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NEW HETEROCYCLIC MODIFIERS OF OXIDATIVE DRUG METABOLISM—II

STERIC FACTORS IN THE INTERACTION OF ISOMERIC 2-(NAPHTHYL)METHYLBENZIMIDAZOLES WITH RAT HEPATIC MICROSOMAL CYTOCHROME P-450 AND MONOOXYGENASE ACTIVITIES

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Abstract—The inhibitory potency of the two isomeric 2-(naphthyl)methylbenzimidazoles towards three monooxygenase activities (aminopyrine *N*-demethylase, 7-ethoxycoumarin *O*-deethylase and aniline *p*-hydroxylase) was assessed in hepatic microsomal fractions from untreated, phenobarbitone-induced and β-naphthoflavone-induced rats. The isomers were essentially equipotent with each other as inhibitors of the phenobarbitone-induced monooxygenases (the ratio of the I_{50}s of the isomers was about 1.0 in each case) but differences between the isomers were noted in the inhibition potencies against three monooxygenase activities from β-naphthoflavone-induced liver. The isomer 2-(1′-naphthyl)methylbenzimidazole was approximately twice as potent as the 2′-naphthyl isomer against 7-ethoxyresorufin *O*-deethylase activity, whereas the opposite was observed with respect to 7-ethoxycoumarin *O*-deethylase inhibition; aniline *p*-hydroxylase was poorly inhibited by both isomers. The binding affinity and extent of binding, assessed from double-reciprocal plots of spectral binding studies, of the 1′-isomer was much greater than that of the 2′-isomer in β-naphthoflavone-induced microsomes. Inhibition data in untreated hepatic microsomes were more complex and the finding of principal interest was that the 1′-isomer was poorly inhibitory towards aniline *p*-hydroxylase activity whereas the 2′-isomer enhanced this activity. These studies suggest (1) that the steric conformations of the isomeric naphthylmethylbenzimidazoles at the cytochrome P-450 active centre determines the extent to which the inhibitors modulate a specific monooxygenase activity, and (2) that multiple binding sites with the capacity to interact to different extents with benzimidazole derivatives are present in P-450 in β-naphthoflavone-induced hepatic microsomes. The apparent importance of steric conformation as a determinant of inhibition and enhancement of aniline *p*-hydroxylase in untreated microsomal fractions may well reflect specific interactions with multiple binding sites.

The oxidative biotransformation of a wide range of lipophilic drug substrates and endogenous compounds to more hydrophilic metabolites is catalysed by the cytochrome P-450 (P-450)-mediated monooxygenase system present in the microsomal fraction of mammalian liver. Although the P-450 system is unusual in that it possesses a very low substrate specificity, it is now clear that this characteristic is attributable to the relatively large number of isozymic forms of P-450 present in the microsomal fraction. Indeed, at least twelve distinct isozymes have been isolated from untreated and differently-pretreated rat hepatic microsomes [1–3].

Several classes of organic compounds are active inhibitors of the hepatic monooxygenase system and it is now clear that many inhibitor classes interact directly with the cytochrome at or near the catalytic centre [4–8]. Nitrogen heterocycles include some of the most potent inhibitors of the P-450 system. Imidazole and benzimidazole derivatives have been the subject of intensive interest regarding the mode of their inhibitory action [4–7]. Small heteroaromatic ring systems, typified by the imidazoles, appear to interact, at least in part, with the P-450 haem iron via its sixth axial coordination position [4, 9, 10]. This interaction produces a transition of the spin equilibrium that exists in ferric P-450 towards the low spin ($S = 1/2$) configuration and results in a decreased rate of reduction of the ferric P-450-substrate complex by electrons transferred from NADPH [11]. Nevertheless, more potent imidazole-based inhibitors probably interact with hydrophobic binding regions on the P-450 apoprotein in addition to the haem interaction [4]. In contrast, most benzimidazoles, which may be considered as imidazole derivatives that possess a fused carbocyclic ring system, do not appear to interact directly with the P-450 haem. Instead, interaction with hydrophobic binding sites adjacent to the P-450 haem would seem to be largely responsible for the noted inhibition potency of substituted benzimidazoles [5, 6, 12, 13]. Consistent with this assertion, most structure–activity relationships have emphasised the primary importance of hydrophobic character to inhibitory potency by benzimidazoles and other monooxygenase inhibitors [4, 5, 13–15]. Studies of this type have imparted little information regarding the steric factors that influence inhibition potency. In view of this deficiency, and in the light of recent studies that have strongly suggested that multiple binding sites may exist in the P-450 catalytic centre [8, 16], the

