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Imidazole derivatives as inhibitors of cytochrome P-450-dependent oxidation and activators of epoxide hydrolase in hepatic microsomes from a marine fish

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A constantly expanding class of microsomal monooxygenase (MFO) inhibitors comprises the nitrogen heterocycles. Monocyclic (imidazoles [1–7]; oxazoles and thiazoles [8]), bicyclic (benzimidazoles [9, 10]), and tetracyclic (ellipticines [11, 12]) derivatives can inhibit MFO activity in microsomes from variously pretreated rats and insects. Values for the spectral binding constants (K_s) correlated well with I_{50} values for a homologous series of 1-alkylimidazoles [2] as well as for other compounds [11], suggesting that the binding of nitrogen heterocycles to cytochrome P-450 is directly responsible for the observed inhibition [2, 11]. To further explore the relationship between binding and inhibition, we studied the ability of a mono-, bi- and tricyclic nitrogen heterocycle to bind to (K_s) and inhibit (I_{50}) the metabolic activity of cytochrome P-450 in control and induced hepatic microsomes from the rat* and a marine fish. The compounds selected were 4(5)-phenylimidazole (4PI)†, benzimidazole (BI), and naphtho(2,3,4′,5′)-imidazole (NI). In the rat, the different cytochrome P-450 isozymes present in control, PB-induced or PAH-induced hepatic microsomes are known to selectively oxidize different sites of the BP molecule [13-17]. We felt that investigation of the effects of 4PI, BI and NI on the pattern of BP metabolites formed by control and induced hepatic microsomes might indicate whether or not total heterocyclic ring size is a determinant of inhibitory selectivity towards the different cytochrome P-450 isozymes present. This report concerns our findings for the marine teleost sheepshead, *Archosargus probatocephalus*.

Studies with fish are complicated by the problem of defining "control" activity in animals captured from natural, possibly polluted environments. In our laboratory, we routinely use α-NF in benzo[*a*]pyrene hydroxylase (AHH) incubations for its ability to differentiate between control (activated) and PAH-induced (inhibited) MFO activities [18–20]. The description of compounds having specificities different from that of α-NF would provide another useful tool in this regard, if the basis for the effect on MFO is understood. In addition, selective inhibitors of cytochrome P-450 isozymes may provide useful probes for studying the mechanisms of microsomal oxidation; the nitrogen heterocycle, ellipticine, has been used successfully in this manner [21].

BI was prepared as described previously [9]. NI was synthesized similarly, by the condensation of naphtho-2,3-diamine (Aldrich Chemical Co., Milwaukee, WI) and formic acid, and 4PI was purchased from Aldrich and recrystallized from aqueous ethanol. Ellipticine was supplied by Mr. L. H. Kedda of the National Cancer Institute, N.I.H., Bethesda, MD.

Sheepshead (500–700 g) were netted near Marineland, FL, and maintained at Whitney Marine Laboratory [20]. Sheepshead MFO activities were induced by injecting a corn oil suspension of 3-MC (20 mg/kg, i.p.) 5 days before sacrifice [22].

Hepatic microsomes were prepared and 7-EC, BND and AHH activities were determined as described previously [20, 23]. I_{50} values were determined in the usual manner [9] from incubations of at least six concentrations of each compound. Apparent spectral binding dissociation constants (K_s) were determined in duplicate from linear regression analysis of double reciprocal plots of ΔA (peak to trough) versus ligand concentration for at least five concentrations of ligand. Spectra were recorded with an Aminco DW-2 spectrophotometer (American Instrument Co., Silver Springs, MD). Epoxide hydrolase (EH) was measured using styrene oxide (SO) as substrate [24, 25]. Nitrogen heterocycles were added to EH incubations from stock solutions in 0.025 N HCl (4PI, BI and NI) or DMSO (ellipticine); solvent was added to control incubations. The amount of acid added did not alter the pH of the buffered incubation mixture and neither the acid nor the compounds themselves increased the non-enzymatic hydrolysis of SO.

