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Biochemical Pharmacology, Vol. 31, No. 9, pp. 1795–1798, 1982.
Printed in Great Britain.

0006-2952/82/091795-04 $03.00/0

Inhibitors of hepatic mixed function oxidases—V*. Inhibition of aminopyrine *N*-demethylation and enhancement of aniline hydroxylation by benzoxazole derivatives

(*Received* 28 *July* 1981; *accepted* 16 *December* 1981)

Many nitrogen heterocycles are potent inhibitors of microsomal mixed function oxidases (MFO) [1–12]. Studies with imidazole derivatives (including benzimidazoles) have described the dependence of inhibitory activity on hydrophobicity [2, 7] and the influence of steric factors on inhibitory activity and binding affinity to cytochrome P-450 [3]. In these investigations of potential inhibitors, some compounds have been found to enhance microsomal MFO activity *in vitro*. In an investigation of oxazoles and thiazoles, Smith and Wilkinson [9] found that benzoxazole stimulated the *N*-demethylation of *p*-chloro-*N*-methylaniline in rat liver microsomes. However, Holder *et al.* [7] had previously reported that two benzoxazole derivatives inhibited the *N*-demethylation of aminopyrine and stimulated the hydroxylation of aniline. To investigate more fully the interaction of benzoxazoles with microsomal oxidases we have studied the effects of a series of benzoxazoles on aminopyrine *N*-demethylase (APDM) and aniline *p*-hydroxylase (AH) activities in hepatic microsomes from phenobarbitone (PB)-treated rats. These enzyme substrates were chosen as compounds which afford cytochrome P-450-difference spectra representative of the two principal types, namely type I (aminopyrine) and type II (aniline) [13, 14].

Materials and methods

Alkylbenzoxazole derivatives (compounds I, III–VII and IX, Table 1) were synthesised by refluxing equimolar quantities of *o*-aminophenol or 2-amino-4-methylphenol with the appropriate organic acid [15]. Compound VII was purified by recrystallisation from petroleum ether/toluene and the other benzoxazoles, which were liquids at ambient temperature, were purified by distillation. All synthesised compounds had physical properties similar to reported values and gave satisfactory elemental analyses (Australian Microanalytical Service, C.S.I.R.O., Melbourne, Vic.). Compounds II, VIII and XI were purchased from Eastman Kodak Co., Inc. (Milwaukee, WI); VIII and XI were recrystallised from aqueous ethanol before use. 2-Amino-5-chlorobenzoxazole (compound XII, zoxazolamine) was obtained from K and K Labs, Inc., (Plainview, NY) and recrystallised from methanol/benzene. Biochemicals and cofactors were from Sigma Chemical Co., (St. Louis, MO) and all other chemicals were analytical grade.

Microsomes were prepared from the livers of male Wister rats (100–150 g) that had been treated with phenobarbitone. APDM and AH activities were followed by the formation of formaldehyde and *p*-aminophenol respectively, as previously described [7]. Benzoxazole derivatives did not interfere with the assays for either formaldehyde or *p*-aminophenol. I_{50} values were determined in duplicate from incubation with at least five concentrations of inhibitor. Cytochrome P-450-difference spectra were recorded with an Aminco DW-2 Spectrophotometer (American Inst. Co., Silver Springs, MD) at a microsomal protein concentration of 1 mg/ml. Wavelengths were determined with respect to characteristic absorbance peaks of holmium oxide.

Structure–activity relationships were examined by multiple linear regression analysis, using the Cyber 72 computer at the Sydney University Computer Centre. Partition coefficients (log $P_{octanol/water}$) were derived or calculated from the literature [16, 17] by addition of the summed hydrophobic

* The first four papers in this series were A. Bobik *et al.*, *Xenobiotica* **5**, 65 (1975), reference 7, A Bobik *et al.*, *J. Med. Chem.* **20**, 1194 (1977) and P. J. Little and A. J. Ryan, *J. Med. Chem.* (in press).

