ELSEVIER

Biochemical Pharmacology 63 (2002) 865–869

Biochemical Pharmacology

Phentermine inhibition of recombinant human liver monoamine oxidases A and B

Ravi K. Nandigama, Paige Newton-Vinson, Dale E. Edmondson*

Department of Biochemistry, Emory University School of Medicine, Rollins Research Building, 1510 Clifton Road, Atlanta, GA 30322-3050, USA

Received 30 April 2001; accepted 1 August 2001

Abstract

Recent studies with rat tissue preparations have suggested that the anorectic drug phentermine inhibits serotonin degradation by inhibition of monoamine oxidase (MAO) A with a K_I value of 85–88 μM, a potency suggested to be similar to that of other reversible MAO inhibitors (Ulus *et al.*, Biochem Pharmacol 2000;59:1611–21). Since there are known differences between rats and humans in substrate and inhibitor specificities of MAOs, the interactions of phentermine with recombinant human purified preparations of MAO A and MAO B were determined. Human MAO A was competitively inhibited by phentermine with a K_I value of 498 ± 60 μM, a value approximately 6-fold weaker than that observed for the rat enzyme. Phentermine was also observed to be a competitive inhibitor of recombinant human liver MAO B with a K_I value of 375 ± 42 μM, a value similar to that observed with the rat enzyme (310–416 μM). In contrast to the behavior with rat tissue preparations, no slow time-dependent behavior was observed for phentermine inhibition of purified soluble human MAO preparations. Difference absorption spectral studies showed similar perturbations of the covalent FAD moieties of both human MAO A and MAO B, which suggests a similar mode of binding in both enzymes. These data suggest that phentermine inhibition of human MAO A (or of MAO B) is too weak to be of pharmacological relevance.

Keywords: Phentermine; Human MAO A; Human MAO B; Amines; Anorectics

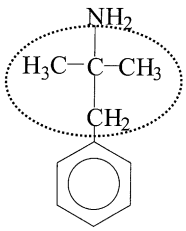
1. Introduction

The anorectic drug phentermine has received considerable attention since its introduction as an appetite suppressant in the clinical treatment of obesity. The drug combination of phentermine and fenfluramine was used extensively to treat obese patients until its recent discontinuation, due to the associated development of cardiac valvulopathy. The therapeutic effects of phentermine are generally thought to be achieved via inhibition of norepinephrine reuptake, although recent publications have suggested that its function may also involve the inhibition of MAO A [1]. Phentermine was reported to inhibit rat liver MAO A with a K_I value of 85–88 μM, a value similar to those of other clinically used reversible MAO inhibitors [2]. The conclusion from these studies was that the drug combination of dexfenfluramine (a serotonin reuptake inhibitor) and phentermine (an inhibitor of serotonin catabolism) could give rise to elevated serum serotonin levels, which could cause pulmonary hypertension, and to cardiac valvular lesions.

Given the widespread use and notoriety of these drugs, confirmation of these conclusions and of the data supporting them is of intrinsic importance. The clinical importance of MAO inhibition by the specific binding of phentermine has not been universally accepted. Plasma phentermine levels of patients receiving a standard dosage have been measured to be ~1 μM [3]. It should also be noted that experiments documenting rat MAO A and MAO B inhibition by phentermine were performed using particle preparations obtained from liver, brain, and lung tissues. Therefore, these preparations contain both MAO A and MAO B, which were assayed using serotonin oxidation for MAO A and phenylethylamine oxidation for MAO B. However, the specificity of each MAO for these substrates is not absolute. Although there are significant homologies in amino acid sequences between the rat and human enzymes, differences exist that may influence the relative

* Corresponding author. Tel.: +1-404-727-5972; fax: +1-404-727-3452.
E-mail address: dedmond@bimcore.emory.edu (D.E. Edmondson).
Abbreviations: MAO, monoamine oxidase; E_s, Taft steric constant.

0006-2952/02/$ – see front matter
PII: S0006-2952(02)00840-7



Scheme 1. Structure of phentermine. The dashed line identifies the alkyl side chain representing the E_s parameter in Eq. (1).

affinities of the two sources of MAO A for reversible inhibitors [4]. Therefore, to determine whether the results obtained with the rat enzymes have relevance to the human forms of these enzymes, it is necessary to measure phentermine inhibition of human MAO A and MAO B.