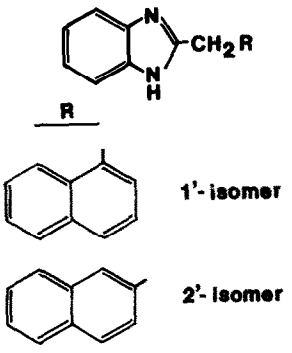

Fig. 1. Structures of the isomeric 2-(naphthyl)methylbenzimidazoles used in the present study.

present study was undertaken to evaluate the importance of steric factors on the interaction of the two isomeric 2-naphthylmethylbenzimidazoles with P-450 and microsomal monooxygenases (Fig. 1).

MATERIALS AND METHODS

Chemicals. The isomeric 2-(naphthyl)methylbenzimidazoles were synthesised by the method of Pool *et al.* [17]. Briefly, 1 m-equiv. of *o*-phenylenediamine (Aldrich Chemical Co., Milwaukee, WI) was refluxed with 2 equivalents of either 1- or 2-naphthylacetic acid (Aldrich) in 2 M HCl for 16 hr. The lower HCl concentration and longer reflux period than the literature method were required for efficient cyclisation to the benzimidazole and as the starting acids were poorly soluble in the acidic medium. After cooling the reaction mixture was neutralised with solid sodium bicarbonate and the resultant precipitate was isolated by filtration, decolourised with activated charcoal in boiling ethanol and recrystallised from aqueous-ethanol. Yields were: 2-(1′-naphthyl)methylbenzimidazole, 41% m.p. 232–235° (*anal*: calc. C 83.7%, H 5.5% N 10.9%; found C 83.7% H 5.8%, N 10.8%) and 2-(2′-naphthyl)methylbenzimidazole, 44%, m.p. 202–204° (*anal*: found C 83.7%, H 5.7%, N 10.9%). Spectroscopic data (i.r., ^{1}H-NMR and CH_4-chemical ionisation MS) were consistent with the assigned structures.

Biochemicals were obtained from Sigma Chemical Co. (St. Louis, MO), and all other reagents and solvents were at least analytical reagent grade.

Animal treatment. Male Wistar rats (200–250 g) were induced with either phenobarbitone (100 mg/kg i.p. once daily for 3 days) or β-naphthoflavone (40 mg/kg i.p.) once daily for 3 days; control rats received no pretreatment. Animals were sacrificed 48 hr after the final treatment.

Microsomal fraction preparation. Washed microsomal fractions were prepared as previously described [18].

Protein was determined according to Lowry *et al.* [19] using bovine serum albumin as standard.

Monooxygenase assays. Aminopyrine *N*-demethylase (APDM) activity was determined at 37° as described elsewhere [14]. Inhibitors were introduced into reaction incubations in 50 μl of dimethylformamide (final concentration in incubation 1.7%); solvent alone was added to control incubations. I_{50} values were obtained from plots of percent inhibition versus log inhibitor concentration. Each plot was constructed from the mean percent inhibition at each of 4–6 different inhibitor concentrations determined at least in duplicate.

7-Ethoxycoumarin *O*-deethylase (ECOD) activity was measured in an Aminco SPF-125 spectrofluorometer by the method of Prough *et al.* [20]. 7-Ethoxyresorufin *O*-deethylase (EROD) activity was measured by literature methods [20] and aniline *p*-hydroxylase (APH) activity was determined as described elsewhere [21]. I_{50}s were determined as described under APDM activity.

Optical difference spectroscopy. Difference spectra were measured at 37° in an Aminco-Chance DW-2a spectrophotometer using 1-cm cuvettes containing 1-ml aliquots of microsomal suspensions (1.0 mg microsomal protein per ml) in potassium phosphate buffer 0.1 M, pH 7.4). Test compounds were added to the sample cuvette in microlitre quantities of dimethylformamide and the difference spectra were recorded between 380 and 500 nm; an equal volume of solvent was added to the reference cuvette. Spectral dissociation constants (K_s) and maximal spectral changes (ΔA_{max}) were determined by established procedures [6].

