2 H Kinetic Isotope Effects and pH Dependence of Catalysis as Mechanistic Probes of Rat Monoamine Oxidase A: Comparisons with the Human Enzyme

ARTICLE *in* BIOCHEMISTRY · AUGUST 2011

Impact Factor: 3.02 · DOI: 10.1021/bi200951z · Source: PubMed

2 AUTHORS, INCLUDING:

Dale E Edmondson
Emory University
241 PUBLICATIONS **9,323** CITATIONS

SEE PROFILE

Published in final edited form as:
Biochemistry. 2011 September 6; 50(35): 7710–7717. doi:10.1021/bi200951z.

^{2}H Kinetic Isotope Effects and pH Dependence of Catalysis as Mechanistic Probes of Rat Monoamine Oxidase A: Comparisons with the Human Enzyme‡

Jin Wang and **Dale E. Edmondson***
Department of Biochemistry, Emory University School of Medicine, Atlanta, GA 30322

Abstract

Monoamine oxidase A (MAO A) is a mitochondrial outer membrane-bound flavoenzyme important in the regulation of serotonin and dopamine levels. Since the rat is extensively used as an animal model in drug studies, it is important to understand how rat MAO A behaves in comparison with the more extensively studied human enzyme. For many reversible inhibitors, rat MAO A exhibits K_i values similar to those of human MAO A. The pH profile of k_{cat} for rat MAO A shows a pKa of 8.2±0.1 for the benzylamine ES complex and pKa values of 7.5±0.1 and 7.6±0.1 for the respective ES complexes with *p*-CF_3-^{1}H and *p*-CF_3-^{2}H-benzylamine. In contrast to the human enzyme, the rat enzyme exhibits a single pKa value (8.3±0.1) with k_{cat}/K_m benzylamine vs. pH and pKa values of 7.8±0.1 and 8.1±0.2 are found for the ascending limbs, respectively, of k_{cat}/K_m vs. pH profiles for *p*-CF_3-^{1}H and *p*-CF_3-^{2}H-benzylamine and 9.3±0.1 and 9.1±0.2 for their respective descending limbs. The oxidation of *para*-substituted benzylamine substrate analogues by rat MAO A exhibit large deuterium kinetic isotope effects on k_{cat} and on k_{cat}/K_m. These effects are pH-independent, and range from 7 to 14, demonstrating a rate-limiting α-C-H bond cleavage step in catalysis. Quantitative structure-activity correlations of log k_{cat} with the electronic substituent parameter (σ) at pH 7.5 and at 9.0 show a dominant contribution with positive ρ values (+1.2 – 1.3) and a pH-independent negative contribution from the steric term. Quantitative structure-activity relationship analysis of the binding affinities of the *para*-substituted benzylamine analogues to rat MAO A show an increased van der Waals volumes (V_w) increases the affinity of the deprotonated amine for the enzyme. These results demonstrate that rat MAO A exhibits similar but not identical functional properties with the human enzyme and provide additional support for C-H bond cleavage *via* a polar nucleophilic mechanism.

Monoamine oxidase A (MAO A) is an outer mitochondrial membrane bound flavoenzyme that regulates the concentrations of important neurotransmitters in cells, such as dopamine and serotonin.[1] Alteration in the physiological levels of MAO A are implicated in neuropsychiatric disorders such as aggressive trait disorder[2,3] and depression[4, 5] and may contribute to cardiomyopathies.[6] Therefore, MAO A is a clinically proven drug target for treatment of neurological disorders and may be a future drug target for cardioprotective drugs.[7]

Rat MAO A is found to be ~90% identical in sequence with the human enzyme. Structural data show that human MAO A crystallizes as a monomer[8] whereas rat MAO A is dimeric.[9] Recent pulsed EPR data show that both human and rat MAO A's exist as dimers in their membrane-bound forms and that either dimer partially dissociates into monomers (~50%) on

‡This work was supported by National Institute of Health Grant GM-29433 (DEE).

*To whom correspondence should be addressed. Dr. Dale E. Edmondson, Department of Biochemistry, Emory University School of Medicine, 1510 Clifton Rd. Atlanta, Georgia 30322. deedmon@emory.edu, Phone: 404-727-5972, Fax: 404-727-2738.

detergent-solubilization and purification.[10] The explanation for their crystallographic differences is that the monomeric form of human MAO A more readily crystallizes, whereas it is the dimeric form of rat MAO A that does so. The high sequence identities of human and rat MAO A serve as the premise for using the rat as an animal model for the human enzyme in drug development studies. Recent comparative studies of *para*-substituted amphetamine analogue binding to rat and human MAO A show differences in their structure-activity correlations.[11] Despite extensive studies on human and rat MAO As, a detailed functional comparison of these two enzymes has not been performed. Mechanistic studies[12] of human MAO A with a series of *para*-substituted benzylamine analogues show: 1. large ^{2}H kinetic isotope effects suggest that α-C-H bond cleavage is rate-limiting in catalytic turnover; 2. steady-state and stopped-flow kinetic studies show that the rate of reduction of the flavin increases with the increasing electron-withdrawing ability of the substituent, and this result has led to a mechanistic description of the reductive half reaction as following a polar nucleophilic mechanism; and 3. a linear correlation of increased binding affinity with the van der Waals volume (V_w) of the *para* substituent is comparable with the monopartite nature of MAO A's active site cavity. Recent pH-dependent studies of human MAO A catalysis show catalytically sensitive pKa values for both ES and E,[13] which may or may not be the same for the rat enzyme.