Incubations for subsequent high pressure liquid chromatography (h.p.l.c.) analysis contained 80 nmoles (0.45 μCi) [^{14}C]BP (Amersham, Arlington Heights, IL), 0.12 mM $MgCl_2$, 2 mM NADPH, inhibitor solution or solvent, 0.1 M Hepes (pH 7.7), and 0.1 to 0.5 mg microsomal protein in a final volume of 1 ml. After incubating for 15 min at 35°, the reaction was stopped by extracting with ethyl acetate (3 × 3 ml). The combined organic phase (>97 percent of the added radioactivity) was dried, concentrated under reduced pressure, taken up in 0.2 ml methanol, and stored under nitrogen in amber vials at −20° until h.p.l.c. analysis. Samples of the concentrated extract (2–3 × 10^5 dpm) were chromatographed on an Altex Ultrasphere ODS 5 μm reverse-phase column with an Altex 322 MP h.p.l.c. using a programmed methanol/water gradient (55–100% methanol) at a flow rate of 0.68 ml/min. The eluate passed through fluorometric and spectrophotometric (280 nm) detectors and was, then, collected in 0.5-min fractions for determination of ^{14}C-content by liquid scintillation counting. Authentic standards of BP metabolites (IIT Research Institute, Chicago, IL) eluted in the order described by Yang *et al.* [13], and their retention times were used to identify metabolite peaks. Total activity was determined from the net radioactivity eluted before BP.

We have studied the binding and inhibitory activity of 4PI, BI and NI with control and 3-MC-induced hepatic microsomes from the marine teleost sheepshead (Table 1). Normally the protocol for these experiments would include the microsomal preparation resulting from treatment of animals with PB. PB-type induction has not yet been

* P. J. Little and A. J. Ryan, unpublished studies.

† Abbreviations: 4PI, the tautomeric compound 4(5)-phenylimidazole; α-NF, 7,8-benzoflavone; 3-MC, 3-methylcholanthrene; PB, phenobarbital; PAH, polycyclic aromatic hydrocarbon; BI, benzimidazole; NI, naphtho (2, 3, 4′, 5′) imidazole; SO, styrene oxide; BP, benzo[*a*]pyrene; AHH, aryl hydrocarbon (BP) hydroxylase; BP-9,10-diol, *trans*-9,10-dihydro-9,10-dihydroxybenzo[*a*]pyrene, 9-HO.BP, 9-hydroxybenzo[*a*]pyrene, BP-9,10-oxide, benzo[a]pyrene 9,10-oxide, BP-1,6-dione, benzo[*a*]pyrene 1,6-quinone, and similarly for other BP dihydrodiols, phenols, oxides and quinones; EH, epoxide hydrolase; 7-EC, 7-ethoxycoumarin *O*-deethylase; BND, benzphetamine *N*-demethylase; DMSO, dimethylsulfoxide; and Hepes, 4-(2-hydropyethyl)-1-piperazine-ethanesulfuric acid.

Table 1. Binding and inhibitory activity of 4(5)-phenylimidazole, benzimidazole, and naphtho(2,3,4',5')imidazole toward cytochrome P-450-mediated mixed-function oxidase activity in control and induced hepatic microsomes from the marine teleost sheepshead, *Archosargus probatocephalus**

	K_s (M) or I_{50} (M)		
	Benzimidazole	Naphtho(2,3,4',5')imidazole	4(5)-Phenylimidazole
Control microsomes			
Binding constant (K_s)	2.85×10^{-4}	5.60×10^{-5}	5.65×10^{-6}
Enzyme inhibition (I_{50})			
7-Ethoxycoumarin O-deethylase	7.9×10^{-4}	2.0×10^{-4}	1.9×10^{-5}
Benzphetamine N-demethylase	2.9×10^{-3}	5.6×10^{-4}	2.2×10^{-5}
Aryl hydrocarbon hydroxylase	2.8×10^{-3}	2.0×10^{-4}	3.0×10^{-4}
3-MC-Induced microsomes			
Binding constant (K_s)	1.28×10^{-3}	2.31×10^{-5}	4.37×10^{-6}
Enzyme inhibition (I_{50})			
Ethoxycoumarin deethylase	1.5×10^{-3}	1.0×10^{-4}	4.3×10^{-5}
Benzphetamine N-demethylase	2.0×10^{-3}	5.4×10^{-4}	2.1×10^{-5}
Aryl hydrocarbon hydroxylase	NI * at 10^{-3} †	6.4×10^{-4}	1.1×10^{-3}

