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NEW HETEROCYCLIC STIMULATORS OF HEPATIC EPOXIDE HYDROLASE

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Abstract—A series of β-carbolines were found to be effective stimulators of rat liver microsomal epoxide hydrolase. The compounds, in order from most active to least active, were: isoquinoline > harman > norharman = ellipticine > 4-azafluorene > 4-phenylpyridine > pyridine > quinoline. Carbazole, 2-aminofluorene, 8-hydroxyquinoline, and napthalene were inactive as stimulators. Compounds possessing a fused bicyclic structure incorporating a pyridinic nitrogen β to the ring fusion were the most potent stimulators. The degree of stimulation by ellipticine was dependent on the type of microsomes studied, and the order from most stimulated to least stimulated was: hepatoma > hyperplastic nodules > normal = phenobarbital induced = 3-methylcholanthrene induced. Sodium cholate-solubilized microsomes were not able to be stimulated, but after removal of cholate by dialysis these microsomes were again able to be stimulated by ellipticine. Reconstitution experiments with purified expoxide hydrolase suggest that a partially dissociable factor in microsomes is necessary for these compounds to stimulate this enzyme.

It is well established that the hepatic metabolism of various drugs and of polycyclic aromatic hydrocarbons involves initial oxidative activation by the microsomal monooxygenase system to reactive species such as epoxides [1–3]. Two major pathways are known for further metabolism of the epoxides: hydrolysis to glycols by microsomal epoxide hydrolase (EC 3.3.2.3) [4, 5] and conjugation to glutathione by glutathione *S*-epoxide transferase [6, 7]. Various compounds are known to stimulate microsomal epoxide hydrolase [8–10]. Some of the more potent of these stimulators are also known to inhibit microsomal monooxygenase activity [9, 10]. Mechanistic studies with carbonyl type stimulators (metyrapone, chalcone oxide, and fluorenone) suggest that these compounds bind to a site distinct from the hydrolase substrate binding site [9]. The β-carbolines harman and norharman, found in cigarette tars [11], some natural foods and charred meats [12], have been reported to be comutagenic with 2-acetylaminofluorene (2-AAF) and its derivatives [13]. Ellipticine has been shown to be a potent inhibitor of microsomal monooxygenase activity [14] and of benzo[*a*]pyrene (BP) mutagenicity in the Ames test [15].

We investigated these heterocyclics and some structurally related compounds for their effects on epoxide hydrolase in various hepatic microsomal preparations from normal rats and rats pretreated with drugs or carcinogens. Ellipticine, harman, and norharman were found to be very potent stimulators of microsomal epoxide hydrolase activity. The degree of stimulation by these compounds was found to be dependent on the drug or carcinogen pretreatment of the rats. Studies with structurally related compounds show a requirement of a pyridinic nitrogen, and the better stimulators possess, in addition, a fused bicyclic ring system.

MATERIALS AND METHODS

Chemicals. All chemicals used were of analytical reagent grade. Harman, norharman, quinoline, 8-hydroxyquinoline, 4-azafluorene, 4-phenylpyridine, and 2-aminofluorene were purchased from the Aldrich Chemical Co. Milwaukee, WI. Carbazole was purchased from Eastman Organic Chemicals, Rochester, NY, and isoquinoline from the K & K Chemical Co., Plainview, N.Y. Ellipticine was a generous gift of Dr. Pierre Lesca, Laboratory of Pharmacology and Toxicology, C.N.R.S. Toulouse, France. Radioactivity was measured using Handifluor scintillation mixture (Mallinckrodt, Inc., St. Louis, MO) in a Packard model B-2450 scintillation spectrometer.

Animals and microsomes. PB and 3-methylcholanthrene (3-MC) induction of male Sprague–Dawley rat liver was initiated by i.p. administration of four doses of the drug [16]. Hyperplastic nodules were obtained by the method of interrupted feeding of 0.05% 2-AAF as described [17]. Hepatomas were resected from animals 14–16 weeks after the last week of feeding 2-AAF (12 weeks). The liver, nodule, or tumor microsomes were prepared by homogenization in 250 mM sucrose, 24 mM KCl, 5 mM $MgCl_2$, and 5 mM Tris·HCl (pH 7.6) and differential centrifugation [16]. The microsomal pellets were resuspended in the above buffer by mild (20 W) ultrasound with ice cooling for 30 sec and recentrifuged. The washed microsomes, resuspended as above, were stored in the above buffer.

