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THE RELATIONSHIP BETWEEN THE BINDING OF 2-*n*-ALKYLBENZIMIDAZOLES TO RAT HEPATIC MICROSOMAL CYTOCHROME P-450 AND THE INHIBITION OF MONOOXYGENATION

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Abstract—The binding of a homologous series of 2-*n*-alkylbenzimidazoles to rat hepatic microsomal cytochrome P-450 has been examined. Type I, Type RI and mixed Type I/RI spectra were observed with control, phenobarbitone or 20-methylcholanthrene-induced microsomal preparations. In general short chain (C_1–C_4) substituted compounds elicited Type RI spectra, whereas C_5–C_9 substituted benzimidazoles gave rise to Type RI/I or Type I spectra. The type of binding spectrum observed was dependent upon the substrate concentration, the source of microsomes and the length of the substituent alkyl chain. As the lipophilic character of the substituent was increased a corresponding increase in Type I nature was noted. However, an optimal chain length of C_7–C_8 carbon atoms was observed for Type I binding; compounds with longer side chains showed a decreased affinity for the Type I site. The apparent spectral binding constants (K_s values) for the Type I site (but not the Type RI site) were closely associated with the K_i and I_{50} values for the inhibition of cytochrome P-450-dependent monooxygenation. From their inhibition properties it seems that even the short chain (C_1–C_4) substituted benzimidazoles also bind to the Type I site and thus compete for the substrate binding site of cytochrome P-450.

A large number of compounds have been shown to bind to hepatic microsomal cytochrome P-450, eliciting the formation of characteristic binding spectra [1, 2]. Several studies have related the structure of such substances to the type of binding spectrum observed [3, 4]. These spectra have been classified into two main groups; Type I (spectral maximum at 385–390 nm, minimum at 415–425 nm) and Type II (max 425–435 nm and min 395–405 nm). Type I compounds are usually lipophilic compounds and are thought to bind to the hydrophobic protein of cytochrome P-450. Type II compounds are typically substances containing a basic nitrogen atom and are considered to bind to the haem moiety of the haemoprotein since competition for CO has been observed [2]. A third type of binding spectrum is the Type RI (reverse (inverse) Type I or modified Type II) spectral change (max 415–425 nm, min 380–395 nm).

Imidazole and benzimidazole are compounds which elicit typical Type II spectral changes with microsomal cytochrome P-450 [4, 5]. Wilkinson, studying a series of substituted imidazoles, has shown that some 4(5)- and 1-substituted imidazoles bound to cytochrome P-450 with a very high affinity ($K_s < 10\ \mu M$). However, substitution in the 2-position of the imidazole decreased the binding affinity by approximately 2 orders of magnitude [6]. All these compounds produced Type II binding spectra when added to microsomes. The substituted imidazoles were shown to be potent inhibitors of microsomal monooxygenase activity and the spectral dissociation constants (K_s values) appeared to closely parallel the I_{50} values for the inhibition of monooxygenation.

Initial studies using substituted benzimidazoles showed that both Type II and Type RI spectra could be elicited depending on the position of substitution and the chemical nature of the substituent [7]. Some benzimidazoles have also been shown to be inhibitors of certain monooxygenases [8]. The present studies were designed to examine the effects of induction by phenobarbitone and 20-methylcholanthrene on the binding of a series of 2-*n*-alkylbenzimidazoles to rat hepatic microsomal cytochrome P-450 and the relationship between this binding and inhibition of monooxygenases.

MATERIALS AND METHODS

Chemicals. Benzimidazole, 2-methyl-, 2-propyl- and 2-nonylbenzimidazole were purchased from Aldrich Chem. Co. Ltd (Gillingham, U.K.). The other 2-*n*-alkylbenzimidazoles used were synthesized by the reaction of *o*-phenylenediamine with the appropriate carboxylic acids [9]. 7-Ethoxycoumarin [10] and 7-ethoxyresorufin [11] were synthesized by published methods. *N*,*N*-Dimethylformamide (Puriss) was obtained from Koch-Light Laboratories (Colnbrook, U.K.) All other chemicals or biochemicals were purchased from Aldrich Chem. Co. Ltd or Sigma Chem. Co. Ltd (London, U.K.).

