Biochemical Pharmacology. Vol. 25. pp. 2747–2750. Pergamon Press, 1976. Printed in Great Britain.

SHORT COMMUNICATIONS

Inhibitors of hepatic mixed function oxidases—II* Some benzimidazole, benzoxazole and benzothiazole derivatives

(*Received* 23 *April* 1976; *accepted* 8 *July* 1976)

The activity of hepatic microsomal mixed function oxidases may be inhibited by a range of compounds including 2-diethylaminoethyl 2,2-diphenylvalerate HCl (SKF 525-A), methylenedioxyphenyl derivatives [1], thiono-sulphur containing compounds [2], phenolic ketones [3], and imidazole derivatives [4–7]. The remarkable potency of many 1- and 4(5)-arylimidazoles led to proposals that the investigation of series of imidazoles may yield information on the nature of the enzymes associated with oxidative drug metabolism. This report describes the effects of nineteen benzimidazole and four structurally related benzoxazole and benzothiazole derivatives on aminopyrine *N*-demethylase (APDM) and aniline *p*-hydroxylase (AH) activities in rat liver microsomes, and on quinalbarbitone sleeping time in mice.

MATERIALS AND METHODS

2-Alkyl and 2-alkyl-5-methylbenzimidazoles were synthesized by the reaction of *o*-phenylenediamine or 3,4-diaminotoluene with the appropriate organic acid [8, 9]. Compounds 20 and 22 were purchased from Eastman Kodak Co. Rochester N.Y., and compounds 21 and 23 from Aldrich Chemical Co., Inc. Milwaukee,-Wis. A sample of SKF 525-A was supplied by Smith, Kline and French Laboratories (Australia) Ltd. Biochemicals were purchased from Sigma Chemical Co., St. Louis, Mo. All other chemicals were analytical grade.

Microsomes were prepared from the livers of immature male Wistar rats (100–150 g) that had been pretreated with phenobarbitone. The livers were removed and homogenized in phosphate buffer (0.1 M, pH 7.4, 7 volumes). The homogenate was centrifuged at 1000 *g* for 10 min, 9,000 *g* for 10 min and the resultant supernatant was centrifuged at 105,000 *g* for 1 hr at 4° to sediment the microsomal fraction. The microsomal pellets were resuspended in phosphate buffer (0.1 M, pH 7.4) using a Potter–Elvehjem homogenizer. Protein was determined by the biuret method [10] using bovine serum albumin as standard. For the determination of APDM activity aminopyrine 1.5 μmole, $MgCl_2$ 7.5 μmole, NADP 1.21 μmole, semicarbazide 22.5 μmole, glucose-6-phosphate 12.5 μmole, glucose-6-phosphate dehydrogenase 10 units and microsomal protein 5.6 mg in phosphate buffer (0.1 M, pH 7.4, 3.0 ml) were used. For the determination of AH activity aniline 15 μmole, $MgCl_2$ 7.5 μmole, NADP 3.03 μmole, glucose-6-phosphate 12.5 μmole, glucose-6-phosphate dehydrogenase 10 units and microsomal protein 8.6 mg in phosphate buffer (0.1 M, pH 7.4, 3.5 ml) were used. APDM activity was followed by the formation of formaldehyde [11] and AH activity by the formation of *p*-aminophenol assayed by the method of Schenkman *et al.* [12] except that *o*-cresol was used in place of phenol and the colour was measured at 610 nm. Incubation times for I_{50} determinations were 15 and 12 min for APDM and AH activities respectively. The test compounds were added to the incubation mixtures from stock solutions in 0.07 N HCl or DMSO:water (4:1 by vol). 2-*n*-Butylbenzimidazole was tested by introduction from each solvent and the I_{50} was identical in both cases.

Quinalbarbitone sleeping time was determined in male Sydney–White mice (25 g), divided randomly into groups, and injected with quinalbarbitone sodium (0.07 mmol/kg, ip) in saline 5 min after receiving either the test compound (0.34 m-mole/kg, i.p.) in DMSO, or solvent alone. Mice were kept at 37° and sleeping time was recorded as the period between the quinalbarbitone injection and the return of the righting reflex. The significance of the results was calculated using Student's *t*-test.