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Enzyme assay. Styrene oxide hydrolase was assayed according to the method of Oesch *et al.* [18] as modified in this laboratory. Routinely, assays were done as follows. To 0.5 ml of 0.25 M Tris·HCl (pH 9.0), containing 50–100 μg protein and the appropriate concentration of the stimulator, was added 10 μl of 25 mM [7-^{3}H]styrene oxide in acetonitrile (0.4 μCi/μmole). After incubation at 37°, unreacted styrene oxide was removed from the aqueous reaction mixtures by two extractions with petroleum ether (boiling range 30–60°), and the aqueous phase containing the radioactive product, styrene glycol, was counted in Handifluor, a mixture designed for use with aqueous solutions. Boiled microsomes or boiled enzyme preparations, both of which were enzymatically inactive, were used as blanks. Stimulators were made water soluble by trituration with glacial acetic acid or concentrated hydrochloric acid and adjusted to 50 mM. Appropriate aliquots of this solution were then used to obtain the desired concentration in the assay.

Purification of normal rat liver epoxide hydrolase. The technique was a modification of a procedure published previously [19]. Washed normal rat liver microsomes were solubilized in 4% sodium cholate at 0° for 30 min, and ribosomes were removed by sedimentation at 110,000 *g* for 2 hr. The clear supernatant solution was dialyzed against 10 mM Tris·HCl (pH 8.5) for 16 hr (4°) and applied to a DEAE cellulose (Whatman DE 52) column, previously equilibrated in the Tris buffer. The column was washed successively with Tris buffer and 0.5% Emulgen 911, and the enzyme was then eluted with 1.0% Emulgen 911. The preparation appeared essentially homogeneous by analytical polyacrylamide gel electrophoresis, and it represented a 60 per cent recovery of epoxide hydrolase protein. Emulgen was removed from the preparation by shaking with washed polystyrene beads (SM-2, Bio-Rad Corp., Richmond, CA) at 4°, to reduce the Emulgen concentration to less than 0.2%. Details of this procedure will be published elsewhere.

Solubilization of microsomes and reconstitution of microsomal components by dialysis. A suspension of PB-induced microsomes (8 mg protein) was solubilized by incubation at 0–4° for 2 hr in 0.5 ml of 5 mM phosphate buffer (pH 7.4) containing 0.1 mM dithiothreitol, 1 mM EDTA, 30% glycerol, and 2.1% sodium cholate. To reconstitute the microsomal components, the clarified microsomal solution thus obtained was dialyzed overnight against 1 litre of phosphate buffer (5 mM, pH 7.4) containing 0.1 mM dithiothreitol, 1 mM EDTA, and 20% glycerol with or without 0.2% cholate. This resulted in a liposome-like suspension which could be observed visually or microscopically.

Purified epoxide hydrolase reconstitution with microsomes and microsomal lipids. Epoxide hydrolase purified as above was mixed with normal rat liver microsomes in 10 mM potassium phosphate (pH 7.5), containing 2.1% sodium cholate, and incubated at 27° for 3 hr. The solubilized preparation was dialyzed (4°) for 16 hr in 10 mM potassium phosphate to remove most of the sodium cholate. A similar technique was used with normal microsomal lipids which were obtained from lyophilized microsomes by extraction with chloroform–methanol (3:1).

RESULTS

Figure 1 shows a typical dose–response curve obtained for stimulation of hyperplastic nodule microsomal epoxide hydrolase by ellipticine, norharman, and harman. Norharman and harman showed maximum stimulation at 1 mM whereas ellipticine showed maximum stimulation at 50 μM. Similar dose–response curves were obtained with PB, 3-MC, hepatoma, and normal microsomes. Concentrations above 1 mM of all stimulators were not investigated because of their limited solubility under the conditions of assay. Table 1 shows the results obtained for ellipticine, harman, norharman, and metyrapone with various microsomal preparations at the optimum stimulator concentration. The degree of stimulation of microsomal epoxide hydrolase was found to be dependent on the induction of liver enzymes by pretreatment with PB, 3-MC or 2-AAF. Epoxide hydrolase activity of 3-MC-induced microsomes was stimulated the least (1.4- to 3-fold over control), whereas hepatoma microsomal hydrolase was stimulated the most (5.1- to 8.7-fold over control). Normal, PB, and hyperplastic nodule microsomes had enzyme which gave intermediate degrees of stimulation. The data presented in Table 1 are representative of the types of microsomes presented, and the assays were routinely performed in quadruplicate. Normal microsomes from rats fed basal diet [17] for at least 1 week were obtained on three separate occasions and the extent of stimulation was