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Animals. Male albino COBS–Wistar rats (150–200 g) were obtained from Charles River Ltd. (U.K.) and allowed food and water *ad lib.* The animals were housed in wire-mesh floored plastic cages at 22–24° with a light period of 0700–1900 hr.

Pretreatment of animals and preparation of microsomes. Animals were treated with either sodium phenobarbitone (80 mg/kg) or 20-methylcholanthrene (20 mg/kg) on each of three consecutive days by intraperitoneal injection. The rats were killed on the fourth day, their livers were excised into ice-cold 1.15% (w/v) KCl and washed microsomes were prepared as described by Netter [12]. Microsomal protein was assayed by the method of Lowry *et al.* [13] using bovine serum albumin standards. Log P values were obtained from the literature [30] or determined by Dr. R. Hyde from the octanol:water partition coefficient.

Spectrophotometry. Difference spectra were recorded at 25° using an Aminco DW-2 UV-VIS spectrophotometer in the split beam mode. Microsomal suspensions containing 1–2 mg protein/ml, were equally divided between sample and reference cuvettes and a baseline of equal light absorbance was established between 350 and 500 nm. Substrates were utilized at 250 mM or 500 mM stock solutions in *N,N*-dimethylformamide and added to the cuvette in μl quantities.

Apparent spectral dissociation constants (K_s values) were calculated from plots of the reciprocal absorbance changes vs the reciprocal substrate concentration [2]. At least five substrate concentrations were used for each determination. Wherever appropriate apparent K_s values were calculated for the Type I spectra using the lower end of the concentration range to avoid interference from R Type I binding.

Cytochrome P-450 concentrations were determined by the method of Omura and Sato [14].

Microsomal enzyme assays. 7-Ethoxycoumarin-*O*-deethylation [10] and 7-ethoxyresorufin-*O*-deethylation [11] were assayed by published methods using a Turner filter fluorometer. Incubation mixtures contained 7-ethoxycoumarin (250 μM), microsomal protein (1 mg/ml), NADPH (50 μM) and 66 mM Tris–HCl (pH 7.4); or 7-ethoxyresorufin (500 mM), microsomal protein (200 μg/ml), NADPH (250 μM) and 66 mM Tris–HCl (pH 7.4). The final incubation vol. was 2.5 ml in each case and the assays were carried out at 25° (i.e., the temperature used for binding spectra measurements).

Inhibitors were added as μl quantities of solutions in *N,N*-dimethylformamide. Concentrations of dimethylformamide up to 10 μl/2.5 ml of microsomal suspension had no effect on the initial rate of 7-ethoxyresorufin-*O*-deethylase but very low volumes of the solvent (0.2 μl/2.5 ml) did reduce 7-ethoxycoumarin-*O*-deethylation. Consequently inhibition of 7-ethoxycoumarin metabolism by the compounds tested was corrected for the inhibition produced by solvent alone.

Inhibition constants (K_i values) were calculated from Dixon plots [15]. I_{50} values (the inhibitor concentrations which produced 50% inhibition of the initial rate of metabolism) were determined from the means of duplicate incubations with at least four different inhibitor concentrations.

RESULTS

The binding of 2-n-alkylbenzimidazoles to control, phenobarbitone and 20-methylcholanthrene-induced microsomes

A detailed investigation on the binding of 2-*n*-alkylbenzimidazoles (Table 1) to cytochrome P-450 was carried out using hepatic microsomal preparations from rats treated with phenobarbitone, 20-methylcholanthrene and vehicles (saline and corn oil) alone. No interference from the dimethylformamide used as solvent for the benzimidazoles was observed for Type I or Type RI spectra regardless of the source of the rat hepatic microsomes. It was

Table 1. Binding of 2-*n*-alkylbenzimidazoles to phenobarbitone, 20-methycholanthrene and control rat hepatic microsomes