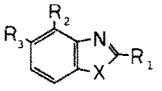
RESULTS AND DISCUSSION

Table 1 shows the effects of nineteen benzimidazole (compounds 1–19), two benzoxazole (compounds 20–21) and two benzothiazole derivatives (compounds 22–23) and the reference compound 2-diethylaminoethyl 2,2-diphenylvalerate hydrochloride (SKF 525-A) (compound 24) on aminopyrine *N*-demethylase (APDM) and aniline *p*-hydroxylase (AH) activities in rat liver microsomes. All compounds inhibited APDM activity with I_{50} values ranging from 108×10^{-5} M (compound 2) to 1.5×10^{-5} M (compound 13). AH activity was inhibited by all but four of the compounds and I_{50} values varied from 360×10^{-5} M (compound 2) to 16×10^{-5} M (compound 11). Compounds 1 and 20–22 stimulated AH activity. Compounds that were inhibitory to both reactions were all more potent (2–20 times) toward APDM activity; a similar difference was observed with SKF 525-A [24] which was approximately fifty times more potent toward APDM activity. The I_{50} values of these inhibitors decrease with increasing lipophilicity due to both extension of the alkyl side chain (compounds $2 \rightarrow 3 \rightarrow 4 \rightarrow 6 \rightarrow 8$, etc.) and modification of the heterocyclic ring (compounds $17 \rightarrow 21 \rightarrow 23$, APDM activity only).

The apparent relationship between lipophilicity and potency for these inhibitors was analysed by the multiple regression approach of Hansch [13–15] using partition coefficients derived from the literature [15, 16]. Table 2 shows the first and second order regression equations for the correlation of octanol/water partition coefficients with I_{50} values for the inhibition of APDM and AH activities by nineteen and seventeen benzimidazole derivatives respectively. The linear analyses (Eqs. 1 and 3) of the inhibition of APDM and AH activities yield statistically significant ($P < 0.01$) correlation coefficients of 0.897 and 0.818 respectively. The inclusion of a $(\log P)^2$ term into the analysis (Eq. 2) produced a significant reduction in the residual sums of squares ($P < 0.05$) for the inhibition of APDM activity. Although the second-order term resulted in a lowering of the variance for the inhibition of AH activity, the improvement was not significant at $P = 0.10$. The second-order regression equations account for 94 per cent and 81 per cent of the variance of pI_{50} in equations

* The first paper in this series was reference [3].

Table 1. The effects of benzimidazole, benzoxazole and benzothiazole derivatives on *in vitro* aminopyrine-*N*-demethylase (APDM) and aniline-*p*-hydroxylase activities in rat liver microsomes and *in vivo* quinalbarbitone sleeping time in mice



Compound	X	R_1	R_2	R_3	$I_{50} \times 10^{-5}$ M APDM	$I_{50} \times 10^{-5}$ M AH	% Increase in sleeping time
1	NH	H	H	H	98	Stimulates	45*
2	NH	CH_3	H	H	108	360	83†
3	NH	C_2H_5	H	H	67	330	73†
4	NH	*n*-C_3H_7	H	H	30	105	101‡
5	NH	*iso*-C_3H_7	H	H	54	115	—
6	NH	*n*-C_4H_9	H	H	12	112	110†
7	NH	*iso*-C_4H_9	H	H	16	160	—
8	NH	*n*-C_5H_{11}	H	H	5.0	49	96†
9	NH	*n*-C_6H_{13}	H	H	3.2	25	152†
10	NH	*cyclo*-C_6H_{11}	H	H	7.0	22	—
11	NH	*n*-C_7H_{15}	H	H	2.6	16	76‡
12	NH	*n*-C_8H_{17}	H	H	2.9	22	55‡
13	NH	*n*-$C_{11}H_{23}$	H	H	1.5	24	63†
14	NH	*n*-$C_{13}H_{27}$	H	H	2.3	N.S.§	57‡
15	NH	$CH_2C_6H_5$	H	H	9.2	46	150†
16	NH	H	H	CH_3	58	190	—
17	NH	CH_3	H	CH_3	48	160	20*
18	NH	C_2H_5	H	CH_3	27	180	154†
19	NH	CH_3	CH_3	CH_3	25	48	—
20	O	CH_3	H	H	76	Stimulates	—
21	O	CH_3	H	CH_3	20	Stimulates	64†
22	S	CH_3	H	H	14	Stimulates	—
23	S	CH_3	H	CH_3	5.7	120	69†
24	SKF525-A				0.82	45	—

* Not significant, † $P < 0.01$, ‡ $P < 0.05$, § N.S. Compound not sufficiently soluble in incubation mixture.

