activity for the oxidative dealkylation of amitriptyline, clomipramine, desipramine, desmethyltamoxifen, FAR 90X2, imipramine, methadol, methadone and promethazine but did not form 455 nm absorbing complex with these compounds.

These results are understandable, as the N-dealkylation of amino compounds (step 1 of Fig. 3), the first step in the formation of P450–iron–nitrosoalkane complexes, is a necessary but not sufficient step in the formation of these complexes. Moreover some exceptions to a relationship between rates of N-dealkylation of aminocompounds and extent of complex formation could be related to the inhibition of N-dealkylation due to complex formation. Particularly in the case of quick and extensive formation of such inhibitory complexes, N-dealkylation could be inhibited to a great extent. This might explain why few compounds such as SKF 525A, which gave high amounts of complex (Table 1), exhibited very low rates of N-dealkylation (Table 3).

Formation of a 455 nm absorbing complex upon oxidation of N-hydroxy-didesmethylimipramine by rat liver microsomes

In order to know whether the last step of the formation of a P450–Fe(II)–RNO complex upon microsomal oxidation of a tertiary amine (step 5 of Fig. 3) varies greatly with the nature of the microsomes, the oxidation of N-hydroxy-didesmethylimipramine by various rat liver microsomes was studied. The reaction of 100 μM N-hydroxy-didesmethylimipramine with DEX-treated rat liver microsomes in the presence of NADPH led to the appearance of an intense difference spectrum characterized by a peak at 455 nm. The maximum absorbance, ΔA (455–490 nm), corresponded to approx. 100% microsomal P450 leading to metabolite complex formation, if one admits an ε (455–490 nm) of 65,000 $M^{-1}\,cm^{-1}$ for such a complex [44, 54, 58] (Table 4). Microsomes from untreated rats and from rats treated with PB, 3MC or CLO gave lower amounts of the 455 nm absorbing complex (63, 67, 46 and 60%, respectively). These results show that, at least in the case of imipramine (step 5 of Fig. 3), the last step in the formation of a P450–Fe(II)–RNO complex is performed by all kinds of rat liver microsomes, DEX-treated rat microsomes being the most efficient. Therefore, the lack of formation of an inhibitory complex upon oxidation of imipramine or desipramine by PB-treated rat microsomes (Table 1) should be due to the absence of the enzymatic activity corresponding to step 3 (Fig. 3) and/or to too slow steps 1 and 2 (Table 3).

Microsomes from yeasts expressing a given human liver P450, 3A4 [48], 1A1 [49] or 2C8, 2C9 and 2C18 [50] were also used for N-hydroxy-didesmethyl-

Table 4. Formation of a 455 nm absorbing P450 metabolite complex upon oxidation of *N*-hydroxy-didesmethylimipramine by microsomal P450s either from liver of differently treated rats or from yeast-expressing human liver P450s

Microsomes		ΔA (455–490 nm).(nmol P450)$^{-1}$.10^3	% of complexed P450
Rat	no	41.0	63
	PB	43.6	67
	3MC	29.9	46
	DEX	65.0	100
	CLO	39.0	60
Yeast	W(R)	ND	ND
	W(R)-1A1	37.7	58
	W(R)-3A4	52.0	80
	W(R)-2C8	63.7	98
	W(R)-2C9	ND	ND
	W(R)-2C18	65	100

Difference spectra were obtained by addition of 100 μM substrate and 500 μM NADPH to microsomal suspensions containing 2 μM P450 from rats either untreated (no) or treated with PB, 3MC, DEX or CLO (they contained 1.1, 2.5, 1.4, 2.0, 1.4 nmol P450/mg protein, respectively), or 0.15 μM P450 from yeast-expressing human P450 1A1 (W(R)-1A1), 3A4 (W(R)-3A4), 2C8 (W(R)-2C8), 2C9 (W(R)-2C9), 2C18 (W(R)-2C18) which contained 206, 37.5, 42.1, 85.7, 42.9 pmol P450/mg protein. The amount of P450 existing in the form of a 455 nm absorbing complex was calculated by assuming an ε (455–490 nm) = 65,000 M^{-1} cm^{-1} for this complex [44, 54]. The reported ΔA (455–490 nm) values obtained after 20 min, were means from three measurements (SD ≈ 15%); ND, not detected.

imipramine oxidation. All the yeast-expressed P450s were able to form a 455 nm absorbing complex (except 2C9) (Table 4). P450 2C18 and 3A4 gave the highest rates of formation of a 455 nm absorbing complex in the first 2 minutes after addition of NADPH (data not shown). The results of Table 4 clearly show that the last step of formation of a 455 nm absorbing complex (step 5 of Fig. 3) is a very easy reaction performed by many P450 isozymes. They suggest that this step should not be limiting for complex formation from metabolic oxidation of tertiary or secondary amines.