With the availability of purified recombinant rat MAO A,[14] it was of interest to determine whether the mechanistic properties insights determined for the human enzyme would also be true for the rat enzyme. As shown in this study, the functional properties of rat MAO A are similar to but are not identical with those of the human enzyme. Both enzyme sources exhibit pH-dependent behaviors consistent with the proposed polar nucleophilic mechanism. These comparative data should be important in future drug development studies using the rat as an animal model for the human.

EXPERIMENTAL PROCEDURES

Materials

Benzylamine, d-amphetamine, isatin, kynuramine, methylene blue, phenylethylamine, serotonin, glycerol, Bis-Tris propane and potassium phosphate were purchased from Sigma-Aldrich. β–Octylglucopyranoside was from Anatrace Inc., and reduced Triton X-100 was from Fluka. Harmane, pirlindole mesylate and tetrindole mesylate were purchased from TOCRIS Bioscience. All benzylamine analogs used in this study were either purchased from Sigma-Aldrich or synthesized in this laboratory as described previously.[15] Recombinant rat liver MAO A was expressed in and purified from cells of *Pichia pastoris* strain KM71 as described previously.[14] Purified rat MAO A was stored in 50 mM potassium phosphate containing 20% glycerol and 0.8% (w/v) β–octylglucopyranoside (pH 7.2). d-Amphetamine, a reversible MAO A inhibitor used for stabilizing the enzyme during purification, was removed prior to all kinetic measurements.

Determinations of k_{cat}, K_m and K_i Values

Steady-state kinetic measurements of rat MAO A were conducted at 25°C in 50 mM potassium phosphate buffer (pH 7.5) containing 0.5% (w/v) reduced Triton X-100 using kynuramine as a substrate. The rate of kynuramine oxidation by MAO A is followed spectrophotometrically by the formation of the product 4-hydroxyquinoline at 316 mm ($\Delta\varepsilon$ = 12,600 $M^{-1}cm^{-1}$). One unit of the enzyme activity is defined as the amount of enzyme required to oxidize 1 μmol of kynuramine to 4-hydroxyquinoline in 1 min. All kinetic measurements were performed at air-saturation (~240 μM O_2), which is assumed to be saturating conditions.

Values for k_{cat} and K_m were obtained by global fits of steady-state kinetic data to the Michaelis-Menten equation using GraphPad-Prism 5.0 software. Competitive K_i values were determined by measuring initial rates of substrate oxidation in the presence of varying concentrations of inhibitor and the data analyzed also using GraphPad Prism 5.0 software. The K_i value for methylene blue was obtained using the equation developed by Morrison[16] for tight-binding inhibitors.

pH-Dependent kinetic experiments were performed in 20 mM Bis-Tris propane buffer containing 0.5% (w/v) reduced Triton X-100, 50 mM NaCl and 20% glycerol. The pH of the buffer was adjusted in the range 6.5–9.5 by the addition of HCl or NaOH.

Steady-State Kinetic Measurements of para-Substituted Benzylamine Analogue Oxidation

All steady-state kinetic measurements of *para*-substituted benzylamine analog oxidation with rat MAO A were also performed either in 50 mM potassium phosphate buffer (pH 7.5) or in 20 mM Bis-Tris propane buffer containing 0.5% (w/v) reduced Triton X-100 at 25ºC. The steady-state rate of benzylamine analogue oxidation to the corresponding benzaldehyde was measured spectrophotometrically. Monitoring wavelength and molar absorption extinction coefficients for each aldehyde are given in Walker and Edmondson.[15] The low oxidation rates of *p*-F-BA, *p*-Me-BA, *p*-MeO-BA as well as α,α-[^{2}H]benzylamine analogues precluded accurate determinations of their rates of product formation by following aldehyde accumulation. Therefore, the Amplex Red-peroxidase coupled assay ($\Delta\varepsilon_{560}$ = 54,000 $M^{-1}cm^{-1}$) was used because it has a 5-fold higher level of sensitivity.

Data Analysis

Values of the substituent parameters σ, π, and E_s were obtained from Hansch et al.[17] and V_w values from Bondi.[18] K_d values for the benzylamine analogues were determined from steady-state ^{2}H kinetic isotope effect data as described by Klinman and Matthews.[19] Multivariate linear regression analysis of rate and binding data as a function of substituent parameters was performed using the StatView software package (Abacus Concepts). Fits of pH-dependent kinetic data were analyzed as described by Dunn et al.[13] by fitting the data to the appropriate equations using GraphPad Prism 5.0.

RESULTS

Competitive Inhibition by MAO A Reversible Inhibitors

The K_i values exhibited by rat MAO A for the reversible MAO A-specific inhibitors, harmane, pirlindole tetrindole, and methylene blue are essentially identical with those exhibited by human MAO A under identical conditions of pH, buffer, and temperature (Table 1). MAO B-specific reversible and irreversible inhibitors bind weakly (K_i values >10 μM) to rat MAO A as they do to the human enzyme with only minor differences in observed K_i values (data not shown). These data demonstrate that rat MAO A exhibits similar affinities as does the human enzyme for reversible MAO A specific inhibitors and the rat enzyme shows no unexpected affinities for any of the MAO B specific inhibitors

pH Effects on Steady-State Kinetic Parameters

Previous pH-dependent steady-state kinetic studies on human MAO A by Dunn et al.[13] demonstrated that a group in the ES complex with benzylamine is deprotonated for optimal activity and that two deprotonation steps (for either free enzyme or free substrate) influence catalytic turnover with both an enhancement and subsequently a decrease in the rate of enzyme in enzyme-catalyzed reaction on increasing the pH of the assay medium. To determine whether rat MAO A exhibits pH-dependent behavior and pKa values similar to that of the human enzyme, the effect of pH on steady-state kinetic parameters for the