Control values ± standard errors (number of observations) were 4.64 ± 0.75 nmoles formaldehyde formed/mg protein/min (20) for APDM activity, 1.20 ± 0.12 nmoles *p*-aminophenol formed/mg protein/min (20) for AH activity, and 29.9 ± 2.1 min. (119) for quinalbarbitone sleeping time.

2 and 4 respectively. The partition coefficient of the benzimidazole derivatives is of major importance to the structure–activity relationships of these inhibitors. The wide range of log *P* values (1.35 to 8.20) spanned by the benzimidazole derivatives revealed the importance of the $(\log P)^2$ term in the relationship.

The optimal partition coefficients ($\log P_0$) of the benzimidazole derivatives as inhibitors of APDM and AH activities were 7.20 and 6.05 corresponding to alkyl chains of 11–12 and 9–10 carbon atoms respectively. Wilkinson *et al.* [6] found a parabolic relationship between inhibitory activity of 1-alkylimidazoles towards aldrin epoxidation and the length of the alkyl chain, the optimum length being 9–10 carbon atoms. The intercepts (k_0) of the regression equations 2 and 4 are 1.62 and 1.06 respectively reflecting the greater intrinsic activity [17] of the benzimidazole derivatives as inhibitors of APDM.

Wilkinson *et al.* [5] also studied the inhibition of the epoxidation of aldrin, hydroxylation of dihydroisodrin and *N*-dealkylation of *p*-chloro-*N*-methylaniline in rat liver microsomes by a series of 4(5)- and 2-substituted imidazoles and proposed that "at least one of the imidazole nitrogens must be free for inhibitory activity". This proposal was supported by the findings that 1-alkylimidazoles, having such an unhindered nitrogen at position 3 flanked by unsubstituted ring positions, were more potent inhibi-

Table 2. Correlation of octanol/water partition coefficient (*P*) with I_{50} values for the inhibition of aminopyrine-*N*-demethylase (APDM) and aniline-*p*-hydroxylase (AH) activities in rat liver microsomes

$$\log (1/I_{50}) = k_0 + k_1 \log P + k_2(\log P)^2$$

Compounds	Microsomal activity	k_0	k_1	k_2	r*	s†	n‡	Eq. no.
1–19	APDM	2.72	0.306	0	0.897	0.274	19	1
1–19	APDM	1.62	0.875	−0.061	0.969	0.158	19	2
2–13, 15–19	AH	2.19	0.263	0	0.818	0.262	17	3
2–13, 15–19	AH	1.03	0.881	−0.072	0.899	0.207	17	4

* Multiple correlation coefficient, † Standard deviation from the regression, ‡ Number of compounds in analysis.

tors of APDM activity than the corresponding 2-alkylimidazoles [7], and that 1-(2-cyanophenyl)- and 1-(2-isopropylphenyl)imidazole were exceptionally potent *in vitro* and *in vivo* inhibitors of hepatic microsomal oxidases [4]. In contrast, the benzimidazole derivatives cannot possess this unhindered ring nitrogen and are generally less potent inhibitors of microsomal oxidases than the imidazole derivatives.

