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INHIBITION OF RABBIT LIVER MONOAMINE OXIDASE BY NITRO AROMATIC COMPOUNDS

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Abstract—Nitrobenzenoid, nitroheterocyclic and cyanobenzoid compounds inhibit type B monoamine oxidase. A partially purified enzyme preparation from rabbit liver mitochondria, oxidizing *p*-dimethylaminobenzylamine as the substrate, was competitively inhibited by nitrobenzoid compounds with K_i values in the range of 0.28 μM for *p*-dinitrobenzene to 0.56 mM for *p*-nitrobenzoic acid. The potencies of nitrobenzoid compounds were positively correlated with the Hammett sigma value for each substituent on nitrobenzene. Dinitro derivatives were slightly more potent than the corresponding mononitro compounds but not as potent as would be expected from their sigma values. For the nitroheterocyclic compounds, inhibition was also competitive; the lowest K_i was 1.3 μM for 5-nitrofurfural semicarbazone (nitrofurazone). Compounds with cyano groups in place of nitro groups were also inhibitory; the most potent was *p*-acetobenzonitrile with a K_i of 1.3 μM. The results of this study indicate that, in addition to nitrobenzoid compounds, other compounds with planar, electron-deficient nuclei are effective inhibitors of type B monoamine oxidase. Although hydrophobic and steric parameters may play some role in inhibition, the predominant factor is the electron-withdrawing power of the ring substituents.

Monoamine oxidase [monoamine:O_2 oxidoreductase (deaminating), EC 1.4.3.4] (MAO) is a flavoprotein which is bound to the outer membrane of the mitochondrion. Substrates for this enzyme include biogenic amines and numerous compounds with similar structures, which are oxidatively deaminated with the electrons being transferred to O_2. In the present studies, directed initially at finding an alternative electron acceptor for this enzyme, it was observed that some nitro aromatic compounds were potent inhibitors.

Nitro compounds are known to react with flavin systems. They are reduced by the hepatic microsomal P-450 system [1], which contains flavoproteins, and by xanthine oxidase [2]. Nitro compounds have been shown to react non-enzymatically with $FMNH_2$ [3, 4] by which they are reduced to hydroxylamines. Only a few compounds containing the nitro or similar group have been studied as inhibitors of MAO. Most of these are nitro derivatives of MAO inhibitors whose potency is no greater than the parent compound [5, 6]. MAO from rat liver and heart is inhibited by glyceryltrinitrate and related nitrate esters of aliphatic polyalcohols [7, 8]. K_i values for these inhibitors are in the range of 0.01 to 1.0 mM. Sodium nitrite, at 0.01 to 0.1 M, is not inhibitory, but rather slightly increases the MAO activity [9].

In the present study, nitro aromatic compounds and cyano compounds were examined for their ability to inhibit MAO partially purified from rabbit liver. The K_i values and reversibility of the inhibitions were determined and correlated with chemical and physical parameters of the compounds.

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MATERIALS AND METHODS

Except where otherwise noted, the chemicals for these studies were all commercially available. The authenticity of each purchased nitro aromatic compound was confirmed by an ultraviolet spectrum and a melting point which were in agreement with literature values. The methyl esters of *p*-nitrobenzoic acid and 2,5-dinitrobenzoic acid were prepared from HCl methanolic solutions of the acids and recrystallized twice. The melting point of these compounds and the ultraviolet and the infrared spectra correspond to the assigned structure of the esters.

Clorgyline was a gift from May & Baker Ltd. (Dagenham, England). Deprenyl was prepared by Dr. J. Knoll (Semmelweiss University, Budapest, Hungary) and supplied to this laboratory by Dr. M. Sandler (Queen Charlotte's Maternity Hospital, London, England).

Enzyme preparation. Female, white New Zealand rabbits, 6 to 8-weeks-old, were deeply anesthetized by an intravenous injection of 70 mg/kg pentobarbitol; the livers were rapidly removed and placed in ice-cold 0.25 M sucrose, pH 7.6. The organs were weighed and used immediately or stored frozen at −20° for 2 weeks or less before use. Using only minor modifications of the method of Yasunobu and Gomes [10], partially purified MAO was obtained by isolating mitochondria, solubilizing with Triton X-100, and fractionating several times with ammonium sulfate. All steps were carried out at 4°. The enzymes were kept in frozen aliquots (−20°) until used. The MAO was stable and no changes in kinetic parameters were seen. The specific activity was 83 units/mg protein.

Protein concentrations were determined using a biuret reagent [11] with bovine serum albumin as the standard.