oxidation of benzylamine and of *para*-CF_3-benzylamine (α,α-^{1}H- and α,α-^{2}H) were determined. The reason for comparison of these two benzylamine analogues is that they exhibit differing pKa values (pKa=0.6) for their unbound forms with benzylamine exhibiting a pKa of 9.3 and the *p*-CF_3 analogue a pKa of 8.8 which provides observable differences in the pH-activity profiles. Since the [O_2] is at saturating concentrations with MAO A at pH 7.5, it was assumed that saturating conditions are maintained at all pH values. As shown in Figure 1A-C (solid line), the k_{cat} values for benzylamine, α, α-[^{1}H]- and α, α-[^{2}H]-*p*-CF_3-benzylamines increase and exhibit sigmoidal dependences with increasing pH, indicating the presence of a single macroscopic ionization with respective pKa values of 8.2±0.1, 7.5±0.1 and 7.6±0.1. These values represent pKa values of their respective ES complexes. The difference in pKa values for the catalytic parameters on comparison of benzylamine with the *p*-CF_3 analogue (0.6) is within experimental uncertainty of the difference in pKa values for the free forms of these substrates. As shown with the human MAO A-catalyzed oxidation of benzylamine, the k_{cat}/K_m data for rat MAO A oxidation of proteo and deutero *p*-CF_3-benzylamines are best fit by a bell-shaped profiles, as described by Dunn et al.[13] (Figure 1B–C), with estimated pKa values of 7.8±0.1 and 9.3±0.1, 8.1±0.2 and 9.1±0.2, respectively (Table 2). A small alkaline shift of the pKa_1 value (0.3–0.4) is observed for ^{2}H-*p*-CF_3-benzylamine relative to the ^{1}H form. In contrast, with benzylamine, the k_{cat}/K_m vs pH profile exhibits a single ionization (8.3±0.1) (Figure 1A, dashed line) in the pH range covered rather than two pKa values observed with both the human enzyme and the *p*-CF_3-benzylamine analogue. Therefore, if a pH-dependent deprotonation occurs that is detrimental to catalysis with the rat enzyme, it must occur in a region more alkaline than pH 9.5. Considering the differences in pKa values found on comparison of other ionizations of benzylamine with the *p*-CF_3 analogue, this pKa is expected to occur at 10 or higher.

Steady-State Kinetic Parameters for Rat MAO A-Catalyzed Oxidation of para-Substituted Benzylamine Analogs and the ^{2}H-Kinetic Isotope Effects

The steady-state kinetic parameters for rat MAO A-catalyzed oxidation of seven *para*-substituted benzylamine analogues and their corresponding α, α-[^{2}H]analogues were determined at pH 7.5, and the respective values for k_{cat} and K_m are shown in Table 3. The pH 7.5 conditions were chosen to allow direct comparison with published values for human MAO A.[12] Turnover numbers (k_{cat}(H)) of rat MAO A determined for each substrate analogue show marked dependence on the nature of the substituent. The k_{cat} values range from 9.2 min^{-1} for *p*-MeO analogue to 333.5 min^{-1} for the *p*-CF_3 analogue. Catalytic rates determined for the α, α-[^{2}H]benzylamine analogues exhibit large isotope effects on both $^D k_{cat}$ and on $^D(k_{cat}/K_m)$ (Table 3). Human MAO A also exhibits large isotope effects on k_{cat} in the range of 6.7–14.3, demonstrating that α-C-H bond cleavage (the reductive half-reaction) is rate limiting in catalytic turnover.[12] The large kinetic isotope effects observed for *para*-substituted benzylamine oxidation by rat MAO A shows this conclusion is also valid for rat MAO A.

The pH-dependence of k_{cat} and k_{cat}/K_m show maximal values at pH 9.0 (Figure 1) and therefore, the substituent effect for benzylamine oxidation was also performed at this pH value. Previous mechanistic conclusions from substituent effect data have been criticized[20] by suggesting that the positive ρ value observed with the human enzyme at pH 7.5 originates from electronic effects on amine pKa values in the ES complex. Performing these measurements at pH 9.0 would minimize this contribution since the ES complex is in its deprotonated form. Values for the kinetic parameters at pH 9.0 for both human and rat MAO A are shown in Table 4. In agreement with data collected at pH 7.5, the k_{cat} values are higher for those analogues with electron-withdrawing substituents. Correlations of k_{cat} values for both the rat and human enzymes at pH 7.5 and 9.0 with electronic, hydrophobic, and steric parameters are presented and discussed below.

Substituent Effects on the Rates of Catalytic Turnover

Previous data on human MAO A[12] show that k_{cat} values and the rates of flavin reduction are mainly dependent on the electronic parameter (σ) of the *para* substituent at pH 7.5. The steady-state kinetic isotope values observed demonstrate that k_{cat} values are good approximations of the intrinsic rate of the C-H bond cleavage step. These data constitute the most powerful argument that supports the C-H bond cleavage step in MAO A catalysis as occurring via a H^+ abstraction that is part of a polar nucleophilic mechanism.[12] Correlation analysis using k_{cat} values to assess the influence of substituent parameters on the rates of benzylamine analogue oxidation was performed using the data in Table 4 and are presented in Table 5. The statistical F values for these correlations allow for identification of the most appropriate substituent parameters (electronic, hydrophobicity, or steric). Statistical analysis of the rat and human MAO A k_{cat} data at pH 7.5 and at 9.0 (Table 5) shows that the best single parameter correlation is found with the electronic parameter σ, although the rat MAO A data at pH 9.0 also shows a good correlation with the Taft steric parameter (E_s). Two parameter correlations of log k_{cat} values of human and rat MAO A show marked improvements in the statistical F values for a combination of electronic and steric parameters (Table 5).