The correlation between log P and I_{50} values against APDM and AH activities of the benzimidazoles and that between K_s, the spectral binding constant of cytochrome P-450, and I_{50} values against aldrin epoxidation and dihydroisodrin hydroxylation of imidazoles suggested that a correlation ought to be found between the K_s values and I_{50} values, and K_s and log P for the benzimidazoles studied. However, Dickens *et al.* [18] studying eight benzimidazoles including compounds 1, 2, 3, 15 and 16, found no correlation between log P and K_s for these compounds. This is perhaps not surprising because, although agreement was observed between K_s values of the imidazoles and their I_{50} values against epoxidation and hydroxylation (5), no correlation was found with *p*-chloro-*N*-methylaniline demethylation activity. Since the present study examined a demethylation, absence of K_s and log P correlation is reasonable in the presence of I_{50} and log P correlations. Additionally, the oxidized cytochrome P-450 difference spectra of the benzimidazoles were of two types, both II and RI whereas the 1-, 2-, and 4(5)- substituted imidazole derivatives gave only type II difference spectra. The binding spectra recorded for the present compounds* confirm the proposal of Dickens *et al.* [18] that the addition of 2-alkyl substituents to benzimidazole alters the binding spectrum with oxidized cytochrome P-450 from type II to type RI.

The I_{50} values of the benzoxazoles (compounds 20 and 21) and benzothiazoles (compounds 22 and 23) tested against APDM activity were found to be close to those predicted by substitution of the appropriate log P values into the regression equation (Eq. 2) established for benzimidazole derivatives.

Although compounds 1 and 20–22 were inhibitors of APDM activity, they stimulated AH activity. Compounds 1 and 20 caused a gradual increase in AH activity (above control levels) as the concentration was increased from zero to saturating concentrations (approx. 1 mM); AH activities at 1 mM were 112 and 153 per cent of control activity respectively. Compounds 21 and 22 showed maximum stimulation (113 and 123 per cent of control activity respectively) at 0.02 mM and were weakly inhibitory at concentrations of 1 mM. This stimulation of AH activity is not without precedent. In addition to acetone [19], 2,2′-bipyridyl [20], and compounds 1 and 20–22, benzoxazole and four other alkylbenzoxazoles* have been found to stimulate this microsomal reaction.

Compounds which inhibit microsomal oxidases *in vitro* may prolong the pharmacological activity of drugs *in vivo*. Methylenedioxyphenyl derivatives such as piperonyl butoxide prolong hexobarbitone narcosis and zoxazolamine paralysis in mice [1]. 1-Alkyl [6, 7], 1-aryl [4] and 4(5)-substituted [5] imidazoles greatly prolong hexobarbitone sleeping time in mice without exhibiting any intrinsic hypnotic activity. Table 1 shows the per cent increase of sleeping time for mice administered (0.34 m-mole/kg, i.p.) of the test compounds 5 min before a dose of quinalbarbitone (0.07 m-mole/kg, i.p.) in saline. Compound 9 exhibited no hypnotic activity when administered (0.25 m-mole/kg, i.p.) to untreated mice, or mice that had just regained the righting reflex following a hypnotic dose of quinalbarbitone; administered 5 min before quinalbarbitone, compound 9 caused a 152 per cent increase in the mean sleeping time. Apart from compounds 1 and 17, all the compounds tested caused a significant ($P < 0.05$) increase in quinalbarbitone sleeping time in mice; these benzimidazole, benzoxazole and benzothiazole derivatives thus inhibit hepatic microsomal oxidases *in vivo* as well as *in vitro*. Although the 1-alkylimidazoles [6] are more potent than the 2-alkylbenzimidazoles toward the prolongation of barbiturate narcosis, the structure–activity relationships for the two homologous series are very similar. For both series the activity increases with increasing alkyl chain length to a maximum at 8 and 6 carbon atoms respectively, falls abruptly upon addition of the next carbon atom, and remains approximately constant for the four highest members tested for each homologous series. The prolongation of barbiturate sleeping times caused by imidazole derivatives with an unhindered ring nitrogen are greater than those caused by the benzimidazole derivatives tested. This observation is compatible with the conclusions suggested by the *in vitro* results.

The benzimidazoles described in this report represent another group of imidazole derivatives which have been shown to inhibit hepatic microsomal oxidases *in vitro* and *in vivo*. The replacement of the heterocyclic NH group of benzimidazoles by an oxygen or sulphur atom produces compounds which are more potent on a molar basis due to the increase of lipophilicity associated with the substitution. The observation that imidazole derivatives with an unhindered ring nitrogen are the most potent inhibitors of microsomal oxidases so far discovered, suggests that oxazoles and thiazoles substituted only at position 5 should be potent inhibitors. The stimulation of microsomal AH activity has received less interest than the inhibition of this and other microsomal reactions. That the two benzoxazoles described in this report both stimulate AH activity may allow a more intensive study of the relationship between compounds which inhibit, stimulate, or act as substrates for hepatic microsomal mixed function oxidases.