		Control microsomes			Phenobarbitone			20-Methylcholanthrene		
Substituent	Log P	Type	Apparent K_s	ΔE_{max}	Type	Apparent K_s	ΔE_{max}	Type	Apparent K_s	ΔE_{max}
2-Methyl	1.96	RI	1.54 mM	0.114	RI	0.42, 0.18 mM	0.038	RI	0.38 mM	0.048
2-Ethyl	2.48	RI	2.5 mM	0.111	RI	0.71 mM	0.044	RI	2.5 mM	0.034
2-Propyl	2.96	RI	15.0 mM	0.20	RI	1.0 mM	0.083	RI	10.0 mM	0.330
2-Butyl	3.48	RI	2.5 mM	0.012	RI	0.33 mM	0.045	RI	3.3 mM	0.066
2-Pentyl	3.96	RI	0.18 mM	0.013	RI	0.38 mM	0.046	I	1.10 μM	0.023*
2-Hexyl	4.48	I	0.77 μM	0.007*	I/RI	0.40 μM*	0.012*	I	0.83 μM	0.038*
2-Heptyl	4.96	I	0.29 μM	0.007*	I/RI	0.33 μM*	0.012*	I	1.0 μM	0.048*
2-Octyl	5.48	I	0.17 μM	0.010*	I/RI	0.37 μM*	0.016*	I	0.3 μM	0.036*
2-Nonyl	5.96	I	0.48 μM	0.011*	I	0.40 μM	0.031*	I	0.56 μM	0.041*

ΔE_{max} expressed per mg protein. Values represent the means of at least two experiments on separate microsomal preparations with <10% variation between values for each compound.

* Values refer to those of Type I binding spectrum.

Phenobarbitone = liver microsomes derived from phenobarbitone pretreated animals (1.3 nmoles cytochrome P-450/mg protein).

20-Methylcholanthrene = liver microsomes derived from 20-methylcholanthrene pretreated animals (1.2 nmoles/mg protein).

Controls = liver microsomes containing 0.6 nmoles/mg protein. No significant difference was observed between saline and corn oil pretreated control animals.

noted from earlier experiments [7] that substitution of the 2-position of benzimidazoles produced a Type RI spectrum, the only exceptions being derivatives containing relatively polar moieties.

2-Methyl, 2-ethyl-, 2-propyl- and 2-butylbenzimidazole all produced typical RI spectra with control microsomes (Table 1). 2-Methylbenzimidazole had the lowest apparent K_s value (1.54 mM) and 2-propylbenzimidazole the highest apparent K_s (15.0 mM).

Derivatives containing 2-substituents with longer alkyl chains, i.e., 2-pentyl- and 2-hexylbenzimidazole, produced mixed Type I/Type RI spectra. Type I spectra were observed at low substrate concentrations; as the concentration was increased mixed Type I and Type RI spectral interactions were noted, whereas at high concentrations only Type RI spectra were apparent. As the alkyl chain length was increased, the degree of Type I character also increased. This was deduced from the concentration of the compound which could be added before the observed spectrum became intermediate between Types I and RI. 2-Pentylbenzimidazole gave rise to Type I spectra at concentrations up to 0.2 μM. The elicited spectral change was too small for a K_s value for the Type I component to be determined in the usual manner. The apparent K_s for the Type RI component was found to be 0.18 mM. 2-Hexylbenzimidazole produced Type I binding spectra up to a concentration of 200 μM. However, this compound produced additive Type I spectra up to a final concentration of 150 μM. Concentrations greater than 200 μM of 2-hexylbenzimidazole gave rise to a decreased Type I spectrum indicative of a Type I spectrum containing a Type RI component, resulting in a decreased Type I spectrum. A high affinity apparent Type I binding constant was calculated for 2-hexyl benzimidazole of 0.77 μM. Turbidity due to insolubility of the compound in aqueous microsomal suspensions prevented the titration of concentrations necessary to produce Type RI spectral changes.

2-Heptyl-, 2-octyl- and 2-nonylbenzimidazole all elicited Type I binding spectra with control microsomal cytochrome P-450 (Table 1). The apparent K_s values for these compounds were 0.29 μM, 0.17 μM and 0.48 μM respectively. These three derivatives did not produce Type RI spectra at any concentration tested up to 500 μM. From these results, it appeared that the 2-heptyl and 2-octyl derivatives (containing C_7 and C_8 alkyl chains) bound most avidly to the Type I binding site.