Analysis of k_{cat} values with the various substituent parameters for data taken at pH 9.0, which provides conditions for maximal catalytic rate, are also presented in Table 5. The best single parameter correlation for rat MAO A is with the electronic substituent parameter with a ρ value of + 1.8±0.3 and is also true for with human MAO A where a ρ value of + 1.6±0.4 is determined. Substantial increases in F values are observed when 2-parameter correlations are performed with the inclusion of steric terms (either E_s or V_w). The following two parameter equations are presented to describe rat and human MAO A k_{cat} values at pH 9.0:

$$\text{Rat:}\quad \log k_{cat} = +1.2(\pm 0.1)\sigma - 0.3(\pm 0.1)\,E_s + 1.55(\pm 0.03) \qquad F_{2,5}=335,\ P=<0.0001$$

$$\text{Human:}\quad \log k_{cat} = +0.8(\pm 0.1)\sigma - 0.4(\pm 0.1)\,E_s + 1.01(\pm 0.03) \qquad F_{2,5}=337,\ P=<0.0001$$

Both enzyme systems show a correlation with positive ρ values and a negative steric effect for the oxidation of the deprotonated substrates. There is a small decrease in ρ value observed for the human enzyme on increasing the pH but no change is observed for the rat enzyme. In both cases, the coefficient for the steric term remains constant at both pH values. Plots of log k_{cat} corrected for steric contributions versus σ are shown in Figure 2. Because the reaction is facilitated by the *para* electron withdrawing groups, these data provide further support for the polar nucleophilic mechanism[12] in which the pro-R benzyl proton is abstracted as a H^+. If C-H bond cleavage were to occur by a Hydride transfer mechanism, one would expect to observe a negative ρ value as shown for the oxidation of benzylamine analogues (ρ = −1.72 to −2.03)[21,22] in model system studies using hexamethylenetetramine-bromine or cetylmethylammonium permanganate as hydride acceptors. Recent ^{15}N-kinetic isotope effect measurements of human MAO B oxidation of benzylamine show that C-H bond cleavage and N-rehybridization are not concerted in the reaction and which also argues against a Hydride mechanism for MAO B.[23]

Quantitative Structure-activity Relationships the Binding of para-Substituted Benzylamine Analogues to Rat MAO A

The K_d values for rat and human MAO A binding to the deprotonated forms of the *para*-substituted benzylamine analogues were compared. The kinetic isotope effects observed for these benzylamine analogues (Tables 3) permit the calculation of substrate dissociation

constants (which are a function of all pre-isotopically sensitive steps) and have been shown to correlate well with Ks values obtained from reductive half reaction stopped flow data.[12,15] Dissociation constants for all of the benzylamine analogue were calculated from the data in Table 6, as described by Klinman and Matthews:[19]

$$\frac{{}^{D}k_{cat} - 1}{{}^{D}(k_{cat}/K_m) - 1} = K_m/K_d \qquad \text{Equation 1}$$

where K_d is the amine substrate dissociation constant from all complexes occurring prior to the isotope-sensitive C-H bond cleavage step.

Early studies suggested the deprotonated form of the amine[24, 25] binds to the active site of MAO A, and the pH of enzyme assay buffer used in this study is 7.5, we found it necessary to correct the calculated K_d values to reflect the concentration of the deprotonated amine in order to observe reasonable correlations. Equation 2 is used to correct dissociation constants of each benzylamine analogue with MAO As:

$$K_{d(corrected)} = \frac{K_{d(calculated)}}{1 + antilog(pKa - pH)} \qquad \text{Equation 2}$$

where pH is that of the assay buffer for each analogue and the corrected K_d value for each analogue is listed in Table 6.

Linear regression analysis of these K_d values with substituent parameters show the best correlation to involve the steric parameter (V_w) (Table 7). No additional improvement is observed with two-parameter analysis. Essentially identical correlations of increased binding affinity with increased size of the *para* substituent are observed with the log K_d versus V_w plots previously observed in human MAO A[12] and shown with rat MAO A (Figure 3). The following equations do show that the rat enzyme binds these benzylamine analogues with a ~10-fold higher affinity than does the human enzyme but that both are largely dependent on the steric parameter, V_w:

$$\text{Rat:} \quad \log K_d = -0.8(\pm 0.2)(0.1V_w) - 5.2\,(\pm 0.2) \quad F_{1,6}=25, P=0.007$$

$$\text{Human:} \quad \log K_d = -0.9(\pm 0.2)(0.1V_w) - 4.4(\pm 0.3) \quad F_{1,6}=16, P=0.016$$

DISCUSSION

Although rat MAO A is ~90% identical in sequence with the human enzyme, a number of differences in properties warranted this comparative functional study. The rat enzyme crystallizes as a dimer[26] while the human enzyme crystallizes as a monomer,[8,27] even though both enzymes are found to be dimeric in their membrane bound forms.[10] Other differences include a more solvent accessible active site for the human enzyme[28] and different dependencies of *para* substituent size in the binding of *para*-thioalkyl amphetamines.[11] Finally, the altered surface charge of the rat enzyme relative to the human enzyme is evident by its weaker binding to anion exchange columns on purification[14] than observed for the human enzyme.[29] In spite of these observed differences in properties, the results in Table 1 show that the rat MAO A specificities and affinities for the “MAO A”

selective reversible inhibitors are similar to those of human MAO A. This behavior is consistent with available structural data showing the monopartite active site cavities of the two enzymes being quite similar.[8,26]

