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Imidazole derivatives—A new class of microsomal enzyme inhibitors*

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Several groups of materials are recognized for their ability to inhibit the microsomal drug-metabolizing enzymes *in vitro* and to potentiate the action of drugs and insecticides *in vivo*. These include the classical inhibitor 2-diethylaminoethyl-2,2-diphenylvalerate hydrochloride (SKF 525-A) as well as other materials such as the 1,3-benzodioxoles, phenyl-2-propynyl ethers and 1,2,3-benzothiadiazoles, which have been extensively studied as a result of their activity in synergizing insecticides to insects.[1–3] As a part of our continuing search for new inhibitors of drug metabolism, we have examined the effects on rat liver microsomal enzyme activity of a series of imidazole derivatives which have previously been described as inhibitors of histidine decarboxylase.[4] The epoxidation of aldrin, the hydroxylation of dihydroisodrin and the *N*-dealkylation of *p*-chloro-*N*-methylaniline have been used as indicators of microsomal enzyme activity.

The imidazole derivatives (II–XVII) were obtained as crystalline hydrochlorides from Abbott Laboratories, North Chicago, Ill. Aldrin, dieldrin, dihydroisodrin and 6-*exo*-monohydroxydihydroisodrin were supplied by the Shell Development Co., Modesto, Calif., and were recrystallized prior to use. SKF 525-A was furnished by Smith Kline & French Laboratories, Philadelphia, Pa. *Para*-chloro-*N*-methylaniline, *p*-chloro-aniline and all biochemicals were purchased from CalBiochem, Los Angeles, Calif., and imidazole (I) was purchased from the Aldrich Chemical Co., Cedar Knolls, N.J. All other chemicals and solvents were analytical reagent grade.

Livers from male Sprague–Dawley rats, purchased from Blue Spruce Farms Inc., Altamont, N.Y., were homogenized in a Waring blender in cold 1·15% KCl (1:4, w/v) and microsomes were sedimented from the post-mitochondrial supernatant (20,000 *g* for 20 min) by centrifugation at 100,000 *g* for 1 hr. For enzyme assay, the microsomal pellet was resuspended in 1·15% KCl to a concentration of about 0·6 mg/ml. The incubation mixture and the conditions employed were identical to those previously described[5–7] as were the assays for epoxidation, hydroxylation and *N*-demethylation.[5–8] Imidazoles were added to the incubations in 10 μl water and the I_{50} values for each reaction were determined from the means of duplicate incubations with at least four inhibitor concentrations. Difference spectra were recorded with a Norelco Unicam SP-800 spectrophotometer provided with a scale expansion device and a scavenger recorder; the microsomal suspension employed for this purpose contained 1–2 mg protein/ml in 67 mM phosphate buffer, pH 7·4.

Hexobarbital sleeping time was determined in male CF_1 mice from Carworth Farms which were divided into groups at random. The animals had free access to food and water up to the time of injection. Drugs were dissolved in saline and injected intraperitoneally 0·5 hr prior to the administration of hexobarbital in saline (100 mg/kg, i.p.). Student's *t*-test was used in statistical analysis of the results.

Table 1 shows the molar I_{50} values for the imidazoles (I–XVII) against each of the three microsomal reactions investigated. Although imidazole (I) itself exhibits only slight inhibition at 10^{-4} M, all the 4(5)-substituted derivatives (II–XIV) were potent inhibitors of both epoxidation and hydroxylation,

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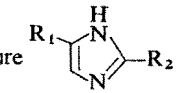
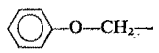
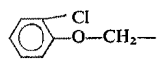
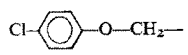
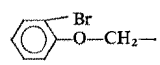
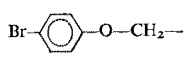
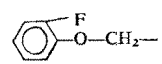
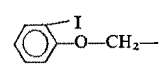
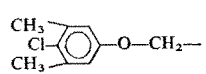
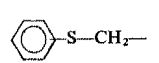
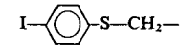
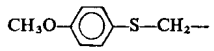
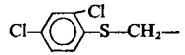
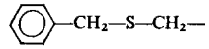
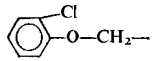
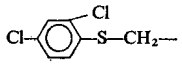
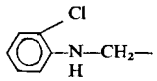
TABLE 1. INHIBITION OF RAT LIVER MICROSOMAL ENZYMES BY IMIDAZOLE DERIVATIVES