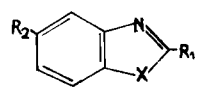
substituent (π) values to the log P value of the parent structure as described [16].

Results and discussion

Twelve benzoxazole derivatives and a reference compound, 4(5)-phenylimidazole (XIII) were tested against APDM and AH activities in hepatic microsomes from PB-treated rats and the results are shown in Table 1. Benzoxazole (I) was a weak inhibitor of APDM although it has previously been found to enhance the *N*-demethylation of *p*-chloro-*N*-methylaniline in rat liver microsomes [9]. For the 2-alkylbenzoxazole series (compounds I–VI) inhibitory potency towards APDM activity increased as the number of carbon atoms in the alkyl side chain increased. The inhibitory potency of the parent compound, benzoxazole (compound I, APDM $I_{50} = 1.5 \times 10^{-3}$ M) was increased 26 times by 2-alkyl substitution. The most potent 2-alkylbenzoxazole derivative tested against APDM activity was 2-n-heptylbenzoxazole ($I_{50} = 5.8 \times 10^{-5}$ M). Substitution of a methyl group into the 2 or 5 position of the benzoxazole nucleus (compounds II and IX, respectively) produced a 2–3-fold increase in inhibitory potency in each instance. Methyl substitution of both the 2 and 5 positions (2,5-dimethylbenzoxazole, APDM $I_{50} = 2.0 \times 10^{-4}$ M) resulted in a seven-fold increase in potency relative to the parent compound. 2-*o*-Hydroxyphenylbenzoxazole (compound VIII, APDM $I_{50} = 5.3 \times 10^{-5}$ M) was the most potent benzoxazole derivative tested, being 30 times more potent than benzoxazole as an inhibitor of APDM activity. This compound would be expected to have a rigid structure resulting from hydrogen bonding between the hydrogen of the hydroxyl substituent and the oxygen of the five-membered ring of the heterocycle. The hydrogen bonding would tend to interfere with the interaction of the oxygen of the heterocycle with cytochrome P-450, and the low I_{50} value for this compound adds to the evidence that these inhibitors act by an interaction between the heterocyclic nitrogen and a binding site on cytochrome P-450 [2, 11]. Zoxazolamine (XII) inhibited both APDM and AH activities in rat liver microsomes. Zoxazolamine has been used to study the *in vivo* activity of MFO inhibitors (prolongation of 'zoxazolamine paralysis time') [18] and is a known substrate of microsomal monooxygenases [19]. It is suggested that zoxazolamine was inhibitory because it acted as an alternative substrate.

The data for the inhibition of APDM activity by ben-

Table 1. Effect of benzoxazole derivatives on aminopyrine-*N*-demethylase (APDM) and aniline-*p*-hydroxylase (AH) activities in phenobarbitone-induced rat liver microsomes



Compound	X	R_1	R_2	I_{50} (M) or E(%)* APDM	AH
I	O	H	H	1.5×10^{-3}	E (2)
II†	O	CH_3	H	7.6×10^{-4}	E (20)
III	O	C_2H_5	H	3.8×10^{-4}	E (37)
IV	O	*iso*-C_4H_9	H	1.2×10^{-4}	E (86)
V	O	n-C_5H_{11}	H	8.5×10^{-5}	E (51)
VI	O	n-C_7H_{15}	H	5.8×10^{-5}	E (47)
VII	O	n-$C_{11}H_{23}$	H	NS‡	E (18)
VIII	O	*o*-HO·C_6H_4	H	5.3×10^{-5}	E (8)
IX	O	H	CH_3	5.4×10^{-4}	E (2)
X†	O	CH_3	CH_3	2.0×10^{-4}	E (13)
XI	O	CH_3	C_6H_5	6.8×10^{-5}	E (15)
XII	O	NH_2	Cl	2.9×10^{-4}	6.7×10^{-4}
XIII		4(5)-Phenylimidazole		7.4×10^{-6}	1.1×10^{-5}

* Enhancement (% AH activity above control level in the presence of 0.1 mM (VII and VIII) or 0.2 mM (I–VI and IX–XI) of benzoxazole derivative in the incubation medium). Enhancement was greater or lesser at higher concentrations (see text).