Our laboratory has succeeded in the high level expression of human liver MAO B [5], has worked extensively with recombinant human liver MAO A [6], and has purified each of them to homogeneity in their fully functional forms. Using these enzyme preparations, we found that the binding affinities of various phenylalkylamines to human liver MAO A increase with the Taft steric parameter of the alkyl side chain linking the phenyl ring with the deprotonated form of the amine [7] by the following relationship:

$$\log K_D = 4.11\,(\pm 0.56)E_s + 0.24\,(\pm 0.97) \quad (1)$$

where E_s is the Taft steric constant, which is a measure of the van der Waals radius of the alkyl side chain [8]. This correlation predicts that phentermine (alkyl side chain $E_s = -2.78$) (Scheme 1) would bind to human liver MAO A by over 4 orders of magnitude tighter than the K_I value observed with amphetamine. This prediction also suggests that phentermine binds to MAO A with a K_D of $\sim$10 nM, which is $\sim 10^4$ times tighter than the value observed experimentally with the rat enzyme (85–88 μM) [2]. We undertook the quantitative determination of the binding affinity of phentermine to purified human liver MAO A and MAO B in an attempt to achieve three goals: (a) to provide experimental support for the predicted tight binding of phentermine to MAO A by the above equation, (b) to determine if phentermine binds as tightly to MAO B, and (c) to provide experimental data that would test the hypothesis proposed by Wurtman and coworkers [2] from their experiments on rat MAO.

2. Materials and methods

Human liver MAO A was expressed in *Saccharomyces cerevisiae* under the control of a galactose promoter and purified to electrophoretic homogeneity as described by Weyler *et al.* [6]. Human liver MAO B was expressed in *Pichia pastoris* under the control of an alcohol oxidase promoter and purified to homogeneity as described [5]. Each enzyme preparation exhibited >95% functionality as judged from turnover numbers and by the level of flavin reduction when incubated with substrate under anaerobic conditions.

Phentermine HCl, benzylamine HCl, and kynuramine were purchased from the Sigma and used directly without further purification. All assays were performed spectrophotometrically in 50 mM potassium phosphate, pH 7.5, in the presence of 0.5% (w/v) reduced Triton X-100 at 25°. The oxidation of *p*-CF_3-benzylamine by MAO A was monitored at 243 nm (absorption maximum for the aldehyde product, extinction coefficient = 11,800 M^{-1} cm^{-1}), and the oxidation of benzylamine by MAO B was monitored at 250 nm (absorption maximum for benzaldehyde, extinction coefficient = 12,800 M^{-1} cm^{-1}) [9,10]. The oxygen concentration in all assays was at air-saturation ($\sim$240 μM), which is saturating for MAO A and at the K_M value for MAO B. All spectral data were obtained using a Perkin-Elmer Lambda 2 UV–VIS double-beam spectrophotometer with thermostatted cuvette holders. The difference spectra for the interaction of phentermine with MAO A and MAO B were obtained by subtracting the spectral contributions of phentermine and MAO A or MAO B from the spectra of phentermine and MAO A/MAO B mixtures. The spectra were then corrected for 100% occupancy of phentermine at MAO A or MAO B binding sites based on the experimentally obtained K_I values.

Kinetic data were evaluated and plotted using Microcal Origin or Sigma plot software running on a Gateway PC.

3. Results and discussion

3.1. Interaction of phentermine with recombinant human MAO A

Preliminary experiments (data not shown) showed no detectable oxidation of phentermine by either MAO A or MAO B, demonstrating that it is not a substrate for either enzyme. This is expected since there are no α-hydrogens on the alkylamine side chain. Competitive binding experiments demonstrated that phentermine is a competitive inhibitor of MAO A when the substrate is *p*-trifluoromethylbenzylamine. The data in Fig. 1A show intersecting lines in Lineweaver–Burk plots as expected for a competitive inhibitor. A plot of the K_M (apparent) vs. phentermine concentration (Fig. 1B) shows a linear relationship, which permits the determination of a K_I value of 498 ± 60 μM. This value is approximately 6-fold higher than the K_I value of 85–88 μM determined for rat MAO A [2]. The higher affinity of rat MAO A for phentermine relative to human MAO A reflects their differences in inhibitor specificity. The differences in values are not due to differences in assay conditions since the pH conditions of the measurements were essentially identical. The differences in temperature in the two studies (25° in our assays and 37° in the rat