General structure 

Compound	R_1	R_2	Molar I_{50}		
			Epoxidation	Hydroxylation	*N*-demethylation
I	H	H	$> 10^{-4}$	$> 10^{-4}$	$> 10^{-4}$
II		H	$8{\cdot}8 \times 10^{-7}$	$1{\cdot}35 \times 10^{-6}$	$\sim 10^{-4}$
III		H	$1{\cdot}15 \times 10^{-6}$	$3{\cdot}3 \times 10^{-7}$	$\sim 10^{-4}$
IV		H	$2{\cdot}4 \times 10^{-6}$	2×10^{-6}	4×10^{-5}
V		H	$6{\cdot}4 \times 10^{-7}$	$3{\cdot}2 \times 10^{-7}$	$\sim 10^{-4}$
VI		H	$2{\cdot}1 \times 10^{-6}$	$1{\cdot}9 \times 10^{-6}$	$7{\cdot}6 \times 10^{-5}$
VII		H	$2{\cdot}2 \times 10^{-6}$	$1{\cdot}1 \times 10^{-6}$	$1{\cdot}6 \times 10^{-5}$
VIII		H	$5{\cdot}2 \times 10^{-7}$	$2{\cdot}4 \times 10^{-7}$	$1{\cdot}8 \times 10^{-5}$
IX		H	$1{\cdot}45 \times 10^{-6}$	$5{\cdot}4 \times 10^{-6}$	$2{\cdot}6 \times 10^{-5}$
X		H	8×10^{-7}	$2{\cdot}3 \times 10^{-6}$	$> 10^{-4}$

XI		H	$8{\cdot}6 \times 10^{-7}$	$3{\cdot}7 \times 10^{-7}$	5×10^{-6}
XII		H	$5{\cdot}0 \times 10^{-6}$	$2{\cdot}1 \times 10^{-6}$	$7{\cdot}5 \times 10^{-6}$
XIII		H	$7{\cdot}4 \times 10^{-7}$	$7{\cdot}4 \times 10^{-7}$	$1{\cdot}8 \times 10^{-5}$
XIV		H	$5{\cdot}5 \times 10^{-7}$	$4{\cdot}5 \times 10^{-7}$	$1{\cdot}4 \times 10^{-5}$
XV	H		$> 10^{-4}$	$> 10^{-4}$	$> 10^{-4}$
XVI	H		$4{\cdot}2 \times 10^{-5}$	6×10^{-5}	$> 10^{-4}$
XVII		H	$5{\cdot}8 \times 10^{-5}$	$\sim 10^{-4}$	*

* Material interferes with colorimetric assay procedure.

and showed similar activity in each case. They were uniformly less potent as inhibitors of *N*-demethylation and, in some cases, were practically inactive toward this reaction. The reason for this is not known and it remains to be seen to what extent different substrates influence the degree of inhibition with each of the reactions studied. Inhibitory activity was greatly reduced or eliminated in the 2-substituted isomers, as can be clearly seen by comparing XV with III and XVI with XIII. Compound XVII, the nitrogen isostere of III, was also practically inactive. While it is possible that the small difference in basicity between 4(5)- and 2-isomers[9] might account for the marked changes in inhibitory potency, it seems more likely that steric hindrance prevents the interaction of the 2-isomers with the microsomal complex. Although 1-phenyl-imidazoles and 4(5)-phenyl-imidazoles are also excellent inhibitors of microsomal oxidation, it is of interest that 4,5-dimethyl- and 4,5-diphenyl-imidazole are almost completely inactive.* In the latter two, both nitrogens are hindered, as in the 2-derivatives reported here, so that it appears that at least one of the imidazole nitrogens must be free for inhibitory activity.