† Data reported previously [7].

‡ NS, Not sufficiently soluble in incubation medium.

Control values ± S.E. (number of observations) were 4.64 ± 0.75 nmoles formaldehyde formed/mg protein/min (20) for APDM activity and 1.20 ± 0.12 nmoles *p*-aminophenol formed/mg protein/mg (20) for AH activity.

Table 2. Regression analysis of the inhibition of aminopyrine *N*-demethylase activity in phenobarbitone-induced rat liver microsomes by benzoxazole derivatives

	r*	S.D.†	Equation
(1) Six 2-alkylbenzoxazoles (Table 1, compounds I–VI)			
$pI_{50} = 0.417 \log P + 2.29$	0.967	0.162	1
$pI_{50} = 1.109 \log P - 0.104 (\log P)^2 + 1.29$	0.997	0.055	2
(2) Eleven benzoxazoles (Table 1, compounds I–VI and VIII–XII)			
$pI_{50} = 0.412 \log P + 2.40$	0.947	0.166	3
$pI_{50} = 1.273 \log P - 0.130 (\log P)^2 + 1.13$	0.985	0.096	4

* Multiple correlation coefficient.

† Standard deviation from the regression.

zoxazoles were fitted to linear and parabolic equations correlating inhibitory potency (pI_{50}) with hydrophobicity (log P). The computed equations are shown in Table 2. The linear analysis (equation 1) of the inhibition of APDM activity by six 2-alkylbenzoxazoles (Table 1, compounds I to VI) gave a correlation coefficient of 0.967. Inclusion of the $(\log P)^2$ term into the analysis (equation 2) produced a significant ($P < 0.05$) improvement in the fit of the data to the model; the correlation coefficient of 0.997 showed that 99% of the variance of pI_{50} in the data was accounted for by the parabolic model correlating inhibitory potency and hydrophobicity (this relationship is depicted graphically in Fig. 1). The optimum log P value ($\log P_0$) was determined by setting the partial derivative of the second order equation equal to zero. The $\log P_0$ value for the relationship described by equation 2 was 5.33, for which the corresponding I_{50} value would be 5.68×10^{-5} M. Thus, 2-n-heptylbenzoxazole (compound VI, log P = 5.09, APDM $I_{50} = 5.8 \times 10^{-5}$ M) was approximately the most potent compound which would be obtained from a 2-alkylbenzoxazole series, based on predictions from the proposed model (Table 2, equation 2). Inclusion of the I_{50} and log P values for the five additional benzoxazole derivatives in Table 1 (compounds VIII to XII) into the analysis, showed that the quality of the fits of the data to the equations (equations 3 and 4) was almost as good as that for the analysis of the 2-alkylbenzoxazoles alone. The second order analysis in log P (equation 4) gave a correlation coefficient of 0.985. Thus, for the variety of substituents shown in Table 1, 97% of the variance of pI_{50} in the data was explained by a parabolic model solely in terms of the hydrophobic characteristics of the inhibitory compounds. Throughout our work we have used log P values, rather than π substituent values, as a measure of hydrophobocity. The use of log P values allows for direct comparisons of the relationship between hydrophobicity and inhibitory potency amongst series of compounds (e.g., benzimidazoles and benzoxazoles) which are based on parent structures having different partition coefficients. Figure 1 presents graphically a comparison of the computed regression line for the inhibition of APDM activity by 2-alkylbenzoxazoles (i.e. Table 2, equation 2) with the regression equations for the inhibition of APDM and AH activities by 2-alkylbenzimidazoles reported previously by us [7]. At log P values of less than 4, equihydrophobic 2-alkylbenzoxazole and 2-alkylbenzimidazole derivatives would be predicted to be approximately equipotent as inhibitors of rat liver microsomal APDM activity. At greater log P values, benzimidazole derivatives would be more potent than equihydrophobic 2-alkylbenzoxazoles. The optimum log P_0 value for the 2-alkylbenzoxazoles was lower than that for the 2-alkylbenzimidazoles (Fig. 1, log $P_0 = 7.20$) [7]. At log P values greater than log P_0, inhibitory potency decreases as hydrophobicity increases. Therefore, it is not surprising that the poorly soluble compound, 2-undecylbenzoxazole (compound VII, log P = 7.09), was only slightly inhibitory at saturating concentrations in the incubation medium, and that an I_{50} value could not be determined.