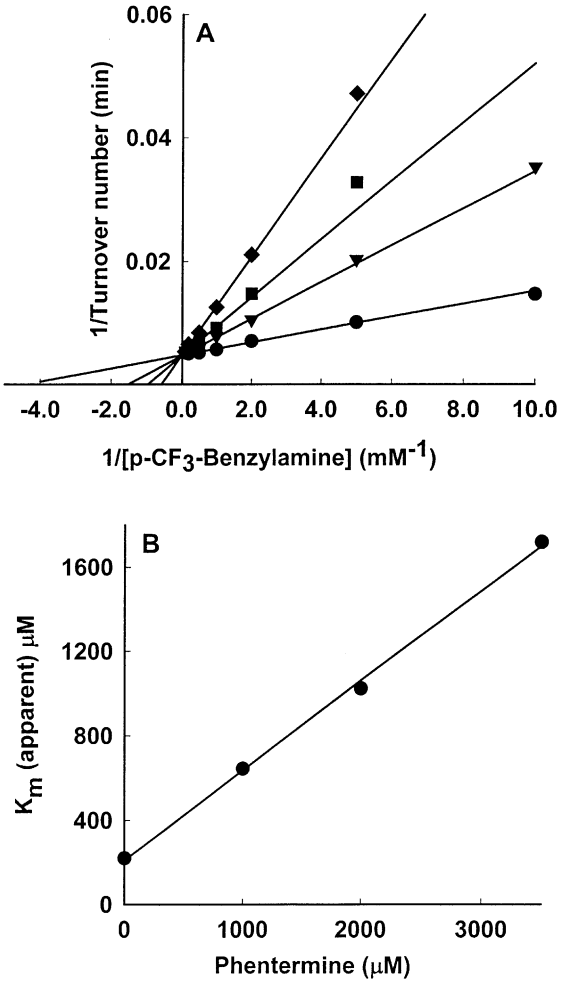


Fig. 1. Determination of the K_I value of phentermine binding to MAO A. Panel A shows double-reciprocal plots for the oxidation of p-CF_3-benzylamine by MAO A in the presence of increasing concentrations of phentermine. The concentrations of phentermine used were (μM): 0 (●), 1000 (▼), 2000 (■), and 3500 (◆). Panel B shows the effect of phentermine on the apparent K_M for the MAO A-catalyzed oxidation of p-CF_3-benzylamine. The solid line is a linear best fit of the experimental data yielding a calculated K_I of 498 ± 60 μM.

enzyme assays) were not expected to result in such a large difference in values. The observed binding affinity of phentermine with human MAO A contrasts with that predicted on the basis of the Taft steric constant of the alkyl side chain of arylalkylamines [7]. While this relationship (Eq. (1)) demonstrates a strong interaction of the alkyl side chain with the enzyme in contributing to the total binding affinity, there are known instances where steric limitations in the binding site cannot accommodate substitutions with too large a size. This has been documented with para-substituents in the binding affinities of phenethylamine substrate analogues with human liver MAO A [7], where a substituent that is too large reduces the binding affinity. It appears that the alkyl side chain of phentermine (Scheme 1) exceeds the size limit that can be accommodated optimally in the substrate binding site of human MAO A. On the other hand, rat MAO A may be able to accommodate the increased size of the phentermine alkyl side chain and thus be able to bind phentermine more tightly than human MAO A. Hence, the inhibitor specificity of rat and human enzymes may be different, and thus results obtained from the rat enzyme system may not be quantitatively extended to the human enzyme system.

3.2. Interactions of phentermine with recombinant human MAO B

Similar experiments were performed with recombinant human liver MAO B using benzylamine as a substrate. The results in Fig. 2A show that, as observed with MAO A, phentermine is also a competitive inhibitor of MAO B oxidation of benzylamine with a K_I value of 375 ± 42 μM. This value is similar to the range of values determined for