Lineweaver–Burk reciprocal plots of the inhibition of aldrin epoxidation by compound XI indicate competitive kinetics, although similar plots of dihydroisodrin hydroxylation suggest more of a mixed type of inhibition, as has been previously observed with other types of imidazoles.* It is possible therefore that the imidazoles are acting, at least in part, as alternative substrates for the microsomal enzymes in a manner similar to SKF 525-A.[10] Although no metabolic investigations have yet been made with the imidazoles described here, their structures would suggest a number of possible metabolic transformations.

Each of the imidazoles investigated exhibited a distinct type II difference spectrum with a peak at 430–431 nm and a trough at 390–393 nm. The difference spectra were identical in either oxidized or NADPH-reduced microsomes. Double reciprocal plots of Δ O.D. 430–490 vs. imidazole concentration yielded straight line plots, and spectral dissociation constants (K_s) were calculated from their intercept on the abscissa. These constants represent the inhibitor concentration required for half-maximal spectral change and consequently are indicative of the binding capacity of the imidazole.

TABLE 2. SPECTRAL DISSOCIATION CONSTANTS (K_s) OF IMIDAZOLE DERIVATIVES IN RAT LIVER MICROSOMES

Compound	K_s (M)*
I	$2{\cdot}1 \times 10^{-4}$
V	$5{\cdot}2 \times 10^{-6}$
VI	$6{\cdot}3 \times 10^{-6}$
VII	$4{\cdot}3 \times 10^{-6}$
VIII	$2{\cdot}8 \times 10^{-6}$
XII	$7{\cdot}7 \times 10^{-6}$
XIII	$3{\cdot}1 \times 10^{-6}$
XV	$1{\cdot}1 \times 10^{-4}$

* Values are based on a protein concentration of 1 mg/ml.

The results in Table 2 clearly show that the 4(5)-imidazoles (III, V, VI, VIII, XII and XIII) bind to the microsomes at concentrations which are several orders of magnitude lower than those usually required with substrates such as aniline or hexobarbital.[11] Imidazole (I) and the 2-substituted derivative (XV) had binding constants almost two orders of magnitude higher than the 4(5)-imidazoles. These data correlate extremely well with the inhibition data in Table 1, and strongly suggest that the potent inhibitory activity of the 4(5)-substituted compounds results from their extremely high affinity for cytochrome P-450. Presumably the greater lipophilicity of the 4(5)-imidazoles facilitates penetration of lipid membranes and hydrophobic bonding to P-450 and accounts for their markedly enhanced potency compared with imidazole. With most of the compounds evaluated, the K_s and I_{50} values toward epoxidation and hydroxylation are very similar, and indicate a close relationship

* C. F. Wilkinson, unpublished data.

between these two parameters. It has been suggested that the type II spectral change indicates binding to the fifth or sixth ligand of the heme moiety of P-450, which is the same as that involved in binding oxygen and CO.[11] In agreement with this, the type II imidazole difference spectrum obtained in NADPH-reduced microsomes was found to be completely displaced by CO to give the usual P-450–P-420 spectrum. Furthermore, in contrast to the effects reported with aniline,[11] the CO difference spectrum was not displaced after the addition of relatively high concentrations (10^{-4} M) of the 4(5)-imidazoles, although a slight decrease in the magnitude of the 450 nm peak was observed. In view of the considerable background information on the interaction of imidazole with iron-heme proteins,[12–14] it would seem that derivatives such as those described here could be useful as type II mode compounds in studies of ligand interaction with P-450.

