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BENZYLHYDRAZINE—A SELECTIVE INHIBITOR OF HUMAN AND RAT BRAIN MONOAMINE OXIDASE

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Abstract—Benzylhydrazine ($BzNNH_2$) was shown to inhibit irreversibly human brain type A and type B monoamine oxidase (MAO) as measured by 5-hydroxytryptamine (5-HT) and phenylethylamine (PEA) deamination, respectively. The concentrations required to inhibit these isoenzymes *in vitro* were 2.7×10^{-7} M and 1.7×10^{-8} M, respectively. Inhibition of 5-HT deamination was essentially non-competitive, whereas that for PEA approached uncompetitive kinetics. $BzNNH_2$ also inhibited rat brain MAO *in vivo*, 50 per cent inhibition of the A and B forms of MAO occurring at injected doses of 0.72 and 0.39 mg/kg respectively. These results indicate that $BzNNH_2$ preferentially inhibits the B form of both human and rat brain MAO.

In the past several years, considerable attention has been focused on the structural features of the amine substrates which determine their relative affinities for either the A or B form of the mitochondrial enzyme, monoamine oxidase (MAO). These studies have revealed that phenylethylamine (PEA) and benzylamine are deaminated predominantly by the B form of MAO, whereas the hydroxylated derivatives of PEA, tyramine and dopamine, are metabolized by both forms of the oxidase or, as in the case of norepinephrine, predominantly by the A form [1, 2].

In contrast, relatively few studies have been designed to determine systematically the structural requirements of MAO inhibitors which dictate selectivity for either form of the oxidase. From the studies that have been performed, it is difficult to predict inhibitor specificity based on a structural similarity to substrate. For example, the PEA derivative, amphetamine, has a higher affinity for the type A oxidase [3], whereas the hydrazine derivative of PEA displays little selectivity for either form of MAO [2]. These data suggest that the structure of the amine side chain is critical in determining binding specificity.

The selective type B MAO inhibitor, pargyline, is an *N,N*-disubstituted derivative of benzylamine. Based on this and the fact that benzylamine is a highly specific substrate for the B oxidase, it was considered possible that the hydrazine derivative of this amine, benzylhydrazine ($BzNNH_2$), may be a selective inhibitor of the B form of MAO. Several reports have indicated that $BzNNH_2$ is a potent inhibitor of brain and liver MAO in animals [4, 5], and Popou *et al.* [6] have suggested that this hydrazine derivative may, in fact, be a selective inhibitor of the B form of rat brain MAO, *in vivo*. Accordingly, the present study was undertaken to determine whether benzylhydrazine would also be a selective inhibitor of human brain MAO.

MATERIALS AND METHODS

Mitochondria from the frontal lobe of human brain were isolated as described previously [7] except that the specimens were homogenized in a Waring blender for 1 min in 4 vol. of 0.1 M potassium phosphate buffer, pH 7.4, containing 0.25 M sucrose. The MAO assay was a modification of the procedure used previously in this laboratory [8]. Reaction mixtures contained either ^{14}C-labeled 5-hydroxytryptamine or [^{14}C]phenylethylamine and varying concentrations of $BzNNH_2$ and enzyme, in a total volume of 0.4 ml of 0.05 M potassium phosphate buffer, pH 7.4. Benzylhydrazine was preincubated in the presence of enzyme for varying lengths of time depending on the nature of the experiment. Reactions were initiated with the addition of substrate and terminated by the addition of 50 μl of 0.4 M HCl. Aliquots (0.2 ml) of the reaction mixture were passed through cation exchange resin columns (Bio-Rex 70, sodium form, 100–200 mesh) and collected in scintillation counting vials. The columns were washed with 2.8 ml water which was also collected in these vials. Ten ml Aquasol was added to each vial and the radioactivity was measured by liquid scintillation spectrometry. Blank values were determined in the presence of 10^{-3} M pargyline. Except where indicated, all values for $BzNNH_2$ concentration are based on the final concentration after substrate was added.