Acknowledgements—We wish to thank Dr. I. K. Hotson of Merck, Sharp & Dohme (Australia) Pty. Ltd., for supplying a sample of 2,4,5-trimethyl benzimidazole, and Miss C. Seeto for assistance with the animal experiments.

Department of Pharmacy,
The University of Sydney,
Sydney, N.S.W. 2006
Australia

GERALD M. HOLDER
PETER J. LITTLE
ADRIAN J. RYAN
THOMAS R. WATSON

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

REFERENCES

1. K. Fujii, H. Jaffe, Y. Bishop, E. Arnold, D. Mackintosh and S. S. Epstein, *Toxic. appl. Pharmac.* **16,** 482 (1970).
2. A. L. Hunter and R. A. Neal, *Biochem. Pharmac.* **24,** 2199 (1975).
3. A. Bobik, G. M. Holder, A. J. Ryan and L. I. Wiebe, *Xenobiotica* **5,** 65 (1975).
4. K. C. Leibman, *Chem.-Biol. Interact.* **3,** 289 (1971).
5. C. F. Wilkinson, K. Hetnarski and T. O. Yellin, *Biochem. Pharmac.* **21,** 3187 (1972).
6. C. F. Wilkinson, K. Hetnarski, G. P. Cantwell and F. J. Di Carlo, *Biochem. Pharmac.* **23,** 2377 (1974).
7. E. D. Palmer and M. A. Cawthorne, *Xenobiotica* **4,** 209 (1974).
8. E. C. Wagner and W. H. Millett, in *Organic Syntheses* Coll. Vol. 2, p. 65 J. Wiley and Sons, New York (1943).
9. W. O. Pool, H. J. Harwood and A. W. Ralston, *J. Am. chem. Soc.* **59,** 178 (1937).
10. H. W. Robinson and R. Hogden, *J. biol. Chem.* **135,** 707 (1940).
11. T. Nash, *J. biol. Chem.* **55,** 412 (1953).
12. J. B. Schenkman, H. Remmer and R. W. Estabrook, *Molec. Pharmac.* **3,** 113 (1967).

13. C. Hansch and T. Fujita, *J. Am. chem. Soc.* **86,** 1616 (1964).
14. C. Hansch, *Drug Design.* Vol. 1 (ed. E. J. Ariens), p. 271. Academic Press, New York (1971).
15. A. Leo, C. Hansch and D. Elkins, *Chem. Rev.* **71,** 525 (1971).
16. T. Fujita, J. Iwasa and C. Hansch, *J. Am. chem. Soc.* **86,** 5175 (1964).
17. G. M. Cohen and G. J. Mannering, *Molec. Pharmac.* **9,** 383 (1973).
18. M. Dickens, C. R. Elcombe, R. H. Nimmo-Smith and J. W. Bridges, *Biochem. Soc. Transact.* **3,** 970 (1975).
19. M. W. Anders, *Archs Biochem. Biophys.* **126,** 269 (1968).
20. M. W. Anders, *Biochem. Pharmac.* **18,** 2561 (1969).

Biochemical Pharmacology, Vol. 25, pp. 2750–2752. Pergamon Press, 1976. Printed in Great Britain.

Response of microperoxisomes in rat small intestinal mucosa to CPIB, a hypolipidemic drug

(*Received* 19 *January* 1976; *accepted* 26 *March* 1976)

Since their identification and chemical characterization, peroxisomes (microbodies) of rat liver have been studied rather extensively [1–4], while those in other organs and tissues have received relatively less attention.