Cytochrome P-450 was quantitated by the method of Omura and Sato [22] using an extinction cooefficient of 91 $mM^{-1}cm^{-1}$ for the ferrocytochrome P-450–carbonyl complex.

RESULTS

Inhibition of hepatic microsomal monooxygenase activities from untreated and induced rats

From the data in Table 1 it is apparent that the isomeric 2-naphthylmethylbenzimidazoles were quite potent inhibitors of several monooxygenase activities included in the present study. Throughout the study the I_{50} ratio (ratio of the I_{50} of the 2′-isomer against a particular monooxygenase activity to the I_{50} of the 1′-isomer against the same activity) was employed to assess the relative potencies of the isomers (Fig. 2).

In hepatic microsomes from untreated rats ECOD activity was equally sensitive to inhibition by both isomers (I_{50} = 13 μM for the 1′-isomer and 15 μM for the 2′-isomer) whereas APDM activity was inhibited preferentially by the 2′-isomer; this compound was about 2.5-times more potent than the 1′-isomer against APDM from untreated rat liver (Table 1). Thus it is apparent that the specific monooxygenase activity selected is a determinant of the relative potency of these isomeric inhibitors (Fig. 2).

The effects of phenobarbitone and β-naphthoflavone pretreatment on inhibitory potency was also considered. Induction of rats with these agents produces well-documented alterations in the profile of monooxygenase activity. In this study phenobarbitone-inducible ECOD and APDM were

Table 1. Inhibition of rat hepatic microsomal monooxygenase activities by isomeric 2-naphthylmethylbenzimidazoles

	Microsomal monooxygenase activity (I_{50}; μM)*					
	Control microsomes		Phenobarbitone-induced microsomes		β-Naphthoflavone-induced microsomes	
Inhibitor	ECOD	APDM	ECOD	APDM	ECOD	EROD
2-(1′-naphthyl)methylbenzimidazole	13	100	21	33	67	1.5
2-(2′-naphthyl)methylbenzimidazole	15	42	17	29	39	3.4

* Control activities were: in control microsomes, ECOD (7-ethoxycoumarin *O*-deethylase): 1.0 nmole umbelliferone/min/mg protein; APDM (aminopyrine *N*-demethylase): 1.7 nmole formaldehyde/min/mg protein. In phenobarbitone-induced microsomes, ECOD: 2.8 nmole umbelliferone/min/mg protein; APDM: 4.3 nmole formaldehyde/min/mg protein. In β-naphthoflavone-induced microsomes, ECOD: 8.9 nmole umbelliferone/min/mg protein; EROD (7-ethoxyresorufin *O*-deethylase): 1.9 nmole resorufin/min/mg protein.

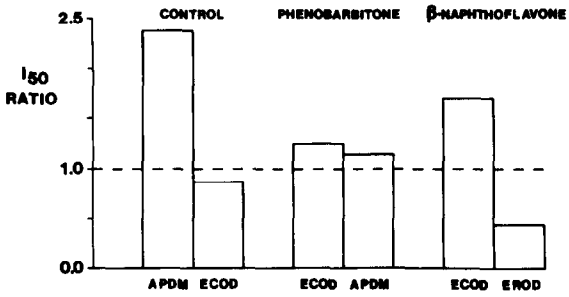

Fig. 2. Histogram showing the I_{50} ratio (relative potencies of the isomers) as a function of the monooxygenase activity evaluated. The broken line represents an I_{50}-ratio of 1.0 (equipotent inhibition by the isomers). The ratio takes a value greater than 1.0 when the 2′-isomer is a more potent inhibitor than the 1′-isomer.

equally susceptible to inhibition by the isomeric benzimidazole derivatives (Table 1; Fig. 2). In contrast, induction by β-naphthoflavone produced quite different effects. The 2′-isomer proved to be approximately twice as potent as the 1′-isomer as an inhibitor of ECOD activity after β-naphthoflavone induction whereas the 1′-isomer was twice as potent as the 2′-isomer against EROD activity. Superficially it may have been anticipated that these two *O*-dealkylation reactions in β-naphthoflavone-induced microsomes may be similarly susceptible to inhibition by the two benzimidazoles. This was clearly not the case and therefore the data have clear implications for the nature of substrate binding at the catalytic centre of the P-450 haemoprotein.