Formation of P450–Fe(II)–nitrosoalkane complexes in vivo

Diltiazem, tamoxifen and imipramine, three drugs which were previously found to produce P450–iron–metabolite complexes *in vitro*, were administered to rats pretreated with various P450 inducers in order to evaluate complex formation *in vivo*. As shown in Fig. 4, three daily administrations of 100 mg/kg imipramine, diltiazem or tamoxifen to rats failed to give rise to any significant formation of a 455 nm absorbing complex in rat liver. Naturally, a single administration of these three drugs (100 mg/kg) to rats 36 hr before death did not produce any complex formation. An identical result was obtained when this single dose was administered to rats pretreated with 3MC or CLO (Fig. 4). On the contrary, livers from rats first treated with PB or DEX and then with a single administration of imipramine contained significant amounts of 455 nm absorbing complex (18 and 17%, respectively). In the case of diltiazem, livers from rats pretreated with DEX contained a higher amount of P450–metabolite complexes than those from rats pretreated with PB (20% of total microsomal P450 instead of 8%). However, the most spectacular result was obtained with tamoxifen, as livers from rats pretreated with DEX before administration of a single dose of tamoxifen were found to contain about 70% of their P450 in the form of a 455 nm absorbing complex, although livers from PB-pretreated rats contained only 5% P450–metabolite complex (Fig. 4).

CONCLUSION

The formation of inhibitory P450–Fe(II)–nitrosoalkane–metabolite complexes upon oxidation of drugs containing a tertiary amine function is a complex process involving four oxidation steps (Fig. 3) [29, 31]. The 21 compounds studied belong to very different chemical series and exhibit very different structures; 13 of them were able to give significant amounts of 455 nm absorbing complexes with PB-treated and/or DEX-treated rat liver microsomes. DEX-treated rat liver microsomes were found to be able to give such complexes in most cases (10 of the 21 compounds studied), and gave much higher amounts of inhibitory complexes than PB-treated rat liver microsomes. In fact, in the case of eight compounds whose oxidation by microsomes from rats treated with several inducers was studied, only DEX-treated rat microsomes, and to a lesser extent, PB-treated rat microsomes, were able to lead to significant amounts of 455 nm absorbing complexes.

In the case of amiodarone, only very low amounts of 455 nm absorbing complex were detected with microsomes from PB- and DEX-treated rats. This is in agreement with a previous report [38] which showed the appearance of a very small 455 nm absorbing spectrum upon reaction of amiodarone with PB- and DEX-treated rat liver microsomes. However, it is noteworthy that the amount of 455 nm