To measure inhibition of the A and B forms of MAO activity in rat brain *in vivo*, varying concentrations (0.15 to 1 mg/kg) of $BzNNH_2$ in isotonic saline were injected i.p. into 400–500 g rats. Control rats received the vehicle only. After 1 hr the animals were decapitated and the brains were removed as quickly as possible. Brains were homogenized in 4 vol. of cold 0.1 M potassium phosphate buffer, pH 7.4, in a Teflon glass homogenizer. Homogenates were centrifuged at 600 *g* for 10 min and type A and B MAO activities were measured in the resulting supernatant solution essentially as described above.

Radioactively labeled [^{14}C]5-hydroxytryptamine (49.3 mCi/m-mole) and phenylethylamine (50.98 mCi/m-mole) were obtained from New England Nuclear, Boston, MA; unlabeled 5-HT and PEA from the Sigma Chemical Co., St. Louis, MO; and pargyline from Abbott Laboratories, Chicago, IL. Bio-Rex 70 was obtained from Bio Rad Laboratories, Richmond, CA. Benzylhydrazine was purchased from Pfaltz & Bauer,

Stamford, CT. All chemicals used were of the purest grade available from commercial sources.

RESULTS

The ability of $BzNNH_2$ to inhibit the type A and B MAO substrates, 5-hydroxytryptamine (5-HT) and PEA, respectively, is illustrated by the data presented in Fig. 1. When $BzNNH_2$ was preincubated with enzyme for 10 min, 50 per cent inhibition of PEA and 5-HT was achieved at preincubation concentrations of approximately 1.7×10^{-8} M and 2.7×10^{-7} M respectively. At 2.7×10^{-7} M $BzNNH_2$, PEA deamination was inhibited more than 95 per cent.

To determine whether the inhibition of PEA and 5-HT deamination by $BzNNH_2$ is reversible or irreversible, mitochondria were preincubated in the presence and absence of the hydrazine derivative for 10 min. Aliquots were removed for determination of type A and B MAO activities. The remaining portion was centrifuged at 15,000 *g* for 20 min and washed three times with 0.1 M potassium phosphate buffer, pH 7.4, as described previously [9]. Results of these experiments, shown in Table 1, indicate that repeated washing of the $BzNNH_2$-treated mitochondrial preparations did not reduce the per cent inhibition of either PEA or 5-HT deamination. As expected, $BzNNH_2$ was a more effective inhibitor of PEA deamination than of 5-HT deamination.

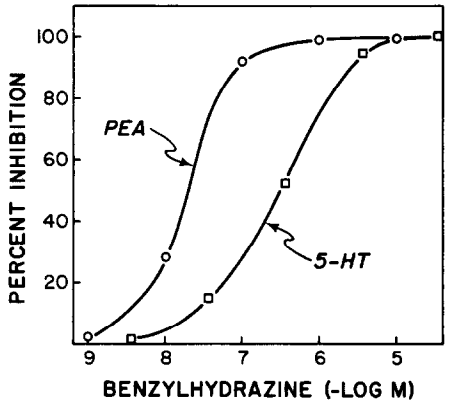

Fig. 1. Benzylhydrazine ($BzNNH_2$) inhibition of 1×10^{-4} M 5-hydroxytryptamine (5-HT) and 2.5×10^{-6} M phenylethylamine (PEA) deamination by human brain mitochondria. Reaction mixtures containing 74.2 or 44.5 μg protein, respectively, and varying amounts of $BzNNH_2$ in a total volume of 0.3 and 0.4 ml of potassium phosphate buffer, pH 7.4, were preincubated for 10 min at 37° prior to the addition of ^{14}C-labeled 5-HT (100 μl) or PEA (10 μl). Incubations were continued for 60 and 5 min, respectively, followed by the addition of 50 μl of 0.4 M HCl. Deaminated products formed during the reaction were separated by cation exchange chromatography.