Assay of enzyme. A spectrophotometric assay using *p*-dimethylaminobenzylamine as the substrate was employed. Absorbance at 355 nm, which indicated the production of dimethylaminobenzaldehyde, was continuously monitored for 20 min. The assay mixture contained 67 mM potassium phosphate buffer, pH 7.2: 0.033% Lubrol WX; 83.2 units of enzyme; and various concentrations of dimethylaminobenzylamine (DAB) in a total volume of 3 ml. With the use of cuvettes open to air, the concentration of oxygen was maintained at the level of an air-saturated solution (approximately 200 μM). The reaction was initiated by adding DAB following a 20-min preincubation of the other components, cooled to 20°. A unit of enzyme activity was defined as that amount of enzyme required to deaminate 1 nmole *p*-dimethylaminobenzylamine/min (when saturated with DAB). The reaction was linear with respect to time and enzyme concentration.

Deprenyl and clorgyline sensitivities of rabbit liver MAO with DAB as the substrate. Stock solutions of deprenyl and clorgyline, which were 10 mM in water, were diluted to give the following concentrations in the assay: clorgyline, 0.01 mM to 10 mM (in five steps); and deprenyl, 0.05 μM to 10 mM (in nine steps). The drugs were preincubated for 20 min with the enzyme before addition of substrate. The percent inhibition was based upon an assay with no inhibitor present.

Inhibition studies. Compounds to be tested were each dissolved in methanol to 10 mM. Some of the weaker inhibitors were used at this concentration, but the more potent inhibitors were serially diluted with methanol to obtain inhibitions between 20 and 80%. The final concentrations of the inhibitors (1–100 μM) were achieved by adding 0.03 ml to the 3-ml assay mixture along with the normal assay components. The percent inhibition was calculated on the basis of the control mixtures containing 0.03 ml methanol. This concentration of methanol does not inhibit the enzyme. Two methods were used to check for the reversible nature of the inhibition. In addition to the usual 20-min preincubation of the enzyme with buffer and the inhibitors, the assays were also conducted by adding each inhibitor just prior to the substrate. Also, a solution of enzyme that had been incubated with the inhibitor, *m*-dinitrobenzene, with and without substrate, was assayed after an overnight dialysis to remove any non-covalently bound inhibitor.

Data analyses. The kinetic parameters of the uninhibited enzyme were determined from measurements where the substrate concentration ranged from 0.2 to 3.3 mM. The analyses were either of the graphical or mathematical form of the Lineweaver–Burk double-reciprocal equation. Inhibited activities were determined at four substrate concentrations, each at four concentrations of the compounds tested. The Lineweaver–Burk equation was used to analyze the type of inhibition with respect to the amine substrate and to determine the K_i values. Data points presented are the arithmetic means of at least two pairs of duplicates. Regression lines were calculated by least squares analyses of all data points unless noted otherwise.

Hammett linear-free energy relationships. Inhibitors of MAO activity were tested for correlation with the chemical potential or reactivity of the various nitro compounds by means of a Hammett linear-free energy relationship [12]. The Hammett relationship is usually expressed as the equation: $\log K = \rho\sigma + C$, where σ, the substituent constant, is interpreted as a measure of the ability of the substituent to change the electron density at the reaction center or binding center; ρ, the reaction constant, reflects the sensitivity of the equilibrium or rate constant to an alteration in electron density; and C is the reference constant. In Results, the data are presented in a graphic form where the negative logarithm of the competitive inhibition constant K_i is plotted against σ with the slope being ρ.

RESULTS

Sensitivities to clorgyline and deprenyl. Rabbit liver MAO is much more sensitive to deprenyl than it is to clorgyline when DAB is the substrate. *In vitro* dose–response curves for both drugs are of single sigmoidal shape, indicating that single enzyme form is being inhibited [8]. The I_{50} values are 1 μM and 0.28 mM for deprenyl (MAO B inhibitor) and clorgyline (MAO A inhibitor) respectively. This set of data is consistent with DAB being a substrate for the B form of rabbit liver MAO [13]. Thus, the following results are applicable only to that species of enzyme.

Kinetic studies. The characteristics of rabbit liver monoamine oxidase with DAB as the substrate and those of the inhibition *p*-nitrobenzonitrile (as a representative compound) are illustrated in Fig. 1. The K_m under the conditions described was 0.21 mM and