* Control microsomal values (N) were: 7-ethoxycoumarin *O*-deethylase (7-EC) 0.19 ± 0.15 (6) (nmole · mg^{-1} · min^{-1}), benzphetamine *N*-demethylase (BND) 1.01 ± 0.51 (6) (nmoles · mg^{-1} · min^{-1}) and aryl hydrocarbon hydroxylase (AHH) 3.1 ± 1.8 (6) (F.U. · mg^{-1} · min^{-1}); induced microsomal values (N) were: 7-EC 1.1 ± 0.25 (5) (nmoles · mg^{-1} · min^{-1}), BND 1.1 ± 0.3 (5) (nmoles · mg^{-1} · min^{-1}), and AHH 23.9 ± 4.8 (5) (F.U. · mg^{-1} · min^{-1}). One fluorescence unit equals 0.05 nmoles of 3-HO.BP.

† No inhibition.

demonstrated in marine species [26, 27]; specifically, we have found that PB treatment has no effect on the microsomal MFO system of sheepshead.*

As predicted from other studes [28] and as described previously [9, 10], interaction of the non-bonded electrons of the heterocyclic nitrogen atoms of NI, BI and 4PI with the heme of cytochrome P-450 resulted in type II difference spectra in control and 3-MC-induced sheepshead hepatic microsomes. Rank order for the binding strength of the nitrogen heterocycles was the same for both control and 3-MC-induced microsomes and was influenced both by the degree of steric hindrance at the binding nitrogen atom and by the lipophilicity of the nitrogen heterocycle [2, 3]. The K_s values for the strongest binding compound, 4PI, were similar to the value of 5.2×10^{-6} M that was reported for the binding of 4PI to the cytochrome P-450 of control rat liver microsomes [3].

As inhibitors of 7-EC, each compound showed similar activity in both control and 3-MC-induced microsomes. The order of decreasing potency was 4PI > NI > BI. A similar result was obtained for the inhibition of a classic PB-inducible (in mammals) microsomal MFO activity, BND. In contrast, this order of inhibitory potency was not maintained against AHH activity, a reaction which is mildly induced (in mammals) by PB treatment and strongly induced (in mammals and fish) by 3-MC or other PAH treatment [14-17, 22, 29]. As an inhibitor of AHH activity, 4PI was approximately equally potent to NI in control microsomes but 4PI was 2-fold less active than NI in 3-MC-induced microsomes and BI was not inhibitory (Table 1). Rogerson *et al.* [3] found that 4PI was a potent inhibitor of aldrin epoxidation in both control rat liver microsomes ($I_{50} = 4.6 \times 10^{-6}$ M) and an armyworm gut preparation ($I_{50} = 1.3 \times 10^{-5}$ M). We have also found that 4PI was a good inhibitor ($I_{50} = 3.3 \times 10^{-5}$ M) of AHH activity in PB-induced rat liver microsomes.†

Guenthner *et al.* [21] propose that inhibition of hepatic microsomal MFO by ellipticine involves direct interaction of the nitrogen heterocycle and cytochrome P-450 and depends upon the extension of a hydrophobic portion of the nitrogen heterocycle onto the NADPH-cytochrome P-450 reductase binding site. Polycyclic nitrogen heterocycles are known to inhibit both cytochrome P-450 and cytochrome P-448 [11, 12], and numerous examples exist of the potent inhibitory activity of monocyclic nitrogen heterocycles with cytochrome P-450 (control or PB-induced microsomes) [2, 3, 7], but we are not aware of reports of the interaction of monocyclic nitrogen heterocycles with cytochrome P-448. In the present study, 4PI bound strongly to cytochrome P-448 of 3-MC-induced fish liver microsomes, but did not inhibit the metabolism of BP. One explanation for this lack of inhibition is that 4PI does not bind to cytochrome P-448 in the presence of BP. Another explanation, in terms of the proposed model [21], is that, although 4PI binds strongly to the heme of cytochrome P-448 giving a type II difference spectrum, it is not large enough to interfere with the hydrophobic binding site of NADPH-cytochrome P-450 reductase to cytochrome P-448. It would follow that the binding of 4PI to cytochrome P-448 does not, in itself, cause inhibition of metabolic function. BI, which when bound to the heme of cytochrome P-448 would extend less far, was a less potent inhibitor than 4PI, and the tricyclic derivative, NI, which would extend further than 4PI (and is more lipophilic), did inhibit AHH activity in microsomes from 3-MC-induced sheepshead. Even in fish, control and induced microsomes probably contain more than one form of cytochrome P-450 [22]. A third explanation of these results is that the difference spectra result from interaction with the major form of cytochrome P-450 present in microsomes, whereas metabolic activity toward a particular substrate is mediated by a form that is a minor constituent.