The apparent K_s values for Type RI binding of 2-methyl-, and 2-ethyl-, 2-propyl- and 2-butylbenzimidazole observed with phenobarbitone microsomes were considerably lower than the apparent K_s values established for control microsomes (Table 1). 2-Pentylbenzimidazole produced Type I spectra up to a final concn of 0.6 μM, but intermediate Type I/Type RI spectra were observed at higher concentrations. Type RI spectra only were observed at concentrations above 50 μM. As the length of the alkyl side-chain was increased, the degree of the Type I character increased. Type I spectra only were noted for 2-hexylbenzimidazole (up to 2.4 μM final concn); 2-heptylbenzimidazole (up to 4.0 μM) and 2-octylbenzimidazole (up to 10.0 μM). At concentrations above these values, mixed Type I/Type RI spectra were apparent (Fig. 1). The 2-nonyl derivative produced Type I spectra up to a final concn of 150 μM and intermediate binding spectra were not observed. The 2-heptyl and 2-octyl derivatives had the highest affinity for cytochrome P-450.

With microsomal preparations, from 20-methylcholanthrene-induced rats, the apparent K_s values for the Type RI spectra for 2-methyl, 2-ethyl, 2-propyl and 2-butyl derivatives were similar to those obtained with control microsomes (Table 1). In contrast to both microsomes from phenobarbitone pre-

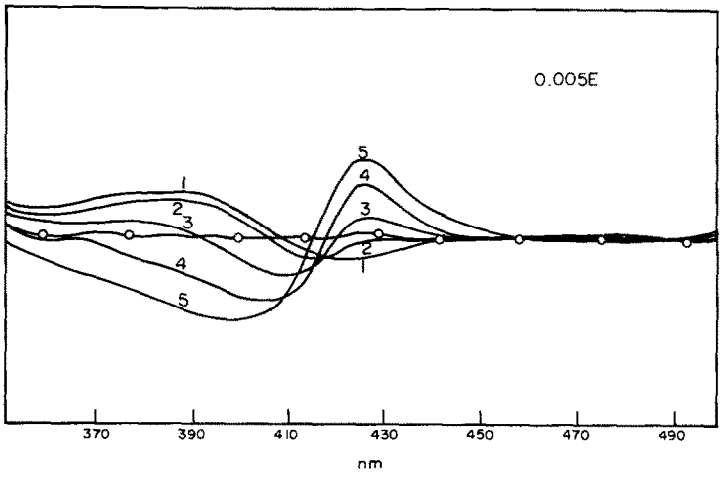

Fig. 1. Effect of concentration on the binding spectrum elicited on the addition of 2-hexyl-benzimidazole to phenobarbitone-induced rat hepatic microsomes. Phenobarbitone-induced microsomes (1 mg protein/ml) in 66 mM Tris–HCl (pH 7.4) were divided equally between two cuvettes and a baseline of equal light absorbance was established between 350 and 500 nm (O—O). Various concentrations of 2-hexylbenzimidazole were added to the sample cuvette and the binding spectra were recorded. Spectrum 1, 0.8 μM; 2, 2.8 μM; 3, 6.8 μM; 4, 150 μM; 5, 400 μM.

treated and control animals, the pentyl to nonyl derivatives all produced Type I binding spectra only over a wide concentration range.

Since the magnitude of the spectral change resulting from substrate interaction is dependent on the concentration of cytochrome P-450 present [16] the theoretical maximal ΔE values were calculated (Table 1). The results showed that the maximal Type I spectral changes observed were for microsomes from 20-methylcholanthrene pretreated microsomes showing that the cytochrome P-450 of these microsomes bound more of the benzimidazole derivatives than either microsomes from control or phenobarbitone pretreated rats.

The inhibition of cytochrome P-450 dependent monooxygenation by 2-n-alkyl-benzimidazoles

2-*n*-Alkylbenzimidazoles were found to inhibit the cytochrome P-450 dependent *O*-dealkylation of 7-ethoxycoumarin and 7-ethoxyresorufin (Table 2).

2-Methyl, 2-ethyl-, and 2-propyl- and 2-butylbenzimidazole showed greater inhibitory character as the substituent chain length (and hence log P value) was increased. The inhibition pattern produced by the C_5–C_9 (pentyl–nonyl) derivatives seemed to be closely related to their affinity for the Type I site. The K_i and I_{50} values for the inhibition shown by these compounds indicated that maximal inhibition of both the monooxygenase reactions studied was given by the C_7 (heptyl) compound. Molecules with longer or shorter side chains than 2-heptylbenzimidazole were weaker inhibitors.