pH –Dependent Activity Profiles

Dunn et. al.[13] have deduced from the pH- k_{cat}/K_m profiles for human MAO A that there are two distinct ionizations influencing catalytic activity with benzylamine analogues: an enhancing deprotonation occurring at a pKa of 8.5±0.1 and an inhibitory ionization with a pKa of 9.2±0.1. A similar pH-dependent k_{cat}/K_m profile with a pKa of 7.5±0.1 has been reported by Jones et al.[30] for the oxidation of kynuramine by human MAO A which is somewhat lower (but probably within the error involved in fitting the data) than the value of 8.0±0.2 reported by Dunn et al.[13]. The conclusions by Jones et al. [30] that the protonated form of the substrate binds to the enzyme and undergoes a deprotonation before reaching the active site for oxidation is an interesting suggestion and requires additional investigation for verification. The hydrophobicities of the active sites of rat and human MAO A would favor entrance of a neutral form of the substrate rather than its protonated form. As shown in this study, substituent effects on the pKa of the ES complex and the perturbation of the macroscopic pKa (by ~2 pK units) provides evidence for a deprotonation step of the ES complex which must involve an initial binding of the protonated amine substrate prior to the chemical steps in catalysis. As stated above, the detailed molecular basis for this deprotonation step requires further investigation.

Rat MAO A exhibits a single activity-enhancing pKa of 8.3±0.1 with benzylamine while the second (unobserved) inhibitory ionization presumably occurs with a pKa>10. Thus, on the free enzyme there are small differences in macroscopic pKa values for groups that influence catalytic activity. Similar differences are observed with the single ionizations (due to the ES complexes with benzylamine) observed in k_{cat} vs pH profiles, which show a pKa of 7.9±0.1 for the human enzyme and 8.2±0.1 for the rat enzyme. These observations are consistent with the conclusion by Dunn et al.[13] that binding of the amine substrate to the active site of either MAO A perturbs the pKa of the amino group by 1–2 pKa units. Rat MAO A oxidation of *para*-CF_3-benzylamine exhibits a pKa for the ES complex (k_{cat}) that is ~ 0.6 units lower than that with benzylamine. This result is consistent with the influence of the substituent on the pKa of the free amine. This behavior shows the ability of electronic substituent effects to be transmitted through the benzyl ring and to influence the reactivity of the benzyl protons as well as the amine N in the bound (ES) form of the substrate.

Structure-activity correlations of the rates of benzylamine analogue oxidation by rat MAO A show that the electronic parameter of the *para* substituent is a major determinant of alterations in reactivity with an observed ρ value of ~ +1.0, which is consistent with C-H bond cleavage rate being limited by a H^+ abstraction. If the cleavage mode were to follow a H^- transfer mechanism as suggested by a number of papers,[20,30] then a negative ρ value would be observed as has been observed in model reactions of benzylamine analogue oxidation by hydride ion acceptors.[21,22] In a recent review[30] and in a previous paper on mouse polyamine oxidase,[20] Fitzpatrick and co-authors have argued that the large positive ρ values observed with human MAO A can be attributed to electronic effects of the *para* substituents on the pKa values of the amine group, since the structure- activity data were determined at pH 7.5 rather than at higher pH values where deprotonated amine levels would be independent of the nature of the *para* substituent. The basis for this criticism is that they observe a 0.5 decrease in ρ value (from −0.09 to −0.59) for Polyamine Oxidase on increasing the pH from 6.6 to 8.6.[31] In response to this criticism, our data with rat and human MAO A show the differences in ρ values to be somewhat less, due to increased contributions from steric parameters. However, they exhibit positive values (~+1 or greater) as would be predicted for a mechanism involving a H^+ abstraction. The magnitudes of

measured $^{D}(k_{cat})$ and of $^{D}(k_{cat}/K_{m})$ values are found to be independent of pH and of the nature of the *para* substituent, suggesting no observable changes in mechanism over the pH range used and that the observed kinetic isotope effects are close to being intrinsic.

In conclusion, the results of this study further support the view that the functional properties of rat MAO A are similar to but not identical to those of the human enzyme. These differences include a 10-fold increased substrate binding affinity, 2–3fold higher k_{cat} values for most substrates, and small differences in pKa values for E and ES. QSAR behavior with para-substituted benzylamine analogues are nearly identical for the rat and human enzymes. In addition, this study provides additional support for the view that the reductive half reaction in MAO A catalysis occurs with C-H bond cleavage involving a H^+ abstraction via a polar nucleophilic mechanism as suggested from the work on the human enzyme[12] and by arguments presented in recent reviews.[32, 33]

Abbreviations

MAO A	Monoamine Oxidase A
OMM	outer mitochondrial membrane
QSAR	Quantitative Structure-Activity Relationships
σ	substituent Hammett electronic constant
V_w	substituent van der Waals volume
π	substituent hydrophobicity constant
E_s	substituent Taft steric constant