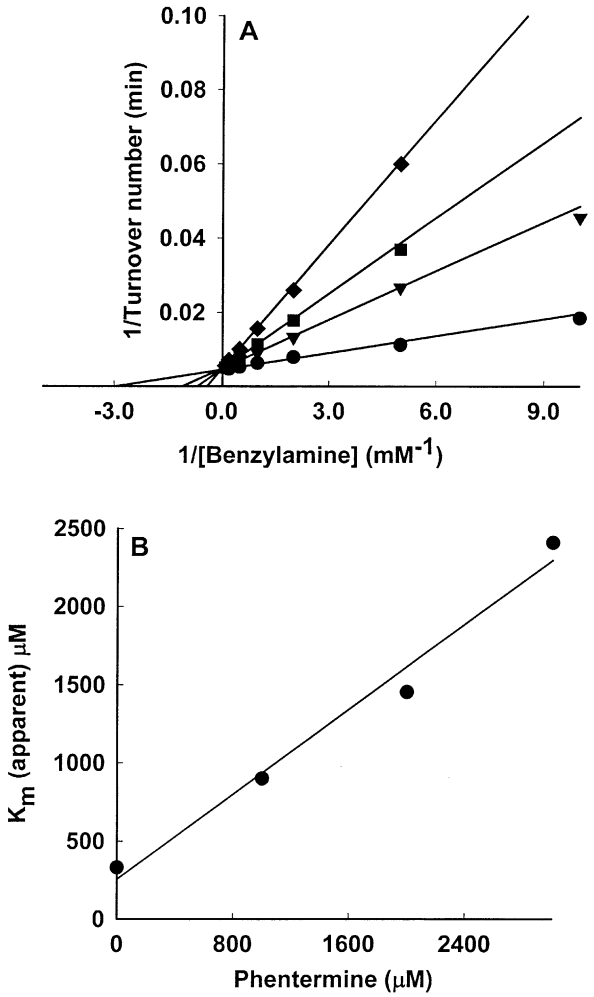


Fig. 2. Determination of the K_I value of phentermine binding to MAO B. Panel A shows double-reciprocal plots for the oxidation of benzylamine by MAO B in the presence of increasing concentrations of phentermine. The concentrations of phentermine used were (μM): 0 (●), 1000 (▼), 2000 (■), and 3000 (◆). Panel B shows the effect of phentermine on the apparent K_M for the MAO B-catalyzed oxidation of benzylamine. The solid line is a linear best fit of the experimental data, yielding a calculated K_I of 375 ± 42 μM.

rat MAO B preparations of 310–416 μM [2]. This agreement in binding affinities between the rat and human enzymes contrasts significantly from that observed with MAO A and deserves comment. Preliminary data in our laboratory showed that the coefficient for the correlation of the Taft steric effect value of the alkyl side chain on the binding affinities of arylalkylamines to human MAO B is diminished to about one-half that observed with human MAO A (see Eq. (1)). Therefore, MAO B should exhibit a lower sensitivity to the nature of the alkyl side chain (Scheme 1) than does MAO A. These differences appear to be a result of differences in protein folding about the substrate binding site in MAO A and MAO B, although both these enzymes share 71% sequence identity in humans. Since the binding affinities of phentermine to MAO B in humans and rats are similar, it appears that the binding sites in rat and human MAO B probably have more similarities than the binding sites of rat and human MAO A, which contributes to the species-based inhibitor specificities as observed.

3.3. Spectral data of phentermine binding to MAO A and to MAO B

We next studied whether any differences could be observed in the spectral perturbations of the 8α-*S*-cysteinylFAD coenzyme induced by phentermine binding to MAO A and MAO B. The difference spectral data in Fig. 3 demonstrate that similar but not identical spectral perturbations of the flavin coenzyme moieties occur upon phentermine binding to each enzyme. The comparative magnitude of the absorption changes indicated are for 100% occupancy of sites. These data demonstrate that the binding of phentermine to MAO A and to MAO B results in similar alterations in the environments of their respective flavin coenzymes. For comparison, the spectral perturbations of MAO A on binding phenylbutylamine (also a competitive inhibitor with a tighter binding affinity, $K_I = 31$ μM) are more intense than those elicited by phentermine binding. Similar data could not be determined with MAO B since phenylbutylamine is a slow substrate and is oxidized during the time required to perform the experiment.

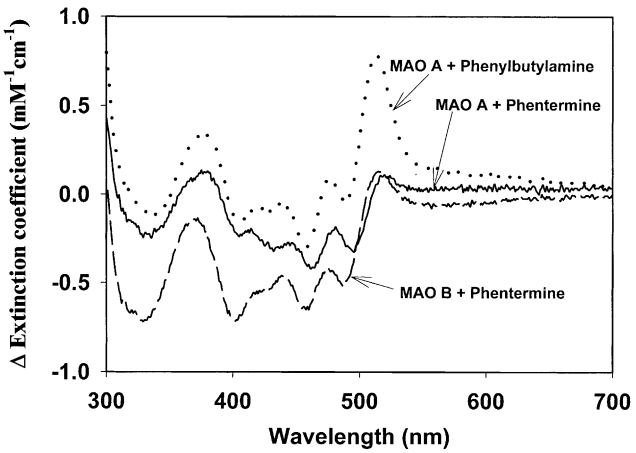


Fig. 3. Difference absorption spectra of the flavin coenzyme moieties for the interaction of phentermine with MAO A and MAO B. The spectra were obtained by subtracting the spectral contributions of MAO A or MAO B from the spectrum of the respective MAO A– or MAO B–phentermine complex. The spectral data were then corrected for 100% occupancy of phentermine at the enzyme site according to the K_I values obtained from Figs. 1 and 2. For comparison, the spectral data for the interaction of MAO A with phenylbutylamine is also included (···).