The nature of the binding to cytochrome P-450 also appeared to affect the inhibition of the reactions studied. In particular, a marked increase in the inhibitory character was noted using microsomes from 20-methylcholanthrene-induced rats. Thus 2-pentylbenzimidazole, which elicited a Type I spectrum with microsomes from 20-methylcholanthrene rats, showed a marked increase in inhibitory character compared with the 2-butyl derivative, a Type RI compound. In contrast, these two compounds produced Type RI binding spectra with the microsomes from phenobarbitone pretreated rats and the difference in the inhibition produced by these two compounds was much smaller (Table 2).

DISCUSSION

Lipophilicity has been shown to be a major factor in determining the affinity of xenobiotics for the Type I binding site of microsomal cytochrome P-450 [17, 18]. For example, several series of aliphatic compounds have been shown to bind with increasing affinity to cytochrome P-450 as the chain length is increased [17–19]. However, a critical point is reached at which further increase in lipophilicity does not produce a concomitant decrease in the apparent K_s value for binding and increasing the chain length further produces an increase in apparent K_s. Thus the results reported here for the binding of 2-*n*-alkylbenzimidazoles to control and microsomes from variously induced rats agree with the work of Yih and van Rossum [17] who studied the binding of 5-ethyl-, 5-*n*-alkyl-substituted barbiturates, in that compounds with C_6–C_8 alkyl chains elicit the lowest apparent K_s values. It is likely that steric factors become increasingly important when the critical chain length is exceeded. Wilkinson [19] tested a homologous series of 1-*n*-alkylimidazoles and found that the optimal chain length for binding was a C_8–C_{10} substituent, possibly because of the smaller physical size of the parent imidazole ring compared with benzimidazole.

A number of workers have reported the binding of a compound to one or more microsomal cytochrome P-450 sites [20–24]. The results from the present study suggest that the nature of the binding of 2-*n*-alkyl-benzimidazoles is dependent both on the lipophilicity of the compound and the source of the microsomal preparation. Clear differences were observed in the binding of the derivatives to microsomes obtained from differently pretreated animals. Thus the Type I character associated with the various microsomal preparations tested increased in the

Table 2. Inhibition of mixed function oxidase activity by 2-*n*-alkylbenzimidazoles

	Ethoxycoumarin deethylase				Ethoxyresorufin deethylase	
	Phenobarbitone		20-Methylcholanthrene		20-Methylcholanthrene	
Substitutent	K_i (μM)	I_{50} (μM)	K_i (μM)	I_{50} (μM)	K_i (μM)	I_{50} (μM)
2-Methyl	240.0	620.0	500.0	4480.0	108.0	170.0
2-Ethyl	330.0	600.0	260.0	845.0	51.0	103.0
2-Propyl	260.0	440.0	100.0	417.5	20.8	35.8
2-Butyl	21.0	84.0	20.0	144.0	8.2	17.4
2-Pentyl	18.8	45.6	3.8	13.2	0.25	0.6
2-Hexyl	5.6	14.7	2.6	7.8	0.17	0.15
2-Heptyl	0.8	8.5	2.5	9.3	0.03	0.37
2-Octyl	1.4	10.4	5.8	17.1	0.06	0.38
2-Nonyl	2.4	10.3	6.0	32.0	0.12	0.54

Phenobarbitone and 20-methylcholanthrene refer to microsomes from rats pretreated with phenobarbitone or 20-methylcholanthrene. Values represent means of at least two determinations on separate microsomal preparations with <10% variation between values for each compound.

order phenobarbitone < control < 20-methylcholanthrene, whereas Type RI character increased in the opposite manner, i.e., 20-methylcholanthrene < control < phenobarbitone.