The effects of 4PI and NI on position specific metabolism of BP in control and 3-MC-induced sheepshead hepatic microsomes are summarized in Table 2. The pattern of BP

* M. O. James and P. J. Little, in press.

† P. J. Little and A. J. Ryan, unpublished data.

Table 2. Effect of 4(5)-phenylimidazole and naphtho(2,3,4′,5′)imidazole on the pattern of benzo[*a*]pyrene metabolites formed in hepatic microsomes from control and induced sheepshead, *Archosargus probatocephalus*

	RATE OF METABOLITE FORMATION*(pmoles/mg protein/min)								
	9,10-Diol	4,5-Diol	7,8-Diol	1,6-Quinone	3,6-Quinone	6,12-Quinone	Phenols 1†	Phenols 2†	Total
Control sheepshead									
Solvent	65.4	13.7	51.7	18.0	19.1	4.8	40.5	30.7	280
4(5)-Phenylimidazole									
5×10^{-4} M	19.9	5.2	23.1	3.8	9.2	6.6	12.2	10.4	90
1×10^{-5} M	37.2	6.7	40.1	5.0	11.4	6.7	19.9	18.7	143
Naphtho(2,3,4′,5′)imidazole									
5×10^{-4} M	28.4	2.0	20.8	0	4.4	6.3	16.2	7.9	86
1×10^{-5} M	41.9	6.8	36.6	10.2	10.4	15.1	27.0	14.3	165
3-Methylcholanthrene-induced sheepshead									
Solvent	332.5	59.9	641.8	89.6	174.7	20.3	261.9	228.1	2410
4(5)-Phenylimidazole									
1×10^{-3} M	416.3	33.1	587.7	84.9	134.4	19.9	123.9	137.4	1940
5×10^{-4} M	424.0	48.4	628.6	71.8	137.8	22.1	157.0	133.9	2060
1×10^{-5} M	332.5	41.7	618.9	112.8	102.9	22.3	232.1	199.9	2290
Naphtho(2,3,4′,5′)imidazole									
5×10^{-4} M	391.2	21.7	534.2	61.6	97.8	19.9	110.5	79.7	1590
1×10^{-5} M	390.2	24.0	523.5	72.1	127.6	20.3	166.5	112.8	1710

* The results shown are from individual control and induced sheepshead. Similar results were obtained with two other control and induced fish. The experimental error was less than 2 percent for individual rates below 50 pmoles · (mg protein)$^{-1}$ · min^{-1} and less than 1 percent for higher rates.

† Phenols 1 includes 9-HO.BP and phenols 2 includes 3-HO.BP; each peak also includes other phenols.

metabolites formed by control sheepshead liver microsomes was different from that of control rat liver microsomes but similar to the pattern of metabolites formed by PAH-induced rat liver microsomes (or 3-MC-induced sheepshead liver microsomes). Relative to untreated fish, 3-MC induction resulted in a decrease (as a percent of total metabolites formed) in the rate of formation of the 9,10-diol (to 13.8 percent of total) and an increase in the rate of formation of the 7,8-diol (to 26.6 percent of total); the rate of formation of BP-4,5-diol increased 4-fold when overall metabolism increased 9-fold, showing a similar response to PAH-induction as observed in the rat [15].