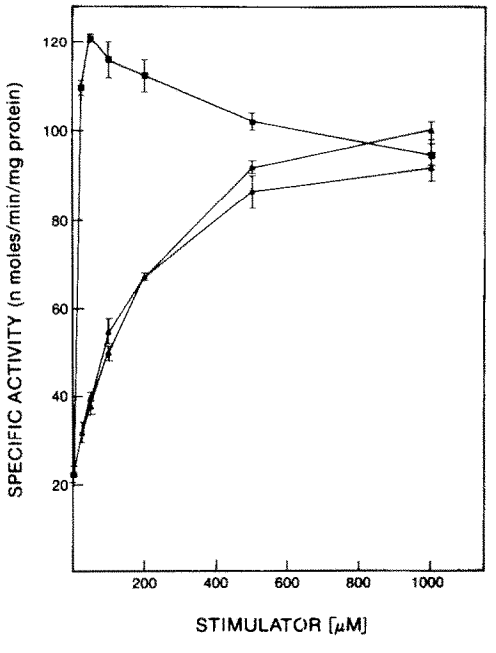


Fig. 1. Dose–response curves for the stimulation of hyperplastic nodule microsomal epoxide hydrolase by ellipticine (—■—■—), harman (—△—△—), and norharman (—▲—▲—). Bars represent S.D. from the mean for quadruplicate determinations.

Table 1. Stimulation of epoxide hydrolase in various types of rat liver microsomes*

Stimulator	Microsomal epoxide hydrolase activity Hepatoma	Hyperplastic nodule	PB-induced	Normal	3-MC-induced
None	6.8	22.3	21.5	4.3	10.5
	(1)†	(1)	(1)	(1)	(1)
Ellipticine (0.05 mM)	58.9	120.8	77.3	15.7	13.0
	(8.7)	(5.4)	(3.6)	(3.6)	(3.0)
Harman (1.0 mM)	50.9	100.0	69.0	21.1	23.5
	(7.5)	(4.5)	(3.2)	(4.9)	(2.2)
Norharman (1.0 mM)	59.2	91.8	69.3	17.4	20.0
	(8.7)	(4.1)	(3.2)	(4.0)	(1.9)
Metyrapone (1.0 mM)	35.0	55.0	46.1	9.8	15.2
	(5.1)	(2.5)	(2.1)	(2.3)	(1.4)

* Units of activity are nmoles·min^{-1}·(mg protein)$^{-1}$. Assays were performed in quadruplicate and the mean is presented.

† Figures in parentheses give the fold stimulation in each case relative to a value of 1-fold for the enzyme activity of the corresponding type of microsome in the absence of stimulator.

nearly the same in each case. PB and 3-MC microsomes were each isolated twice (six rat livers were pooled) and used for determining the degree of stimulation by ellipticine and norharman. Hyperplastic nodules were collected on two separate occasions; at each collection, nodule tissue was pooled from twenty to forty animals. Two types of hepatomas were used for stimulation analysis. One was poorly differentiated and the other well differentiated, as judged by histochemical analysis of 5 μm sections, fixed in neutral-buffered formalin, and stained in hematoxylin-eosin (courtesy of Dr. Kenichi Noda of our laboratory).

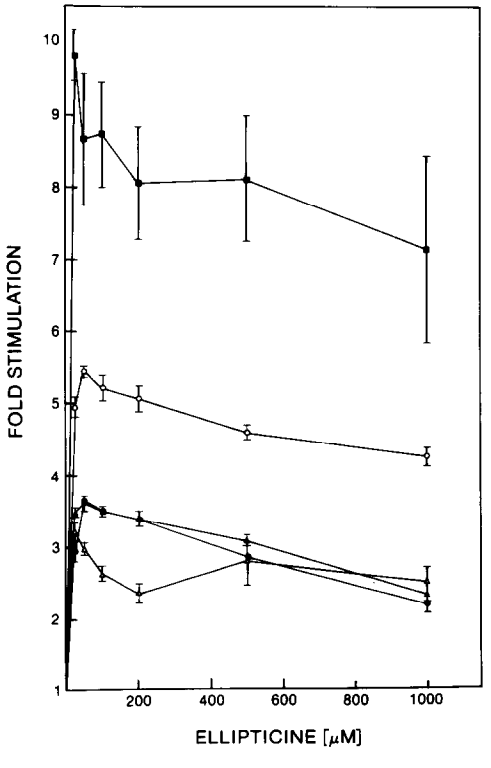
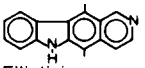
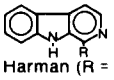
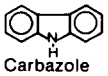
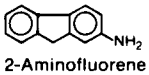
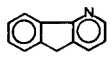


Fig. 2. Comparison of the relative stimulatory effect of ellipticine on microsomal epoxide hydrolase from hepatoma (—■—■—), hyperplastic nodule (—○—○—), PB-induced (—▲—▲—), untreated (—●—●—), and 3-MC-induced (—△—△—) rat liver microsomes. Bars represent S.D. from the mean for quadruplicate determinations.