Effect of naphthylmethylbenzimidazoles on microsomal aniline p-*hydroxylase activity*

The effects of the isomeric naphthylmethylbenzimidazoles on aniline *p*-hydroxylase were assessed in microsomal fractions from untreated rats and rats induced with either phenobarbitone or β-naphthoflavone. From Fig. 3 it is clear that the modifying effects of the compounds depends largely on the nature of the animal pretreatment. Thus, in untreated rat hepatic microsomes, the 1′-isomer was poorly inhibitory (only 15% inhibition at a concentration of 100 μM) whereas the 2′-isomer enhanced the activity. Enhancement was apparently optimal between 20–40 μM; at a concentration of

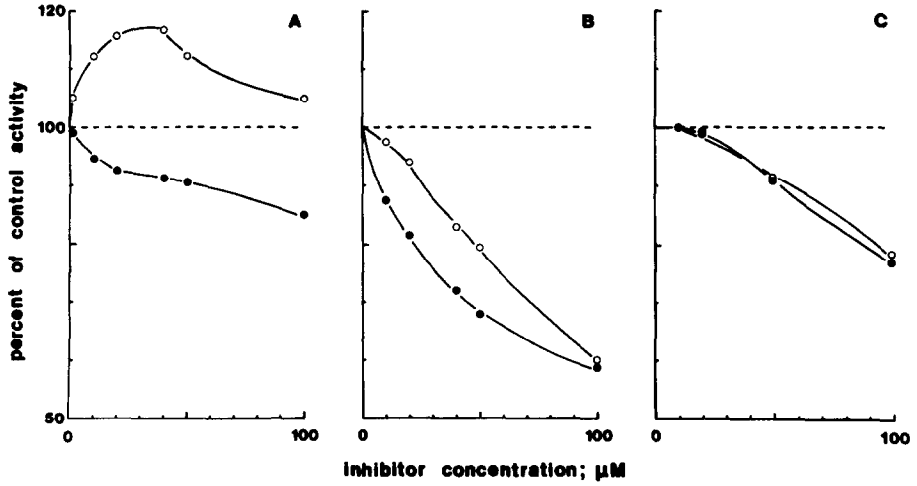

Fig. 3. Effect of the isomeric 2-(naphthyl)methylbenzimidazoles on aniline *p*-hydroxylase activity in hepatic microsomes from (A) untreated, (B) phenobarbitone-pretreated, and (C) β-naphthoflavone-pretreated rats. The broken line represents control activity. (●) 2-(1′-naphthyl)methylbenzimidazole; (○) 2-(2′-naphthyl)methylbenzimidazole.

Table 2. Spectral binding characteristics of isomeric 2-naphthylmethylbenzimidazoles in hepatic microsomes from control and induced rats

	Microsomal type								
	Control			Phenobarbitone-induced			β-Naphthoflavone-induced		
	K_s	$\Delta A_{max} \times 10^3$	$\Delta A/K_s$	K_s	$\Delta A_{max} \times 10^3$	$\Delta A/K_s$	K_s	$\Delta A_{max} \times 10^3$	$\Delta A/K_s$
2-(1′-naphthyl)methyl-benzimidazole	2.8	5.85	2070	6.1	7.26	1190	1.0 ± 0.1	21.2 ± 0.8	22500
2-(2′-naphthyl)methyl-benzimidazole	1.8 ± 0.3	9.87 ± 0.50	5540	0.17 ± 0.05	2.60 ± 0.30	16200	3.1 ± 0.8	15.6 ± 2.1	5030

Data are mean ±SD of three individual estimates; where uncertainties are not shown the data are mean values of duplicate estimates that did not vary by more than 9%.
Units are; K_s, μM; ΔA_{max}, absorbance units/nmole cytochrome P-450; $\Delta A/K_s$, absorbance units/nmole cytochrome P-450/M.