References

1. Weyler W, Hsu YP, Breakefield XO. Biochemistry and genetics of monoamine oxidase. Pharmacol Ther. 1990; 47:391–417. [PubMed: 2290855]
2. Cases O, Seif I, Grimsby J, Gaspar P, Chen K, Pournin S, Muller U, Aguet M, Babinet C, Shih JC, Demaeyer E. Aggressive behavior and altered amounts of brain serotonin and norepinephrine in mice lacking MAOA. Science. 1995; 268:1763–1766. [PubMed: 7792602]
3. McDermott R, Tingley D, Cowden J, Frazzetto G, Johnson DDP. Monoamine oxidase A gene (MAOA) predicts behavioral aggression following provocation. Proc Natl Acad Sci USA. 2009; 106:2118–2123. [PubMed: 19168625]
4. Shih JC, Chen K, Ridd MJ. Monoamine oxidase: from genes to behavior. Annu Rev Neurosci. 1999; 22:197–217. [PubMed: 10202537]
5. Bortolato M, Chen K, Shih JC. Monoamine oxidase inactivation: From pathophysiology to therapeutics. Adv Drug Deliver Rev. 2008; 60:1527–1533.
6. Bianchi P, Kunduzova O, Masini E, Cambon C, Bani D, Raimondi L, Seguelas MH, Nistri S, Colucci W, Leducq N, Parini A. Oxidative stress by monoamine oxidase mediates receptor-independent cardiomyocyte apoptosis by serotonin and postischemic myocardial injury. Circulation. 2005; 112:3297–3305. [PubMed: 16286591]
7. Gentili F, Pizzinat N, Ordener C, Marchal-Victorion S, Maurel A, Hofmann R, Renard P, Delagrange P, Pigini M, Parini A, Giannella M. 3-[5-(4,5-dihydro-1H-imidazol-2-yl)-furan-2-yl]phenylamine (amifuraline), a promising reversible and selective peripheral MAO-A Inhibitor. J Med Chem. 2006; 49:5578–5586. [PubMed: 16942031]
8. De Colibus L, Li M, Binda C, Lustig A, Edmondson DE, Mattevi A. Three-dimensional structure of human monoamine oxidase A (MAO A): relation to the structures of rat MAO A and human MAO B. Proc Natl Acad Sci USA. 2005; 102:12684–12689. [PubMed: 16129825]

9. Ma J, Yoshimura M, Yamashita E, Nakagawa A, Ito A, Tsukihara T. Structure of rat monoamine oxidase A and its specific recognitions for substrates and inhibitors. J Mol Biol. 2004; 338:103–114. [PubMed: 15050826]
10. Upadhyay AK, Borbat PP, Wang J, Freed JH, Edmondson DE. Determination of the oligomeric states of human and rat monoamine oxidases in the outer mitochondrial membrane and octyl beta-D-glucopyranoside micelles using pulsed dipolar electron spin resonance spectroscopy. Biochemistry. 2008; 47:1554–1566. [PubMed: 18198902]
11. Fierro A, Osorio-Olivares M, Cassels BK, Edmondson DE, Sepulveda-Boza S, Reyes-Parada M. Human and rat monoamine oxidase-A are differentially inhibited by (S)-4-alkylthioamphetamine derivatives: Insights from molecular modeling studies. Bioorgan Med Chem. 2007; 15:5198–5206.
12. Miller JR, Edmondson DE. Structure-activity relationships in the oxidation of para-substituted benzylamine analogues by recombinant human liver monoamine oxidase A. Biochemistry. 1999; 38:13670–13683. [PubMed: 10521274]
13. Dunn RV, Marshall KR, Munro AW, Scrutton NS. The pH dependence of kinetic isotope effects in monoamine oxidase A indicates stabilization of the neutral amine in the enzyme-substrate complex. FEBS J. 2008; 275:3850–3858. [PubMed: 18573102]
14. Wang J, Edmondson DE. High-level expression and purification of rat monoamine oxidase A (MAO A) in Pichia pastoris: Comparison with human MAO A. Protein Expr Purif. 2010; 70:211–217. [PubMed: 19883764]
15. Walker MC, Edmondson DE. Structure-activity relationships in the oxidation of benzylamine analogues by bovine liver mitochondrial monoamine oxidase B. Biochemistry. 1994; 33:7088–7098. [PubMed: 8003474]
16. Morrison JF. Kinetics of reversible inhibition of enzyme-catalysed reactions by tight-binding inhibitors. Biochim Biophys Acta. 1969; 185:269–286. [PubMed: 4980133]
17. Hansch, C.; Leo, A. Exploring QSAR-fundamentals and applications in chemistry and biology. ACS; Washington DC: 1995.
18. Bondi A. Van der waals volumes and radii. J Phys Chem. 1964; 68:441–451.
19. Klinman JP, Matthews RG. Calculation of substrate dissociation-constants from steady-state isotope effects in enzyme-catalyzed reactions. J Am Chem Soc. 1985; 107:1058–1060.
20. Pozzi MH, Gawandi V, Fitzpatrick PF. Mechanistic studies of para-substituted N,N ‘-dibenzyl-1,4-diaminobutanes as substrates for a mammalian polyamine oxidase. Biochemistry. 2009; 48:12305–12313. [PubMed: 19911805]
21. Dubey R, Kothari S, Banerji KK. Kinetics and mechanism of the oxidation of substituted benzylamines by hexamethylenetetramine-bromine. J Phys Org Chem. 2002; 15:103–107.
22. Shukla R, Sharma PK, Kotai L, Banerji K. Kinetics and mechanism of the oxidation of substituted benzylamines by cetyltrimethylammonium permanganate. Proc Indian Acad Sci (Chem Sci). 2003; 115:129–134.
23. MacMillar, S.; Edmondson, DE.; Matsson, O. Nitrogen Kinetic Isotope Effects for the Monoamine Oxidase B Catalyzed Oxidation of Benzylamine and (1,1-$^{2}H_{2}$)Benzylamine. Nitrogen Rehybridization and CH Bond Cleavage are Not Concerted. J Am Chem Soc. 2011. http://dx.doi.org/10.1021/ja205629b
24. McEwen CM, Sasaki G, Jones DC. Human liver mitochondrial monoamine oxidase. II. Determinants of substrate and inhibitor specificities. Biochemistry. 1969; 8:3952–3962. [PubMed: 4899582]
25. McEwen CM, Sasaki G, Lenz WR. Human liver mitochondrial monoamine oxidase. I. Kinetic studies of model interactions. J Biol Chem. 1968; 243:5217–5225. [PubMed: 5702044]
26. Ma J, Kubota F, Yoshimura M, Yamashita E, Nakagawa A, Ito A, Tsukihara T. Crystallization and preliminary crystallographic analysis of rat monoamine oxidase A complexed with clorgyline. Acta Crystallogr D Biol Crystallogr. 2004; 60:317–319. [PubMed: 14747710]
27. Son SY, Ma J, Kondou Y, Yoshimura M, Yamashita E, Tsukihara T. Structure of human monoamine oxidase A at 2.2-angstrom resolution: the control of opening the entry for substrates/inhibitors. Proc Natl Acad Sci USA. 2008; 105:5739–5744. [PubMed: 18391214]