Table 2. Stimulation of normal rat liver microsomal epoxide hydrolase by various aromatic compounds*

COMPOUND	% STIMULATION OVER CONTROL
 Ellipticine	265 ± 13.3
 Harman (R = CH_3)	390 ± 19.5
Norharman (R = H)	304 ± 21.3
 Carbazole	0
 2-Aminofluorene	0
 Isoquinoline	485 ± 14.6
 Quinoline	14 ± 6
8-Hydroxyquinoline	0
Napthalene	0
 4-Azafluorene	141 ± 7.3
4-Phenylpyridine	71 ± 8.6
Pyridine	47 ± 10

* All compounds were tested at 1 mM except ellipticine which was optimal at 50 μM.

Table 3. Effect of modifying microsomes with sodium cholate-glycerol on polycyclic stimulators of epoxide hydrolase.

Microsomes*	No activator [nmoles·min^{-1}·(mg protein)$^{-1}$]	Ellipticine†
(A) Unmodified	7.8	24.2
(B) Incubated with 2.1% sodium cholate‡	7.2	7.6
(C) Incubated as in B, then dialyzed overnight verus buffer	5.2	22.0

* These studies were performed with washed liver microsomes from rats pretreated with PB. The experiments were performed twice and gave similar results.
† Ellipticine was used at 50 μM.
‡ The incubations were for 30 min at 4°.

The variation in stimulation of microsomal hydrolase from various liver tissues is illustrated in detail for several concentrations of ellipticine in Fig. 2. Ellipticine showed decreased stimulation at concentrations higher than 50 μM for all microsomal preparations studied. The enzyme from hepatoma microsomes again appeared exceptionally susceptible to stimulation.

Table 2 shows the results of various structurally related compounds tested as stimulators of normal microsomal epoxide hydrolase. 2-Aminofluorene, carbazole, napthalene, and 8-hydroxyquinoline were inactive. Pyridine and quinoline were weakly active (47 per cent and 14 per cent respectively), whereas isoquinoline was highly active (400 per cent). 4-Phenylpyridine and 4-azafluorene showed 71 and 141 per cent stimulation respectively.

As shown in Table 3, a high concentration of sodium cholate abolished the ability of ellipticine to stimulate microsomal epoxide hydrolase (B). The stimulation of hydrolase by ellipticine was regained after 16 hr of dialysis against dialysis buffer (C), or against 0.2% cholate in dialysis buffer (results the same as C, Table 3).

Table 4 shows the results of ellipticine stimulation of purified epoxide hydrolase by itself, reconstituted with normal microsomes, and reconstituted with normal microsomal lipids. The purified, de-emulgenized enzyme, although somewhat stimulated by ellipticine, was stimulated significantly less than the enzyme in normal microsomes (1.4-fold stimulation for purified enzyme, 3.4-fold stimulation for enzyme, reconstituted with microsomes, Table 4). Normal microsomal lipids, extracted by chloroform/methanol, were also reconstituted with purified enzyme at optimum lipid to protein concentration (dose–response curve not shown), and the combination resulted in no greater stimulation than that shown by the purified enzyme itself (1.4-fold stimulation for purified enzyme, 1.3-fold for enzyme reconstituted with lipids, Table 4).

DISCUSSION

Microsomal epoxide hydrolase is known to be stimulated by a variety of compounds and detergents [8–10]. Of special interest in this study were those compounds that have also been reported to be inhibitors of microsomal monooxygenase activity [9, 10]. Mechanistic studies of the effects of aromatic ketones, such as fluorenone, chalcone oxide, and the bis-pyridyl-ketone metyrapone, on the action of epoxide hydrolase indicate that the site responsible for stimulation of enzyme activity is distinct from the

Table 4. Ellipticine stimulation of purified normal rat liver epoxide hydrolase under various conditions.