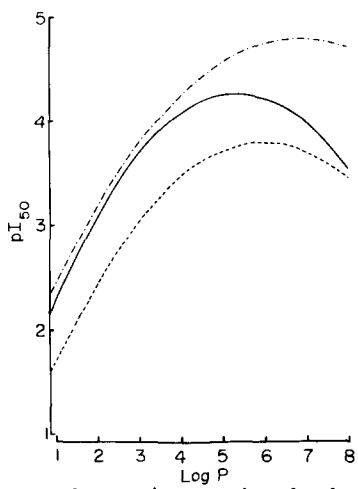


Fig. 1. Computed regression equations for the inhibition of rat liver microsomal mixed function oxidases by benzimidazoles and benzoxazoles. Inhibition of aminopyrine *N*-demethylase activity by 2-n-alkylbenzimidazoles (dash–dot line, $pI_{50} = 0.929 \log P - 0.068 (\log P)^2 + 1.60$, $r = 0.976$, $n = 11$, data from Ref. 7) and 2-alkylbenzoxazoles (solid line, equation 2, Table 2) and the inhibition of aniline-*p*-hydroxylase activity by 2-n-alkylbenzimidazoles (dashed line, $pI_{50} = 1.010 \log P - 0.085 (\log P)^2 + 0.772$, $r = 0.969$, $n = 9$, data from Ref. 7).

4(5)-Phenylimidazole (XIII) is a potent inhibitor of rat liver MFO activity. Rogerson *et al.* [3] reported an I_{50} value of 4.6×10^{-6} M for XIII as an inhibitor of aldrin epoxidation in rat liver microsomes. Compound XIII was a good inhibitor of both APDM activity ($I_{50} = 7.4 \times 10^{-6}$ M) and AH activity ($I_{50} = 1.1 \times 10^{-5}$ M). That XIII was more potent than the benzoxazoles (see Table 1) is consistent with the observation [3, 9] that the steric hindrance inherent in the bicyclic structure should reduce or limit the inhibitory potency of benzoxazoles and related compounds. The evidence that imidazole may be an endogenous ligand of cytochrome P-450 [20] suggests that the marked inhibitory activity of exogenous imidazoles may be related to the optimum nature of imidazole as a ligand.

In contrast to the inhibitory activity shown towards microsomal APDM, 11 of the 12 benzoxazole derivatives studied enhanced AH activity (see Table 1). The response to a range of concentrations of each benzoxazole derivative was determined and although these response curves were not identical, Table 1 gives a measure of the degree of enhancement for each compound at a given concentration (0.1 or 0.2 mM). Compounds I and II at 1 mM caused greater enhancement of AH activity than at 0.2 mM and compounds III, IV, V, VI, X and XI caused less enhancement at 1 mM than at 0.2 mM; IX was slightly inhibitory (5%) at 1 mM. The biphasic response shown by these latter 2-alkylbenzoxazoles is typical of the response described for acetone [21] and metyrapone [22] as activators of AH and acetanilide hydroxylase activities respectively in rat liver microsomes. In other studies, the evaluation of series of compounds (e.g. ketones [21], benzimidazoles and benzothiazoles [7]), has shown that some compounds enhance whilst apparently closely related derivatives inhibit rat liver microsomal AH activity. In our studies with nitrogen heterocycles we have found that benzothiazole, indole and indazole inhibit whereas benzimidazole, benzoxazole and 1H,2,3-benzothiazole enhance rat liver microsomal AH activity *in vitro**. Alkylbenzoxazoles are the first series of closely related compounds shown to enhance microsomal aromatic hydroxylation, however there is no relationship apparent between physical and chemical properties and degree of enhancement of AH activity.