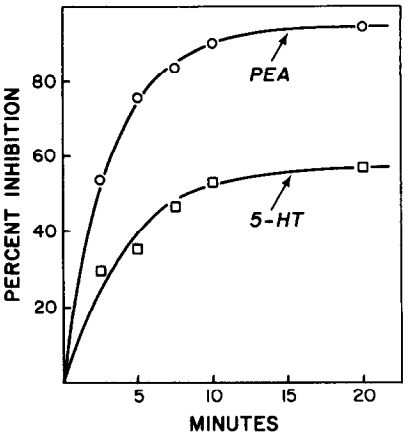

Fig. 2. Effects of preincubation time on inhibition of types A and B brain MAO by $BzNNH_2$. Benzylhydrazine (10^{-7} M final concentration) was preincubated with human brain mitochondria for varying lengths of time, as indicated, prior to the addition of ^{14}C-labeled substrates. See legend to Fig. 1 for details of the incubation conditions.

The effects of preincubation time on inhibition of the two forms of human brain MAO by 10^{-7} M $BzNNH_2$ are illustrated in Fig. 2. By 10 min, inhibition of both forms of MAO had reached a plateau. At this time, inhibition of PEA deamination was greater than 90 per cent, whereas that for 5-HT was slightly less than 55

Table 1. Irreversible nature of benzylhydrazine inhibition of phenylethylamine and 5-hydroxytryptamine deamination by human brain monoamine oxidase*

Sample	Product formed (nmoles/mg protein) PAA	5-HIAA	Per cent inhibition PAA	5-HIAA
Unwashed				
Control	7.7 ± 0.2	27.8 ± 1.3		
Benzylhydrazine	1.9 ± 0.1	19.1 ± 1.1	74.9 ± 0.6	31.3 ± 3.0
Washed				
Control	7.0 ± 0.3	23.0 ± 0.7		
Benzylhydrazine	1.3 ± 0.1	15.4 ± 0.9	81.4 ± 1.2	31.9 ± 4.4

* Human brain mitochondria were preincubated for 10 min in the presence and absence of 10^{-7} M benzylhydrazine prior to centrifugation and washings. Reaction mixtures contained 1.0×10^{-4} M or 2.5×10^{-6} M 5-hydroxytryptamine (5-HT) or phenylethylamine (PEA), respectively, and washed or unwashed mitochondria in a total volume of 0.4 ml of potassium phosphate buffer pH 7.4. Time of incubation was 60 and 5 min, respectively, for 5-HT and PEA. The phenylacetic acid (PAA) and 5-hydroxyindole acetic acid (5-HIAA) formed were assayed by cation exchange chromatography as described in Materials and Methods.

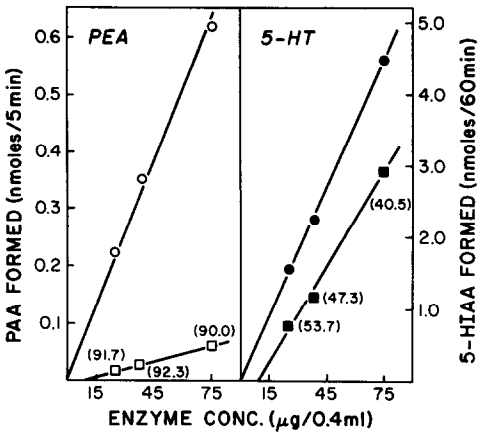

Fig. 3. Effects of enzyme concentration on $BzNNH_2$ inhibition of PEA and 5-HT deamination. Benzylhydrazine (10^{-7} M final concentration) was preincubated for 10 min with varying concentrations of human brain mitochondria prior to the addition of ^{14}C-labeled substrates. See legend to Fig. 1 for details of the incubation conditions. Phenylacetic acid (PAA) and 5-hydroxyindole acetic acid (5-HIAA) formed during the reaction were separated by cation exchange chromatography. Values in parentheses represent per cent inhibition.

per cent. The ratio of per cent inhibition of type B to type A MAO remained relatively constant at each time period assayed and varied between 1.8 and 2.2. When the ratio of per cent inhibition was corrected for preincubation concentrations of $BzNNH_2$ this value ranged from 2.4 to 2.9.