Potentiation of hexobarbital sleeping time was used as a measure of activity against microsomal enzymes *in vivo* and, as shown in Table 3, the results correlated with the findings *in vitro*. Again, it is seen that XIII was more potent than IV, and XV was inactive (2-substitution). On a molar basis, SKF 525-A (mol. wt 353) was twice as potent as IV (mol. wt 174) and equal to XIII (mol. wt 258) in potentiating hexobarbital. Mice injected with these compounds (100 mg/kg, i.p.) were indistinguishable from control animals in overt behavior. Table 4 illustrates the dose–response relationship of XIII in prolonging hexobarbital-induced sleep. Its effect *in vivo* was just detectable at 1 mg/kg

TABLE 3. POTENTIATION OF HEXOBARBITAL SLEEPING TIME BY IMIDAZOLE DERIVATIVES

Treatment*	Sleeping time (min ± S.D.)	Potentiation (%)	P
Saline	37·8 ± 12·6		
SKF 525-A (10 mg/kg, i.p.)	110·2 ± 26·1	290	< 0·001
IV (10 mg/kg, i.p.)	90·4 ± 26·5	252	< 0·001
XIII (10 mg/kg, i.p.)	152·3 ± 21·2	400	< 0·001
XV (30 mg/kg, i.p.)	39·1 ± 10·2		

* Ten mice in each group.

or 4 μmoles/kg. The parent compound of the series, imidazole (I), was inactive at 30 mg/kg but quite good at the next higher log-dose. Assuming uniform distribution of imidazole, this dose is roughly equivalent to a concentration of 2 mM imidazole in the body. Immature male mice (15–20 g) were more sensitive to imidazole (sleeping time: controls, 42·1 ± 11·4; 30 mg/kg, i.p., 75·3 ± 29·9; 100 mg/kg, i.p., 120 ± 21·1).

In addition to their effect on rat liver microsomes, the 4(5)-imidazoles reported here are extremely potent inhibitors of the microsomal enzymes in insects. It is possible that derivatives of this type might have practical utility as insecticide synergists and investigations are currently in progress to evaluate them in this regard.

TABLE 4. DOSE–RESPONSE RELATIONSHIP OF EFFECT OF IMIDAZOLES ON HEXOBARBITAL SLEEPING TIME IN MICE

Treatment	Sleeping time (min ± S.D.)	P
Saline*	31·1 ± 14·4	
I† 30 mg/kg, i.p.	31·5 ± 13·6	
100 mg/kg, i.p.	83·6 ± 28·7	< 0·001
XIII† 1 mg/kg, i.p.	42·8 ± 14·3	< 0·05
3 mg/kg, i.p.	51·3 ± 16·1	< 0·005
10 mg/kg, i.p.	156·3 ± 20·7	< 0·001
30 mg/kg, i.p.	8 hr	< 0·0001

* Thirty-one mice.
† Ten mice/group.

Finally, the current interest in cyclic nucleotide phosphodiesterase and the well known facts that imidazole stimulates and theophylline inhibits this enzyme will undoubtedly lead to an examination of imidazole derivatives and similar compounds in a search for inhibitors.[15] Pharmacological studies of such compounds are likely to center on their ability to potentiate the action of drugs and hormones which are believed to act via cyclic AMP.[16] In view of our results, it would be prudent to rule out effects on drug metabolism when considering the mode of action of imidazole derivatives in studies of that nature.

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Reactivation of isopropyl-methylphosphonylated acetylcholinesterase by α,ω-bis-(4-hydroxyiminomethylpyridinium)-2-trans-butene dibromide. The effect of temperature

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WE HAVE recently studied the interaction of α,ω-bis-(4-hydroxyiminomethylpyridinium)-2-trans-butene dibromide with isopropyl-methylphosphonylated acetylcholinesterase.[1,2] This compound is a strong reactivator of isopropyl-methylphosphonylated acetylcholinesterase.[1]

We have previously studied the influence of pH on the reactivating effect of this oxime.[2] In this study, the influence of temperature on reactivating effect, with the use of isopropyl-methylphosphonylated bovine erythrocyte acetylcholinesterase is described.

α,ω-Bis-(4-hydroxyiminomethylpyridinium)-2-trans-butene dibromide was prepared as reported previously.[1]

Acetylcholinesterase used in this work was prepared from bovine erythrocytes.[3] The specific activity of the lyophilized enzyme preparate was 0·265 μm of acetylcholine per min per milligram at 25°