Enzyme preparation	Intrinsic activity*		Fold stimulation
	No activator	Ellipticine	
Normal microsomes	220	784	3.5
Purified epoxide hydrolase	476	664	1.4
Purified hydrolase reconstituted in microsomes†	302	1014	3.4
Purified hydrolase‡ reconstituted in microsomal lipids	664	858	1.3

* Styrene oxide hydrolyzed [nmoles·min^{-1}·(mg hydrolase protein)$^{-1}$. Hydrolase protein was quantitated by electroimmunoassay [25].
† The species reconstituted were: 3.4 μg purified epoxide hydrolase with 5.4 μg of microsomal hydrolase (135 μg microsomal protein).
‡ When reconstituted in microsomal lipids at optimum enzyme to lipid concentration (approximately 1 to 15).

substrate binding site [9]. Reduction of these carbonyl-type stimulators to the corresponding alcohols results in loss of stimulatory effect on microsomal epoxide hydrolase [8*], suggesting interaction of the carbonyl group at the stimulator site. The new class of stimulators herein reported are pyridine derivatives lacking carbonyl groups (Table 2). Carbazole and naphthalene, which lack pyridinic nitrogens and are structural analogs of norharman and isoquinoline, respectively, were inactive. The aromatic primary amine, 2-aminofluorene, was also without effect. A comparison of the structures of the poorly active compounds (pyridine, quinoline, 4-phenylpyridine, and 4-azafluorene) with those of the active compounds (isoquinoline, norharman, harman, and ellipticine) is revealing in terms of structure–activity relationships. The compounds possessing a fused bicyclic structure incorporating a pyridinic nitrogen β to the bridged carbon atom are the most potent stimulators. The presence of a suitably poised pyridinic nitrogen in these active compounds may allow interaction with either a metal ion or a strong electrophile at the stimulatory site. In this connection it is interesting that ellipticine, a potent stimulator of epoxide hydrolase and an inhibitor of cytochrome P-450, shows a type II difference spectrum with cytochrome P-450 [15], suggesting an interaction involving heme iron.

The existence of a strong interaction between cytochrome P-450 and epoxide hydrolase has been suggested previously [21]. If stimulation of epoxide hydrolase by these heterocyclics results from their binding to cytochrome P-450 in an existing microsomal complex, then the degree of stimulation of epoxide hydrolase would be expected to depend on the level of cytochrome P-450. The degree of stimulation of microsomal epoxide hydrolase by aromatic nitrogen heterocyclics was dependent on the type of microsomes studied (Table 1 and Fig. 2); however, no direct or inverse relationship between the liver microsomal cytochrome P-450 content and the degree of stimulation of hydrolase was observed. In particular, hepatoma microsomes, which have a 70–75 per cent decrease in cytochrome P-450 content compared to normal microsomes [22], had activity that was stimulated 5- to 9-fold, and hyperplastic nodule microsomes, which also have a decreased cytochrome P-450 content [22], showed an increase in stimulation over normal and PB-induced microsomes. PB-induced and normal microsomes were stimulated to similar extents, even though PB-induced microsomes possess twice the normal microsomal P-450 content [20].

Hyperplastic nodules may be putative precursor cells to hepatomas and, though they possess many characteristics of normal liver, share several characteristics with hepatomas [22]. One major characteristic of both nodule and hepatoma is the preneoplastic antigen [23], which has been shown to be epoxide hydrolase [24]. We compared normal, nodule, and hepatoma microsomal epoxide hydrolase stimulation to see if this characteristic of nodule hydrolase was like hepatoma or more like normal liver. It appears that a higher degree of β-carboline stimulation is another characteristic of nodule and hepatoma epoxide hydrolase, in contrast to normal, PB- or 3-MC-induced microsomal hydrolase.

The level of epoxide hydrolase is known, from immunological studies, to be induced 3-fold in PB-induced microsomes [20], 4.5-fold in hyperplastic nodules [20, 25], and 5-fold in hepatoma microsomes [26]. The increase in absolute epoxide hydrolase content does not correlate with the increase in the degree of enzyme stimulation in these microsomes. If heterocyclic stimulators of epoxide hydrolase function by binding to a regulatory site on the enzyme, the variation in degree of stimulation can be most easily accounted for by the existence of multiple forms of epoxide hydrolase, which have altered stimulatory sites. Indeed, multiple forms of rat liver epoxide hydrolase have been reported in normal, PB- and 3-MC-induced livers [27]. Results in Table 3, however, indicate that sodium cholate solubilization abolished stimulation by ellipticine. The stimulatory effect could be restored upon removal of cholate by dialysis.

The experiments with purified epoxide hydrolase by itself and reconstituted in microsomes (Table 4) show that a non-lipid factor in microsomes, which is partially dissociated from the purified enzyme (presumably during purification), is necessary for stimulation.

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