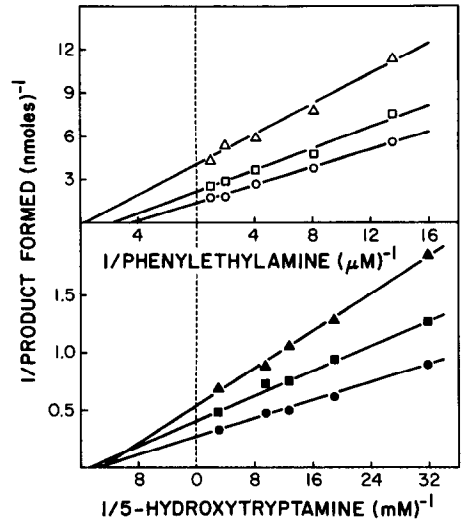

Fig. 4. Lineweaver–Burk plots for benzylhydrazine ($BzNNH_2$) inhibition of 5-hydroxytryptamine (5-HT) and phenylethylamine (PEA) deamination by human brain mitochondria. Reaction mixtures for 5-HT deamination contained varying amounts of [^{14}C]-5-HT, human brain mitochondria, 0, 5×10^{-8} and 1×10^{-7} M $BzNNH_2$ and 20 μmoles of potassium phosphate buffer, pH 7.4, in a total volume of 0.4 ml. $BzNNH_2$ was preincubated for 10 min at 37° prior to the addition of substrate. Incubations were continued for an additional 60 min at which time 50 μl of 0.4 M HCl was added to terminate the reaction. Reaction conditions for PEA were similar except that the concentrations of $BzNNH_2$ used were 1×10^{-8} M and 2.5×10^{-8} M and the substrate incubation lasted for 5 min.

Table 2. *In vivo* inhibition of phenylethylamine (PEA) and 5-hydroxytryptamine (5-HT) deamination in rat brain*

Benzylhydrazine (mg/kg)	Per cent inhibition PEA	5-HT
0.15	6.0	17.8
0.30	52.4	34.1
0.50	57.4	30.4
1.00	86.8	65.7
1.50	89.6	78.6

* See Materials and Methods for experimental details.

The effects of enzyme concentration on $BzNNH_2$ inhibition of PEA and 5-HT deamination are presented in Fig. 3. The rates of deamination of both amines in the absence and presence of inhibitor were linear with enzyme concentration. Per cent inhibition of PEA deamination did not vary with enzyme concentration, whereas 5-HT metabolism decreased approximately 25 per cent as the concentration of protein was raised from 25 to 75 μg/incubation. The plots obtained for untreated and $BzNNH_2$-treated enzymes are not parallel and do not intersect at the abscissa. Only the line from the untreated preparation crosses at the origin. These data are consistent with $BzNNH_2$ being a mixed-type of inhibitor of both the A and B forms of MAO.

The type of inhibition of PEA and 5-HT deamination produced by $BzNNH_2$ is demonstrated by Lineweaver–Burk plots illustrated in Fig. 4. Both graphs display a mixed inhibition pattern, with inhibition of PEA being more uncompetitive in nature and that of 5-HT appearing non-competitive.

The above studies demonstrate that $BzNNH_2$ is a selective inhibitor of human brain type B MAO *in vitro*. The effects of this compound, *in vivo*, on the two forms of brain MAO, when injected i.p. into rats is shown by the data in Table 2. Rat brain deamination of PEA and 5-HT was inhibited at all concentrations of $BzNNH_2$ tested, and at all but the lowest concentration of $BzNNH_2$, per cent inhibition of PEA is statistically greater than that of 5-HT. The ID_{50} values for $BzNNH_2$ inhibition of PEA and 5-HT deamination by rat brain MAO are approximately 0.39 and 0.72 mg/kg respectively.