100 μM enhancement was only 5% over control activity. In view of the potential problems associated with compound solubility, and membrane disruption effects due to the quite high hydrophobic character of these compounds, the inhibitors were not tested at concentrations greater than 100 μM.

Aniline *p*-hydroxylase activity in phenobarbitone-induced rat hepatic microsomes was relatively susceptible to inhibition by the isomeric naphthylmethylbenzimidazoles. Although the 1′-isomer was a slightly more potent inhibitor than the 2′-isomer at concentrations between 0 and 50 μM, by 100 μM the two compounds were essentially equipotent (about 40% inhibition of the control activity catalysed by phenobarbitone-induced microsomes; Fig. 3). β-Naphthoflavone pretreatment was associated with induction of aniline *p*-hydroxylase activity that appeared quite resistant to inhibition by the compounds. At each of the test concentrations the isomers appeared essentially equipotent as inhibitors but even at a concentration of 100 μM only about a 20% decrease in activity was produced.

Spectral binding interactions of 2-(naphthyl)-methylbenzimidazoles in differently-pretreated rat liver microsomes

The capacity of the isomers to interact with ferric P-450 was assessed in hepatic microsomes from untreated, phenobarbitone-induced and β-naphthoflavone-induced rats (Table 2). In all cases, the compounds were found to elicit type I binding interactions of high affinity (low K_s values). However, it was noted that the extent of the binding interaction (ΔA_{max}) was highly dependent upon the nature of the animal pretreatment. In the case of the 1′-isomer, induction by β-naphthoflavone was associated with an approximate 3-fold increase in binding affinity (K_s) and an approximate 3.5-fold increase in the proportion of P-450 undergoing the spectrally-observed interaction (Table 2). In addition, the ratio $\Delta A_{max}/K_s$, a measure of the efficiency of the binding reaction, was more than 10-fold greater in β-naphthoflavone induced microsomal fractions than in fractions from untreated rat liver. In contrast, phenobarbitone induction decreased the affinity of the 1′-isomer for P-450 despite a slight increase in the ΔA_{max} parameter. Overall, in phenobarbitone-induced hepatic microsomes, the binding efficiency of the 1′-isomer was about one-half that in untreated microsomes.

An opposite trend emerged from studies of the binding characteristics of the 2′-isomer. Here, phenobarbitone induction markedly increased binding affinity and, despite the finding that ΔA_{max} was decreased to only one-quarter of that in untreated hepatic microsomes, the binding efficiency was elevated about 3-fold (Table 2). β-Naphthoflavone induction decreased the binding affinity of the 2′-isomer about 2-fold, a finding in direct contrast with the situation regarding the 1′-isomer. The value of the $\Delta A_{max}/K_s$ parameter suggested that exposure of rats to this inducing agent decreased the overall efficiency of binding.

DISCUSSION

The results of the present study add to the growing

body of evidence that different P-450-mediated monooxygenase activities are differently susceptible to the action of a range of inhibitiory compounds [4–7]. A number of existing studies have established that lipophilicity is a major determinant of the affinity of exogenous compounds for the binding site(s) present at the active site of P-450 isozymes [13, 23]. It is clear that lipophilic character also determines inhibitory potency of nitrogen heterocycles such as the imidazoles and benzimidazoles [4, 13]. The importance of steric factors to monooxygenase inhibition has also been proposed [14, 24]. The size of the benzimidazole 2-substituent has been shown to be important in the inhibition of rat hepatic microsomal APDM activity [14].