The perturbations to the control sheepshead metabolic pattern in the presence of inhibitors were: (1) the increased formation of BP-9,10-diol from 23.4 to 33.0 percent of total metabolites in the presence of 5×10^{-4} M NI, (2) the increased formation of BP-7,8-diol from 18.5 to 28.0 percent of total metabolites in the presence of 10^{-5} M 4PI, and (3) the increased formation (as a percent of total metabolites) of BP-6,12-dione in the presence of both concentrations of both 4PI and NI. The effects of several concentrations of 4PI and NI on the pattern of BP metabolites formed by hepatic microsomes from 3-MC-treated sheepshead are shown in Table 2 and Fig. 1. The rate of formation of BP-4,5-diol was decreased by all concentrations of 4PI and NI tested and indicated the need to examine the effects of these compounds on a preparation such as PB-induced rat liver microsomes in which the 4,5-diol is a major metabolite [15]. 4PI and NI at all concentrations tested caused inhibition of the rate of formation of both phenols 1 and phenols 2 and an increased rate of formation of BP-9,10-diol (Table 2). Phenols 1 was often partially resolved into two fluorescent peaks (Fig. 1, peaks I and II) that corresponded to authentic 9-HO.BP and 7-HO.BP respectively. Figure 1 shows that the decreased rate of formation of phenols 1 in the presence of 5×10^{-4} M 4PI was due to a specific decrease in the rate of formation of 9-HO.BP. BP-9,10-diol and 9-HO.BP are formed through the common intermediate BP-9,10-oxide [13]. That the formation of BP-9,10-diol was increased and 9-HO.BP was decreased suggested that the nitrogen heterocycles altered the pathway for the further metabolism of BP-9,10-oxide to favor hydrolysis over isomerization.

There have been several reports [6, 30–32] of compounds that stimulate EH *in vitro*, including one relating to the nitrogen heterocycle, 1-isopropylphenylimidazole [6]. We investigated the effect of 4PI, BI and NI on EH activity in hepatic microsomes from control and 3-MC-treated sheepshead; the potent cytochrome P-450 inhibitor ellipticine was included in this study. The substrate used, SO, is a model EH substrate and may be expected to behave similarly to arene oxides such as BP-9,10-oxide [32]. The EH specific activity of hepatic microsomes from one 3-MC-induced sheepshead was 3.2 nmoles styrene glycol/min per mg protein (mean of duplicate determinations, experimental error < 0.5 percent). In the presence of 1.3×10^{-3} M NI, BI or 4PI, respective specific activities of 13.3, 12.6 and 12.2 nmoles/min per mg protein were measured. Ellipticine (1.5×10^{-5} M) caused activity to increase to 7.8 nmoles/min per mg protein. This experiment was repeated with hepatic microsomes from two different 3-MC-induced sheepshead and three controls, and the extent of stimulation of EH activity by each compound was similar in each preparation. These results suggest that the increased rate of formation of the BP-diols shown in Table 2 was due to the stimulation of EH activity by NI and 4PI. Others have shown that addition of EH to incubations decreases the mutagenicity of BP metabolites [33, 34] and that the relative activities of cytochrome P-450 and EH are more important than the absolute levels of these microsomal enzymes in determining the ultimate metabolic profile of BP [35]. The compounds described in this report move the balance of cytochrome P-450/EH activities to favor EH, not only by inhibiting cytochrome P-450 dependent oxidation, but also by stimulating EH activity.

Because it did not provide a cytochrome P-450 microsomal preparation equivalent to the control or PB-induced rat, the sheepshead was not an ideal model for studying the inhbitory selectivity toward cytochrome P-450 of these nitrogen heterocycles. Indeed, this study provided evidence