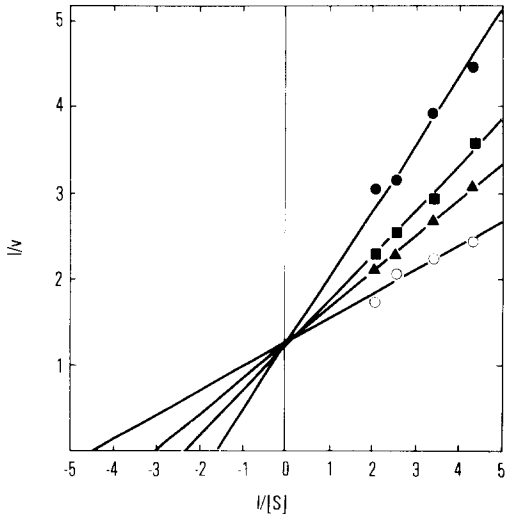

Fig. 1. Lineweaver–Burk plot showing competitive inhibition of rabbit liver MAO by *p*-nitrobenzonitrile. The concentrations of DAB, the substrate, were 5×10^{-4} M, 4×10^{-4} M, 3×10^{-4} M, and 2.3×10^{-4} M. Open circles (○) represent the uninhibited reaction. The concentrations of *p*-nitrobenzonitrile were 6.67×10^{-7} M represented by triangles (▲), 1×10^{-6} M represented by squares (■), and 2×10^{-6} M represented by closed circles (●). The K_i calculated from the slopes of the lines is 1.1×10^{-6}M. The units for reciprocal velocity are (μmoles/10 min/mg protein)$^{-1}$ and for reciprocal substrate concentrations are $(10^{-5}\,\text{M})^{-1}$.

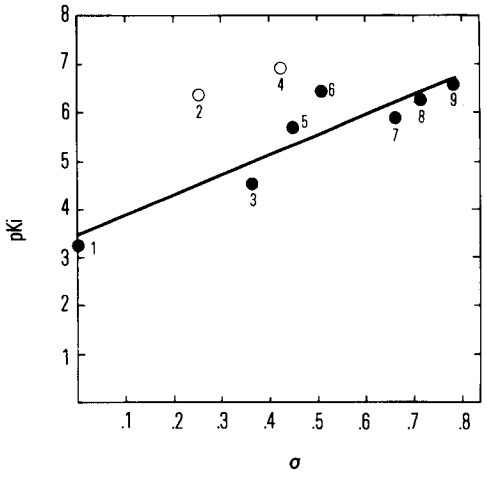

Fig. 2. Correlation of the inhibition of rabbit liver MAO by nitrobenzenoid compounds with the corresponding sigma values (σ). The negative logarithm of the K_i (pK_i) is plotted versus the σ values. The slope of the line (ρ), disregarding points 2 and 4 (open circles), is 4.3 ± 0.8, $r^2 = 0.89$ (see text for explanation). The compounds used were assigned the following numbers: *p*-nitrobenzoic acid (1), *p*-chloronitrobenzene (2), *p*-nitrobenzamide (3), *p*-nitrobenzaldehyde (4), methyl *p*-nitrobenzoate (5), *p*-nitroacetophenone (6), *p*-nitrobenzonitrile (7), *m*-dinitrobenzene (8), and *p*-dinitrobenzene (9).

the V_{max} was 0.83 μmole per 10 min per mg protein. The inhibition was competitive with a K_i of 1.1 μM. The other compounds and their K_i values are listed in Table 1 according to increasing Hammett sigma values [14]. These data are presented graphically in Fig. 2. The drawn line disregards the values for *p*-chloronitrobenzene and *p*-nitrobenzaldehyde. These two compounds appear to be anomalous here and with platelet MAO as well as with studies on the oxidation of hemoglobin studies (unpublished results, A. S. Kimes and D. O. Carr; G. J. Brewer and D. O. Carr). The experiments in this laboratory on platelet MAO and reports by others [15] using different systems also show increased potency of the chloro derivative and suggest it might be due to its hydrophobicity being markedly greater than those of the other compounds. The partition coefficient between hexane and water for *p*-chloronitrobenzene is 120 while the partition coefficients for the other nitro compounds are between 0 and 7.1. MAO is subject to product inhibition and *p*-nitrobenzaldehyde is a product analog. These factors may explain potencies greater than expected from the σ values for these two compounds.

Table 1. Inhibition of rabbit liver MAO by nitrobenzenoid compounds

Compound	σ	K_i (μM)
p-Nitrobenzoic acid	0	560
p-Chloronitrobenzene	0.24	0.43
p-Nitrobenzamide	0.36	16
p-Nitrobenzaldehyde	0.42	0.13
Methyl *p*-nitrobenzoate	0.45	2.2
p-Nitroacetophenone	0.50	0.35
p-Nitrobenzonitrile	0.66	1.1
m-Dinitrobenzene	0.71	0.47
p-Dinitrobenzene	0.78	0.28

Table 2. Inhibition of rabbit liver MAO by dinitrobenzoic acids and ester

Compound	σ	K_i (μM)
2,4-Dinitrobenzoic acid	0.71	300
2,5-Dinitrobenzoic acid	0.73	200
Methyl 2,5-dinitrobenzoate	1.19	1.6

Data for the inhibition of liver MAO by the dinitrobenzoic acids and a methyl ester, along with their sigma values, are shown in Table 2. These compounds are weaker inhibitors than would be predicted from their sigma values or, presented differently, are in the range of the corresponding mononitro compounds. Steric parameters are proposed for the lower potency, but the small number of compounds tested prevents a rigorous analysis.