28. Upadhyay AK, Wang J, Edmondson DE. Comparison of the structural properties of the active site cavities of human and rat monoamine oxidase a and B in their soluble and membrane-bound forms. Biochemistry. 2008; 47:526–536. [PubMed: 18092818]

29. Li M, Hubalek F, Newton-Vinson P, Edmondson DE. High-level expression of human liver monoamine oxidase A in Pichia pastoris: comparison with the enzyme expressed in Saccharomyces cerevisiae. Protein Expr Purif. 2002; 24:152–162. [PubMed: 11812236]

30. Jones TZE, Balsa D, Unzeta M, Ramsay RR. Variations in activity and inhibition with pH: the protonated amine is the substrate for monoamine oxidase, but uncharged inhibitors bind better. J Neur Transm. 2007; 114:707–712.

31. Fitzpatrick PF. Oxidation of amines by flavoproteins. Arch Biochem Biophys. 2010; 493:13–25. [PubMed: 19651103]

32. Edmondson DE, Binda C, Wang J, Upadhyay AK, Mattevi A. Molecular and mechanistic properties of the membrane-bound mitochondrial monoamine oxidases. Biochemistry. 2009; 48:4220–4230. [PubMed: 19371079]

33. Edmondson DE, Binda C, Mattevi A. Structural insights into the mechanism of amine oxidation by monoamine oxidases A and B. Arch Biochem Biophys. 2007; 464:269–276. [PubMed: 17573034]

34. Ramsay RR, Dunford C, Gillman PK. Methylene blue and serotonin toxicity: inhibition of monoamine oxidase A (MAO A) confirms a theoretical prediction. Brit J Pharmacol. 2007; 152:946–951. [PubMed: 17721552]

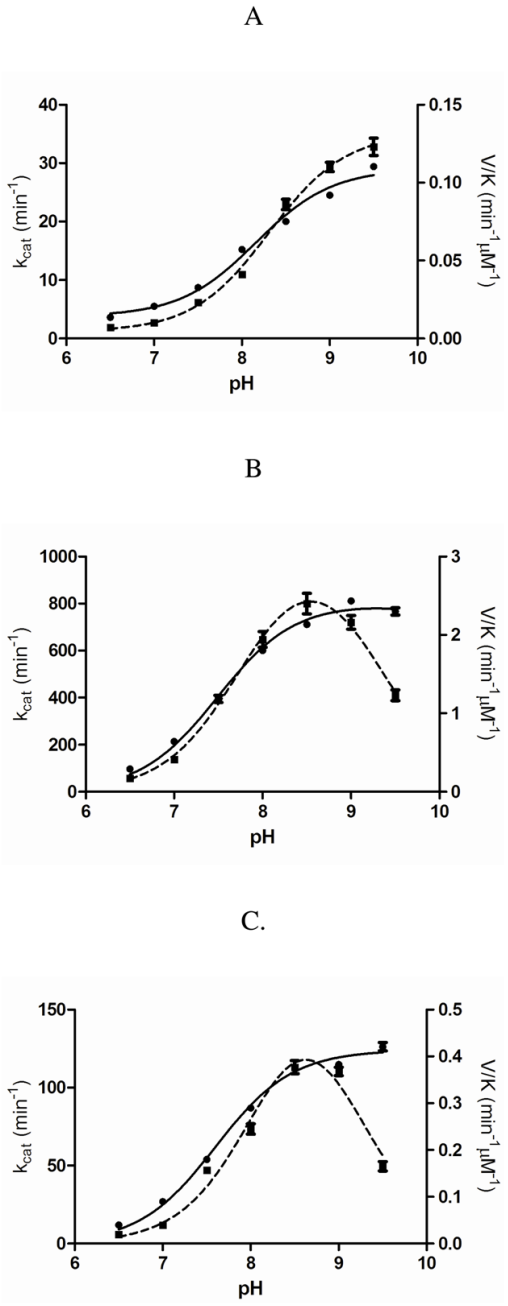

Figure 1.

pH dependence of the steady-state kinetic parameters of rat MAO A-catalyzed oxidation of benzylamine (A), α, α-[^{1}H]-*p*-CF_3-benzylamine (B) and α, α-[^{2}H]-*p*-CF_3-benzylamine (C). Values of k_{cat} and V/K are shown as (—●) and as (---■), respectively.