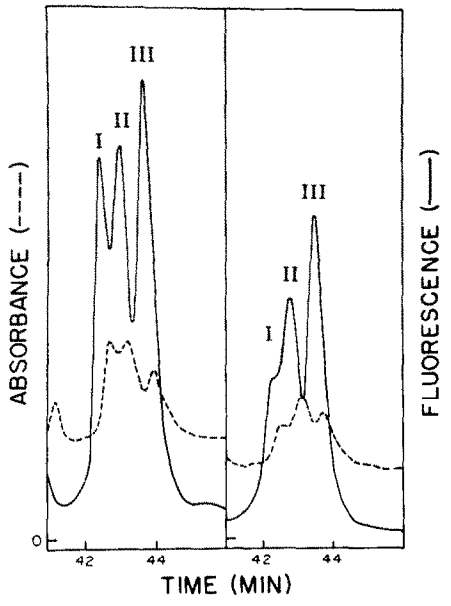

Fig. 1. Effects of 4PI and NI on BP metabolites formed by hepatic microsomes from 3-MC-treated sheepshead. Chromatogram of BP phenols eluting from an Altex Ultrasphere ODS 5 μm h.p.l.c. column after injecting samples resulting from the metabolism of BP by hepatic microsomes from 3-MC-treated sheepshead. Left: control incubation. Right: incubation in the presence of 5×10^{-4} M 4(5)-phenylimidazole. Peak I is 9-HO.BP, peak II is 7-HO.BP, and peak III includes 1- and 3-HO.BP. Column detection was by u.v. absorbance at 280 nm (0.16 absorbance units) and fluorescence (ex. < 390 nm, em > 460 nm, range × 0.5). These standards have been shown previously to elute from a h.p.l.c. column in this order [13].

that the cytochromes of control fish liver microsomes were more similar to the PAH-induced form than is the case for the rat. First, control and 3-MC-induced sheepshead microsomes gave similar patterns of BP metabolites; second, 4PI was 10-fold less active toward the inhibition of control sheepshead AHH activity than against AHH activity in PB-induced rat liver microsomes; and third, the K_s values were similar in control and 3-MC-induced sheepshead microsomes which is not the case in the rat and mouse. Other untreated fish species (scup [36, 37], coho salmon and starry flounder [38], and trout [39]) yield hepatic microsomes which form a pattern of BP metabolites as presently described. However, in these fish, AHH activity is sometimes high and can be inhibited *in vitro* by α-NF, suggesting the possibility of environmental exposure to inducing agents. AHH activity in control sheepshead liver microsomes was relatively high but could be stimulated *in vitro* by α-NF.

In summary, 4PI, BI and NI each gave similar difference spectra and K_s values for binding to the cytochrome P-450 of hepatic microsomes from control and 3-methylcholanthrene-treated sheepshead; 4PI had the highest affinity having a K_s value of 5.65×10^{-6} M with control and 4.37×10^{-6} M with 3-MC-induced microsomes. For 4PI, BI and NI, I_{50} values against control and induced hepatic microsomal 7-ethoxycoumarin *O*-deethylase, benzphetamine *N*-demethylase and benzo[a]pyrene hydroxylase (AHH) activities were determined. Despite its high affinity, 4PI was a weak inhibitor of AHH in control hepatic microsomes ($I_{50} = 3.0 \times 10^{-4}$ M) and 3-MC-induced microsomes ($I_{50} = 1.1 \times 10^{-3}$ M). 4PI and NI increased the rate of formation of BP-9,10-diol while inhibiting the overall rate of formation of BP metabolites by hepatic microsomes from 3-MC-treated sheepshead; this perturbation to the metabolic profile could be due to the activity of these compounds (and BI and ellipticine) to stimulate epoxide hydrolase activity in both control and induced microsomes.

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National Institute of Environmental Health Sciences, c/o C. V. Whitney Marine Laboratory, St. Augustine, FL 32084, *U.S.A., and N.I.E.H.S., Research Triangle Park, NC* 27709, *U.S.A.*

PETER J. LITTLE*
MARGERET O. JAMES†
JOHN R. BEND
ADRIAN J. RYAN

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* Present address: Clinical Research Unit, Baker Medical Research Institute, Alfred Hospital, Commercial Road, Prahran, Vic. 3181, Australia.

† Present address and address for correspondence: Dr. Margaret O. James, Department of Medicinal Chemistry, College of Pharmacy, Box J4, JHMHC, University of Florida, Gainesville, FL 32610, U.S.A.

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