3.4. Reversibility of phentermine inhibition of MAO A and MAO B

Previous data on the membrane-bound rat enzymes showed a time-dependent inhibition by and release of phentermine, which occurred on the time scale of 5 min [2]. To determine whether any time-dependent changes are present in phentermine inhibition of soluble pure recombinant human MAO A or MAO B, enzyme samples were preincubated with saturating concentrations of phentermine (4 mM) for up to 15 min, followed by assay of enzyme activities after dilution of the incubation mixture into the assay buffer containing substrate. The observed catalytic activities of MAO A and MAO B were found to be identical to that observed with control (absence of phentermine) samples. The concentration of phentermine after dilution into the assay solution was several folds lower than the K_I values obtained. It is also interesting to note that the kinetic traces of these catalytic assays were linear in substrate oxidation and devoid of any apparent lag phase, indicating that the release of phentermine from the MAO A/MAO B–phentermine complex is instantaneous (occurring rapidly on a time scale faster than a conventional steady-state assay). When MAO A or MAO B was added to the assay system containing both substrate and phentermine, inhibition of enzyme was instantaneous without any apparent burst phase. These results suggest that association and dissociation of phentermine with MAO A or MAO B occur very rapidly. The small magnitude of spectral flavin perturbations of MAO A and MAO B upon interaction with phentermine (see Fig. 3) precluded using this property to measure the "association on-rate" and "dissociation off-rate" of phentermine from either enzyme site.

In summary, the results in this study resolve the question of the pharmacological relevance of phentermine inhibition of MAO A and MAO B. The inhibitory binding affinity of phentermine to human MAO A is too weak to have any pharmacological influence relative to the measured tight binding of this compound ($K_D \approx 2$–8 μM) [11,12] to noradrenaline and dopamine transporters in the 2–8 μM range, which is within the plasma concentration range expected from a normal dosage [3]. Therefore, the proposed involvement of phentermine in the formation of cardiac valvular lesions must involve interactions other than inhibition of MAO A.

Acknowledgments

This work was supported by a grant (GM-29433) from the National Institutes of Health. The authors wish to thank E. Morgan for his critical reading of the manuscript.

References

[1] Ryan DH. Use of sibutramine and other noradrenergic and serotonergic drugs in the management of obesity. Endocrine 2000; 13:193–9.
[2] Ulus IH, Maher TJ, Wurtman RJ. Characterization of phentermine and related compounds as monoamine oxidase (MAO) inhibitors. Biochem Pharmacol 2000;59:1611–21.
[3] Douglas A, Douglas JG, Robertson CE, Munro JR. Plasma phentermine levels, weight loss, and side-effects. Int J Obestet 1983;7:591–5.
[4] Geha RM, Chen K, Shih JC. Phe^{208} and $Ileu^{199}$ in human monoamine oxidase A and B do not determine substrate and inhibitor specificities as in rat. J Neurochem 2000;75:1304–9.
[5] Newton-Vinson P, Hubalek F, Edmondson DE. High level expression of human liver monoamine oxidase B in *Pichia pastoris*. Prot Expr Purif 2000;20:334–45.
[6] Weyler W, Titlow CC, Salach JI. Catalytically active monoamine oxidase type A from human liver expressed in *Saccharomyces cerevisiae* contains covalent FAD. Biochem Biophys Res Commun 1990;173:1205–11.
[7] Nandigama RK, Edmondson DE. Structure–activity relations in the oxidation of phenylethylamine analogues by recombinant human liver monoamine oxidase A. Biochemistry 2000;39:15258–65.
[8] Hansch C, Leo A. Exploring QSAR. Fundamentals and applications in chemistry and biology. Washington, DC: American Chemical Society, 1995.
[9] Walker MC, Edmondson DE. Structure–activity relationships in the oxidation of benzylamine analogues by bovine liver mitochondrial monoamine oxidase B. Biochemistry 1994;33:7088–98.
[10] Miller JR, Edmondson DE. Structure–activity relationships in the oxidation of para-substituted benzylamine analogues by recombinant human liver monoamine oxidase A. Biochemistry 1999;38:13670–83.
[11] Garratini S, Borroni E, Mennini T, Samanin R. Differences and similarities among anorectic agents. In: Garratini S, Samanin R, editors. Central mechanisms of anorectic drugs. New York: Raven Press, 1978. p. 127–43.
[12] Jenden DJ, Hinsvark ON, Cho AK. Some aspects of the comparative pharmacology of amphetamine and phentermine. In: Garratini S, Samanin R, editors. Central mechanisms of anorectic drugs. New York: Raven Press, 1978. p. 165–77.