Studies with related compounds. Experiments with related compounds were conducted to evaluate the requirements for a benzenoid nucleus in the inhibitor and/or a nitro group on the inhibitor. Results with various nitroheterocyclic compounds are presented in Table 3. These compounds also exhibit competitive inhibitions, but they are generally less potent than the benzenoid compounds. A partial explanation may come from preliminary experiments which show that these compounds are, in general, much more hydrophilic (partition coefficients less than 0.2).

Cyanobenzenoid compounds each had higher K_i values than the analogous nitro compounds (see Table 3). The reasons for these differences are at least 2-fold: the electron withdrawing ability of the cyano group is somewhat less than that of the nitro group (σ value of 0.66 vs 0.78) and the cyano compounds are more hydrophilic. The partition coefficient range for the compounds with nitro groups is 3.5 to 5.5, whereas the range for their cyano analogs is 0.5 to 2.0

Reversibility studies. Studies done to determine if the nitro compounds might form irreversible complexes with the enzyme or might require enzyme activation showed that prior incubation of the

Table 3. Inhibition of rabbit liver MAO by nitroheterocyclic compounds and cyanobenzenoid compounds

Compound	K_i (μM)
Nitrofurans	
5-Nitro-2-furoic acid	340
5-Nitrofuraldehyde	18
5-Nitrofurfural semicarbazone	1.3
Nitroimidazoles	
5-Nitro-2-methylimidazole	9100
Metronidazole	71
Nitrothiazole	
5-Nitro-2-pyridylthiazole	17
Cyanobenzenoids	
p-Acetobenzonitrile	1.3
m-Dicyanobenzene	7.2
p-Dicyanobenzene	3.2

enzyme with the inhibitors did not potentiate the inhibition. Subsequent overnight dialysis of an enzyme preparation that had been inhibited with *m*-dinitrobenzene resulted in an activity equal to that of an uninhibited enzyme preparation treated in a similar manner. Therefore, it is concluded that the competitive inhibition does not require prior activation of the inhibitors nor is there any irreversible complex with the enzyme.

DISCUSSION

The competitive inhibition of type B MAO by nitrobenzenoid and nitroheterocyclic compounds has been characterized. The *m*-dinitro, *p*-dinitro and *p*-chloronitro compounds are potent inhibitors of rat liver MAO; their K_i values are similar to those of tranylcypromine [16], clorgyline [17] and harmaline [18]. The enzyme from rabbit liver, using dimethylaminobenzylamine as the substrate, was more strongly inhibited by the type B inhibitor, deprenyl, than by clorgyline. Future studies will address the utility of nitro compounds inhibiting type A MAO. Furthermore, the potency of the inhibitors is a positive function of the electron deficiency of the benzenoid nucleus. Other investigators, using other types of benzenoid inhibitors [12, 17, 19, 20], have reported similar observations, as well as the smaller contribution of hydrophobic and steric parameters to the potency of the inhibitor.

The reversibility of the inhibition was tested using *m*-dinitrobenzene as a representative compound. With the exceptions of *p*-chloronitrobenzene and *p*-nitrobenzaldehyde which show anomalous results in many systems already mentioned, the inhibition by *m*-dinitrobenzene is kinetically similar to the other nitrobenzenoid, cyanobenzenoid, and heterocyclic compounds tested, making it a good representative compound.

Many of the componds used in the present study have been shown to be radiosensitizing agents and to be cytotoxic; these properties have been shown to be a function of the Hammett sigma value [21]. In addition, such compounds are mutagenic in test systems [22, 23]. These findings bring into focus the problems that may arise in any future use of the compounds *in vivo* [24]. Although the nitrobenzenoid compounds may not be used *in vivo* on a long-term basis or pharmacologically, short-term use in experimental animals may be acceptable and MAO inhibitors are useful in *in vitro* studies to eliminate undesired metabolism of monoamines. Additionally, as shown in one aspect of this report, the cyano group, while not as potent as the nitro group, is quite adequate in generating structures that inhibit MAO. A compound of special interest is *p*-chlorocyanobenzene which is the analog of the unusually potent *p*-chloronitrobenzene.

In conclusion, a new series of MAO inhibitors, based upon nitrobenzene, has been described, but neither the benzene nucleus nor the nitro group is absolutely required. Future studies will be required to delineate the roles and maximize the efficacy of the electron withdrawing group, hydrophobicity, and steric parameters.

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