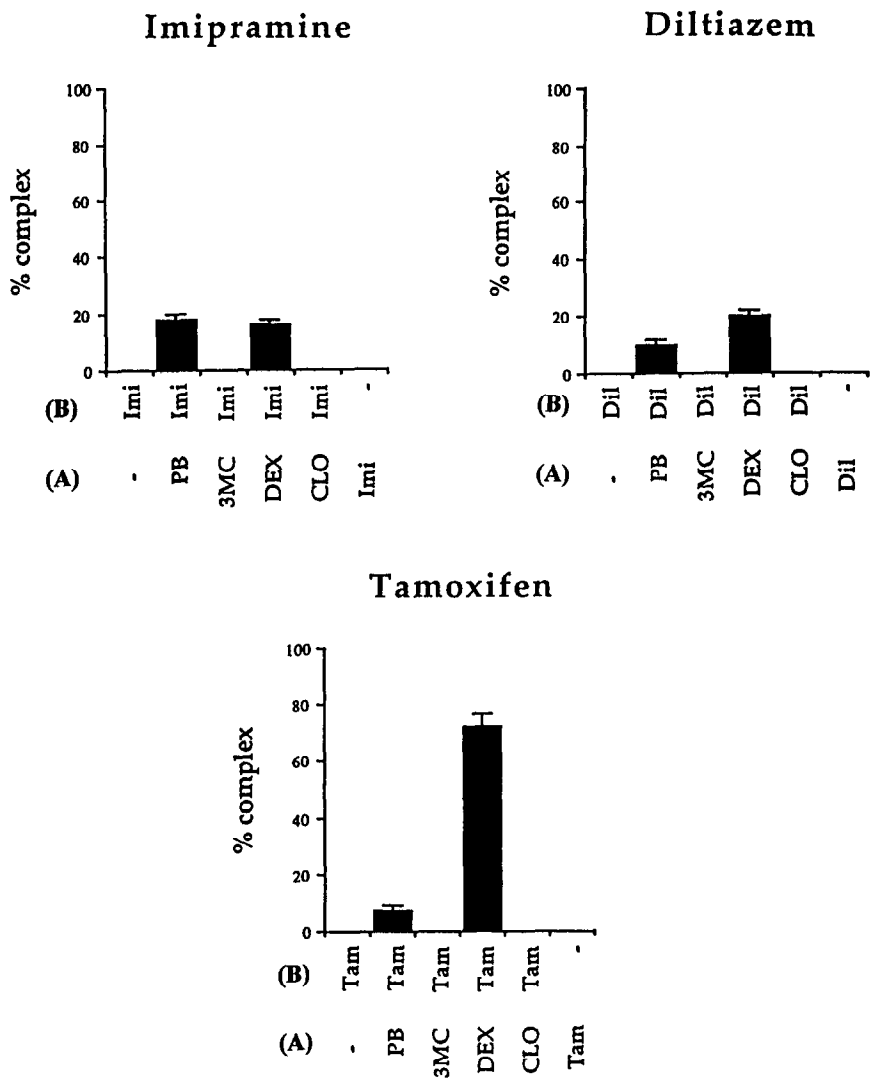

Fig. 4. *In vivo* formation of 455 nm absorbing P450–metabolite complexes upon administration of imipramine, diltiazem or tamoxifen to rats either untreated or pretreated with various P450 inducers. In most experiments rats received two treatments: they were first treated with an inducer (PB, 3MC, DEX, CLO) (A) for 3 days (daily doses indicated in Materials and Methods), and then with a compound under study (B) (single dose of 100 mg/kg) 36 hr before death. They were killed 24 hr after the last injection of the inducer. In some experiments, rats were treated only with the compound to be studied, either with a single dose (100 mg/kg) 36 hr before death (control experiments on untreated rats), or for 3 days (100 mg/kg daily) (as for inducers). Amounts of 455 nm absorbing complexes were measured after treatment of liver microsomes with 50 μM potassium ferricyanide in the sample cuvette in order to dissociate the P450–metabolite complex. They are expressed as per cent of total P450 existing as a 455 nm absorbing complex using ε (455–490 nm) = 65,000 M^{-1} cm^{-1} [44, 54]. Indicated values are means ± SD from three to five experiments.

absorbing complex was found to be much higher in the liver of rats treated *in vivo* by DEX and amiodarone [38].

DEX-treated rat microsomes also led to more intense spectral type I interactions with most of the compounds studied, and very often exhibited highest N-dealkylation activity towards the tertiary or secondary amine function of the compounds used (higher activity with DEX-treated rat microsomes than with PB-treated rat microsomes for 13 of the 21 compounds studied). Generally speaking, there is a qualitative relationship between the ability of P450 3As, induced by DEX, to bind and N-dealkylate a wide variety of amino compounds and their propensity to give 455 nm absorbing complexes. However, this relationship is only qualitative and suffers from the exceptions mentioned above. This is likely explained by the fact that the N-dealkylation of an amino compound is a necessary first step in the formation of 455 nm absorbing complexes but is

not sufficient in itself. The aforementioned results show that P450s of the 3A subfamily are particularly prone to oxidize drugs containing a tertiary or secondary amine function with formation of inhibitory P450–Fe(II)–nitrosoalkane complexes. This was confirmed by *in vivo* experiments in which rats treated with diltiazem, tamoxifen or imipramine accumulated large amounts of 455 nm absorbing complexes in their liver after pretreatment with dexamethasone, a specific inducer of P450 3As (Fig. 4).

This particular ability of P450 3As to oxidize amino drugs with formation of inhibitory P450–iron–metabolite complexes could be of great importance for the appearance of drug interactions in man. P450 3A4 is the most abundant P450 in human liver [4, 5] and is involved in the oxidative metabolism of a surprisingly wide variety of drugs [12, 15–27]. Interestingly, many of these drugs contain an amine function. Therefore, it is likely that the severe problems encountered in patients receiving troleandomycin or erythromycin, a drug able to give large amounts of a 455 nm absorbing complex in human liver, in association with drugs mainly metabolized by P450 3A4, such as dihydroergotamine [33, 39, 40] or cyclosporin [61], could be observed with other amino drugs.

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