The change from Type I to Type RI binding spectra with increasing substrate concentration indicates that the 2-*n*-alkylbenzimidazoles bind to separate binding sites on microsomal cytochrome P-450. Yoshida and Yumaoka [24] have demonstrated the plausibility of compounds binding simultaneously to Type I and Type RI sites. It is likely that even the short chain benzimidazoles (which appear to have exclusive Type RI binding character when judged by spectral changes alone) also bind to the Type I site. Thus we have shown [25] that only compounds with Type I character are capable of dissociating an isosafrole metabolite cytochrome P-450 complex and that such dissociation is elicited by the short-chain (C_1–C_3-substituted) benzimidazoles. Furthermore, preliminary experiments (M. Dickens and J. W. Bridges, unpublished observations) have shown that the use of Type I modifier (cyclohexane), increases the magnitude of the Type RI spectra elicited by the short chain 2-*n*-alkylbenzimidazoles, suggesting the presence of a Type I component. The ability to form a reverse Type I binding spectrum appears to be related to the possession of an SP^2 hybridized N atom and to the position of alkyl and aryl substituents in the imidazole ring [7, 4, 19].

Various imidazoles have been shown previously to be potent inhibitors of microsomal monooxygenation *in vitro* and *in vivo* [5, 6, 8, 19, 26–28]. This inhibitory action has been correlated in two studies with the lipophilic character of the imidazole [8, 19].

In the present study a close agreement between the K_i or I_{50} values for the inhibitory potency of 2-*n*-alkylbenzimidazoles and the apparent Type I K_s values was seen. A good correlation between the log K_i or I_{50} values and the log P values of the compounds was also observed (see Table 3).

In contrast to Type I binding, the ability to bind to the Type RI site (measured by the Type RI K_s values) was apparently unrelated to the inhibitory properties of these compounds. Inhibition apparently due to Type RI compounds may be due to the fact that they are usually also able to bind to Type I sites to a limited extent. Our results indicate that an optimal chain length of 6–8 carbon atoms existed for both maximal Type I binding and maximal inhibition of mixed function oxidase. An increase beyond this critical chain length produced decreased binding and a corresponding reduction in inhibitory potency. Insolubility of these higher alkyl derivatives may partially contribute to this diminished inhibitory potency. By omitting the octyl and nonyl derivatives from the regression analysis, a marked improvement in the correlation was observed. These results suggest that the close correlation between lipophilicity and the ability of these substrates to inhibit monooxygenation is limited to the C_1–C_7-substituted 2-*n*-alkylbenzimidazoles.

Similar optimum molecular dimensions have been observed for the inhibitory properties on mixed function oxidases of 1-alkylimidazoles (optimum side chain C_8 to C_{10}) [19] and for various series of aliphatic compounds which are able to displace the isosafrole adduct from the form of cytochrome P-450 induced by benzodioxoles [25]. The fact that the molecular dimensions for optimal binding are so similar for such a range of structures, although their lipophilicities differ considerably, suggest that these compounds can be used as 'molecular rulers' for the Type I binding site. Based on this concept it may be deduced by taking into conjunction the present data with that of Wilkinson *et al.* [19], Dickins *et al.* [25] and Yih and van Rossum [17] that the spatial geometry for the Type I binding site of at least three different forms of cytochrome P-450, namely those induced by phenobarbitone, 20-methylcholanthrene and isosafrole may have very similar dimensions. This feature may be the means by which fatty acid interactions with these major forms of cytochrome P-450 are minimised [29].

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Table 3. Correlation of log K_i or log I_{50} with the log P values for the inhibition of 7-ethoxycoumarin-*O*-deethylase (ECOD) and 7-ethoxyresorufin-*O*-deethylase (EROD) activities by 2-*n*-alkylbenzimidazoles

Microsomes*	Enzyme activity	Inhibition value	Correlation coefficients (C_1–C_9)†	(C_1–C_7)†
PB	ECOD	K_i	−0.929	−0.947
PB	ECOD	I_{50}	−0.951	−0.975
MC	ECOD	K_i	−0.857	−0.972
MC	ECOD	I_{50}	−0.842	−0.970
MC	EROD	K_i	−0.929	−0.976
MC	EROD	I_{50}	−0.888	−0.948

* PB and MC refer to microsomes from animals pretreated with phenobarbitone or 20-methylcholanthrene.

† (C_1–C_9) and (C_1–C_7) refer to the substituted benzimidazoles used for the regression analysis.

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