It has emerged that benzo[*a*]pyrene hydroxylase in hepatic microsomes from phenobarbitone-induced rats is susceptible to inhibition by small nitrogen heterocycles whereas that activity from 3-methylcholanthrene-pretreated animals is refractory to the same compounds [10, 13]. The inclusion of additional fused or non-fused aromatic groupings into the benzimidazole nucleus produced potent inhibitors of the 3-methylcholanthrene-inducible activity [25]. As equilipophilic alkyl-substituted nitrogen heterocycles generally possess no capacity to modulate this particular monooxygenase activity it is clear that other physiochemical properties can influence overall inhibition potency. Thus a plausible explanation has emerged to account for the activity of the well-studied ellipticines and β-naphthoflavone as inhibitors of 3-methylcholanthrene-induced benzo[*a*]pyrene hydroxylase [26, 27]. In the present study β-naphthoflavone-inducible EROD and ECOD activities differed greatly in their susceptibility to inhibition by the isomeric naphthylmethylbenzimidazoles. Both monooxygenases appear to be catalysed by the P-450 isozyme βNF-B, which may constitute up to about 70% of the total P-450 present in these microsomes [1], yet the 1′-isomer was a potent inhibitor of EROD and the 2′-isomer was more potent against ECOD activity. A possible explanation for this effect may lie in an analysis of the conformation of the two naphthylmethylbenzimidazoles shown in Fig. 4. In the 1′-isomer, rotation about the bonds between the two aromatic systems and the methylene carbon atom is severely restricted by the 8′-hydrogen atom of the naphthyl substituent. The steric interaction between this atom and the π-electron system of benzimidazole results in a relatively rigid molecule and, although not planar, this conformation may promote the superior inhibition of EROD that was observed with the 1′-isomer. Certainly 7-ethoxyresorufin is a much larger molecule than 7-ethoxycoumarin and it remains a possibility that 7-ethoxyresorufin binding involves a greater proportion of the available binding region in the P-450 active site for optimal metabolism. The much more flexible 2′-isomer was a more potent inhibitor of ECOD activity which suggests that the binding of 7-ethoxycoumarin in the active region of β-naphthoflavone-induced P-450 involves a site of smaller dimensions and with greater flexibility than that involved in 7-ethoxyresorufin binding.

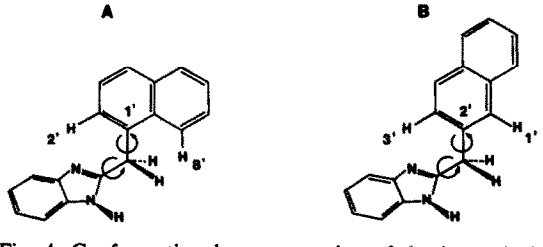

Fig. 4. Conformational representation of the isomeric 2-(naphthyl)methylbenzimidazoles showing bonds around which rotation is theoretically possible. In the 1′-isomer (A) rotation is restricted by the overlap of the naphthyl group 8′-hydrogen atom and the benzimidazole nucleus. In the 2′-isomer (B) steric overlap is much less pronounced.

Inhibition studies in phenobarbitone-induced microsomes revealed the apparently equal potency of the two isomers against each of the three monooxygenase activities. These activities are all probably catalysed by the P-450 isozyme PB-B [1]. The equipotency of inhibition suggests that the same number of sites, perhaps only one, is involved in the binding of all three substrates. The structural, and presumably steric, requirements for inhibition would seem to be the same for both isomers. The fact that I_{50}s versus APH were greater than those against APDM, which were in turn greater than those against ECOD probably relates to the relative affinities of inhibitors and substrates for PB-B under the assay conditions.

Inhibition data obtained using hepatic microsomes from untreated rats are not clear cut and should not be over-interpreted. More than a single isozyme of P-450 would be expected to catalyse APDM activity in these microsomes [1, 28]. Furthermore, ECOD and APH activities are not well-defined with respect to the isozymic forms involved. Instead, these data probably reflect the interaction of the isomeric 2-(naphthyl)methylbenzimidazoles with different P-450 isozymes; any additional statements are probably too speculative to add significantly to this assertion. Nevertheless it is of particular interest that the 2′-isomer enhanced APH activity in untreated microsomal fractions whereas the 1′-isomer elicited no enhancement and was instead weakly inhibitory. Studies are continuing to investigate the structural basis of this phenomenon.

The isolation of individual P-450 isozymes has led to significant advances in the understanding of monooxygenase catalysis. Detailed studies with a range of chemical probes are still required to delineate the subtle differences in binding sites between the cytochromes. Such studies would add considerably to our detailed knowledge of the structural requirements of substrate binding, and catalysis by P-450s and eventually to an explanation of steric factors regarding monooxygenase inhibition and enhancement by nitrogen heterocycles.

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