One benzoxazole derivative tested inhibited AH activity (zoxazolamine, $I_{50} = 6.7 \times 10^{-4}$) and we speculate that this inhibitory activity may be associated with the presence of the 2-amino substituent (and hence a different interaction with cytochrome P-450) or the possibility that it may have acted as an alternative substrate [19]. Due to the short incubation times of the present study it is probable that the observed inhibitory and enhancing activity of the benzoxazoles is mostly associated with the parent structures and not metabolites, however conclusive evidence would only be obtained from detailed investigations using pre-incubations with benzoxazoles and studies with known or potential metabolites.

The interaction of ligands with oxidised microsomal

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

cytochrome P-450 produces characteristic difference spectra of three principal types [13, 14] which can usually be quantified by determination of the spectral binding dissociation (K_s) constant. Alkylbenzoxazoles interacted with PB-induced rat liver microsomes to yield weak difference spectra which coupled with a low solubility in the binding mixture did not allow for the reliable determination of K_s values. The observed peak/trough wavelengths for the cytochrome P-450 difference spectra of compounds I, IX and X were 389/418 nm (type RI), 389/419 nm (type RI) and 384/414 nm (unclassified) respectively. Studies with benzimidazole derivatives [23] have shown that 2-methyl substitution alters binding from type II (benzimidazole) to type RI (2-methylbenzimidazole). 2-Methyl substitution of 5-methylbenzoxazole produced a similar red shift in the absorption maximum of the cytochrome, however in this latter case the difference spectrum became uncharacteristic.

In summary, all benzoxazoles tested inhibited APDM activity in rat liver microsomes and there was an apparent relationship between inhibitory potency and partition coefficient which was best described by a second order equation in log P ($pI_{50} = 1.273 \log P - 0.130 (\log P)^2 + 1.13$, $n = 11$, $r = 0.985$). The maximum inhibitory activity for a 2-alkylbenzoxazole series was lower than that of an arylimidazole reference compound and lower than that demonstrated previously for a 2-alkylbenzimidazole series. Eleven of twelve benzoxazoles enhanced AH activity in rat liver microsomes (and zoxazolamine was inhibitory), however, no relationship was apparent between physico-chemical properties and degree of enhancement of AH activity. Alkylbenzoxazoles represent the first series of compounds to enhance AH activity and may be a useful series for a more detailed study of the mechanism of enhancement of hepatic microsomal aniline hydroxylation.

Acknowledgements—We are grateful to the Pharmaceutical Society of New South Wales for support to P.J.L. during part of this study. We thank Miss C. Harwood for excellent assistance with the preparation of the manuscript.

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Biochemical Pharmacology, Vol. 31, No. 9, pp. 1798–1801, 1982.
Printed in Great Britain.

0006-2952/82/091798-04 $03.00/0

Toxic effect of chlorisondamine in neonatal rat liver*

(*Received* 13 *July* 1981; *accepted* 12 *November* 1981)

Chlorisondamine (CHLOR) is a long-lasting ganglionic blocker that has been used both clinically, in treatment of hypertension, and experimentally, in studies of ganglionic transmission. The physiological and biochemical effects of CHLOR administration are typical of ganglionic blockade: CHLOR decreases blood pressure, inhibits salivary secretion, decreases plasma catecholamines and prevents reflex sympathetic stimulation of a number of a peripheral tissues including the adrenal, heart and blood vessels [1–10]. Although CHLOR is an effective ganglionic blocker, some evidence suggests that this drug possesses other actions which can both oppose and potentiate its ganglionic blocking activity. Acute CHLOR administration causes a transient sympathetic stimulation that induces adrenal tyrosine hydroxylase [7], and chronic CHLOR administration stimulates adrenal catecholamine-synthesizing enzymes by a

* This work was supported by Grant RO1-MH 13688 from the National Institute of Mental Health.