Peroxisomes were first discovered in rodent liver and kidney; subsequently they have been reported in a wide variety of tissues of vertebrates [5, 6] as well as in plants. Recently, Novikoff and Novikoff [7] reported large numbers of peroxisomes in the duodenum, jejunum and ileum of guinea pigs and in rat duodenum. These authors pointed out that peroxisomes of intestinal absorptive cells lacked nucleoids, were of a smaller diameter than those of liver or kidney, and had numerous slender continuities with smooth endoplasmic reticulum membrane. Accordingly, these authors proposed the term "microperoxisome" for this smaller population of organelles. Connock and Kirk [8], using isopycnic centrifugation of homogenates of guinea pig intestinal epithelium, separated a fraction rich in catalase particles that sedimented more slowly than lysosomes and mitochondria and which are probably identical to intestinal microperoxisomes as described in this tissue by the Novikoffs. Pipan and Psenicnik [9] identified microperoxisomes in small intestinal epithelium of 13- to 19-day mouse embryos and found that their number increased with increasing age of the animal and differentiation of the cells in which they were found.

In the present paper, the terms "peroxisome" and "microperoxisome" are used interchangeably and both are regarded as closely related to the term "microbody" as used in earlier literature as a purely morphological term describing hepatic or renal peroxisomes.

It had been shown previously that, in male rat liver, peroxisomes increased in number and one of their principal enzymes, catalase, increased in activity after administration of ethyl alpha-chlorophenoxyisobutyrate (CPIB), a hypolipidemic drug. The results of a number of other studies suggested that peroxisomes may be related to lipid and steroid metabolism [10–15]. Because of the possible relationship of peroxisomes to lipid metabolism and in view of the fact that the intestinal tract may be the major site of cholesterol synthesis in the rat [16, 17], it was desirable to study the response of intestinal peroxisomes to a hypolipidemic agent known to cause significant alterations in lipid metabolism and in hepatic peroxisomes and their enzymes. Moreover, Novikoff and Novikoff [7] suggested that, because of the abundance, size and structure of peroxisomes in intestinal epithelium, they might be favorable for biochemical study. Accordingly, the present study was undertaken as a preliminary step to compare intestinal peroxisomes to those in the rat liver with regard to their response to CPIB.

Materials and methods

F-344 rats weighing 150–200 g were caged individually and given water *ad lib.* Fifteen males and ten females were fed 0.25% CPIB in their diet for 3 weeks; ten rats of each sex served as controls. Animals were fasted overnight and sacrificed between 9:00 and 10:00 a.m. The livers were removed, weighed and samples taken for cytochemical and biochemical determinations. For samples of intestine, the first 10 cm distal to the pyloric sphincter were omitted; the next 20-cm segment of small intestine was removed and designated jejunum. The 20-cm segment of small intestine proximal to the ileocecal valve was removed and designated ileum. Both segments of small intestine were opened longtitudinally and the lumen was flushed rapidly with cold saline. The segments were then placed serosa side downward on parafilm and the mucosa and submucosa were separated from muscularis by scraping gently with the edge of a glass slide. To insure the efficacy of this method for separation of mucosa from remaining intestinal wall, microscopic sections were prepared from the mucosal scrapings and from the remaining tissue.

Biochemical and cytochemical methods. One-g samples of liver and intestinal mucosa were homogenized in 4 ml of M/150 phosphate buffer at 1–4° in a Potter–Elvehjem homogenizer. Catalase activity was measured by the spectrophotometric method of Lück [18]. Total proteins were determined on liver and intestine by the method of Lowry [19]. For cytochemical demonstration of peroxidatic activity of catalase, small portions of liver and intestine were fixed in 2.5% glutaraldehyde buffered with 0.1 M sodium cacodylate, pH 7.4, for 4 hr at 4°. Tissues were rinsed overnight in 0.1 M cacodylate buffer containing 0.2 M sucrose. Slices of minced tissue were incubated at 37° for 45–60 min in the 3,3′-diaminobenzidine medium of Novikoff and Goldfischer [20, 21] modified from Graham and Karnovsky [22]. The incubation medium contained 10 mg of 3,3′-diaminobenzidine tetrahydrochloride (DAB, Sigma Chemical Co., St. Louis, Mo.) in 10 ml of 0.05 M 2-amino-2-methyl-1,3-propanediol buffer, pH 9.4, and 0.2 ml of 1% hydrogen peroxide. Controls consisted of: (1)