DISCUSSION

The data reported in this paper demonstrate that $BzNNH_2$ is a selective inhibitor of the B form of MAO in human and rat brain. Depending on the protein concentration used to assay human brain MAO activity *in vitro*, the concentration of this drug required to inhibit the B form of the oxidase is approximately fifteen times less than that needed for inhibition of the A species. Relative to other type B selective MAO inhibitors, $BzNNH_2$ is less selective than deprenyl [10], but about equal to pargyline [11, 12]. The concentration of $BzNNH_2$ (1.7×10^{-8} M) required to inhibit human type B MAO 50 per cent is similar to that reported for deprenyl (1 to 2.5 $\times 10^{-8}$ M) [13, 14] and pargyline (4×10^{-8} M) [13, 15].

The specificity of $BzNNH_2$ is not surprising considering its structural similarity to PEA, a highly selective substrate for the B form of MAO. Bond distances calculated from computer models for the lowest energy

conformation of both compounds provide evidence for the similarity between the two compounds. For example, the overall lengths of the molecules, as calculated from the distance from the primary amine to the *para* carbon atom of the phenyl ring, differ by less than 10 per cent, being 6.45 and 5.94 Å for PEA and $BzNNH_2$ respectively. In fact, based on dimensions alone, $BzNNH_2$ should predictably have been a more selective inhibitor of the B isoenzyme than the actual results indicate. This suggests that alteration of the α-carbon atom of PEA greatly influences binding selectivity. Thus, changing the α-carbon atom of PEA to a nitrogen atom as in $BzNNH_2$, or substitution on the α-carbon atom, as in amphetamine, greatly reduces the affinity of PEA for the B form of MAO.

An attempt was also made in this study to characterize the nature of the interaction between $BzNNH_2$ and MAO. The results suggest that $BzNNH_2$ irreversibly inhibits both forms of MAO. Inhibition of 5-HT deamination results in non-competitive kinetics, whereas inhibition of PEA metabolism approaches uncompetitive kinetics. Though not shown, the presence of 5-HT or PEA during preincubation periods protected against inhibition by $BzNNH_2$ of the A and B forms of MAO respectively. This suggests that $BzNNH_2$ is metabolized by MAO to an active intermediate which irreversibly binds to the oxidase. Youdim [16] has suggested that inhibition of MAO by the hydrazine derivative of PEA, phenelzine, involves the formation of a stable "reduced-type" of flavin-inhibitor adduct similar to that seen with pargyline. It is difficult to reconcile this mechanism with that of the uncompetitive kinetics observed between $BzNNH_2$ and PEA. Binding of $BzNNH_2$ to the isoalloxazine moiety of flavin would be expected to produce simple non-competitive kinetics. Since this did not occur, at least for PEA deamination, it may be concluded that the site on the B isoenzyme at which $BzNNH_2$ binds irreversibly may be different than that for phenelzine.

Varga and Tringer [17] have suggested that the selective B type inhibitor, deprenyl, may be an effective antidepressant agent. The clinical use of type B MAO inhibitors may have a distinct advantage over the nonselective or A type inhibitors in that the B selective drugs do not produce the hypertensive reaction associated with tyramine or amphetamine administration [18, 19]. Accordingly, the need for new and more highly selective B type MAO inhibitors is justified. The results of this paper demonstrate than $BzNNH_2$ preferentially inhibits the B form of human brain MAO though, relative to deprenyl, $BzNNH_2$ is somewhat less selective. In addition, the data further demonstrate the structural importance of the α-carbon atom of PEA in determining the affinity of drugs for the isoenzymes of MAO.

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