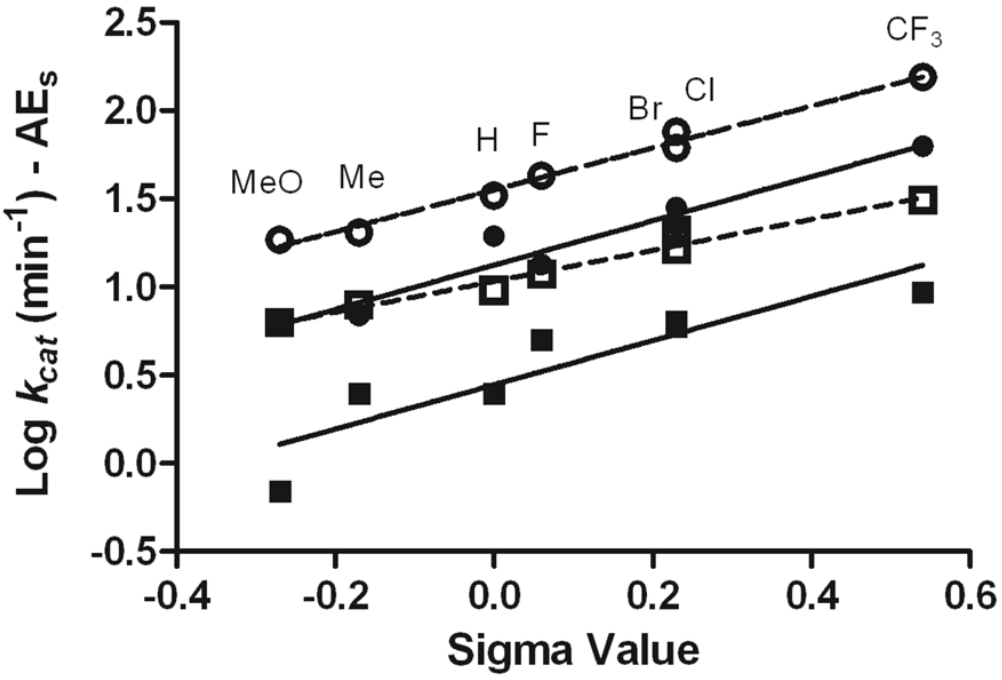

Figure 2.

Correlation of log k_{cat} – AE$_s$ of MAO A turnover of α, α-[^{1}H]-*p*-substituted benzylamine analogues with the substituent electronic parameter (s): rat MAO A at pH 7.5, —●; rat MAO A at pH 9.0, ---○; human MAO A at pH 7.5, —■; human MAO A at pH 9.0, ---□. A is the coefficient determined for the Es contribution and is −0.3±0.1 for the rat enzyme and −0.4±0.1 for the human enzyme.

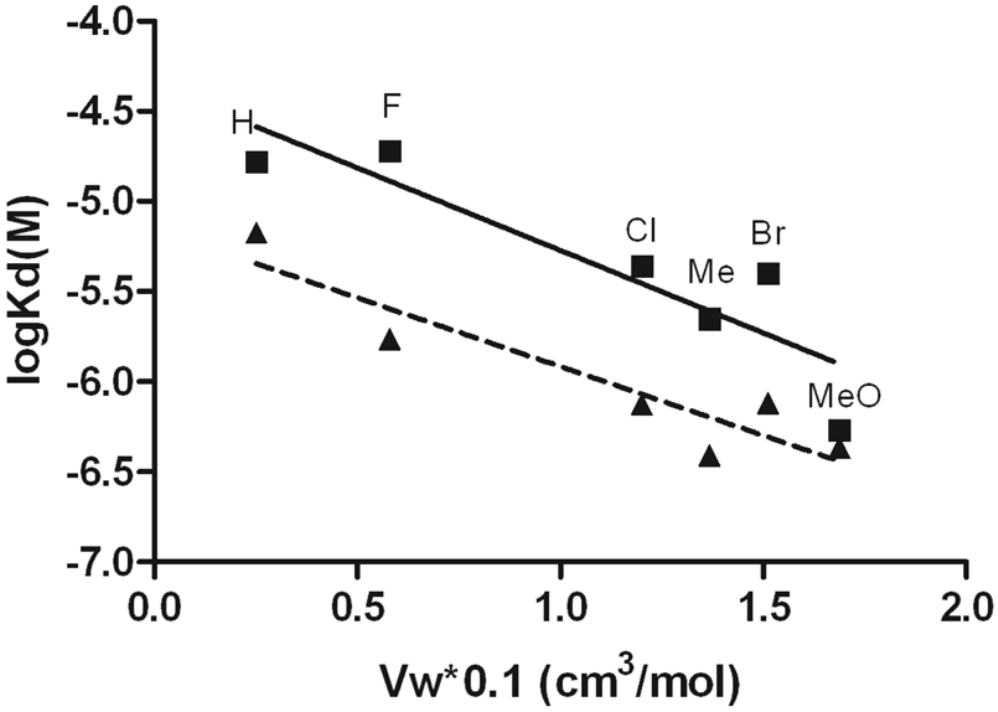

Figure 3.

Comparison of correlations of the binding of *para*-substituted benzylamine analogues to human MAO A (—■) (*12*) and rat MAO A (------▲) with the van der Waals volume (V_w) of the para substituent. All binding constants are corrected for selective binding of the deprotonated amine.

Table 1

Comparison of competitive inhibition constants for human and rat MAO A for a series of reversible MAO A inhibitors.

Inhibitor	Human MAO A	Rat MAO A
	K_i (μM)	
Harmane	0.58±0.02	0.75±0.04
Pirlindole Mesylate	0.92±0.04	0.77±0.02
Tetrindole Mesylate	5.3±0.24	3.6±0.34
Methylene Blue	0.027±0.0029[a]	0.026±0.0015

[a] Data taken from Ramsay et al.[34]

Table 2

pKa values estimated from fits of pH dependence of kinetic parameters of rat MAO A.

Substrate	ES complex	Free E or S	
	pKa	pKa_1	pKa_2
Benzylamine	8.2±0.1	8.3±0.1	-
α, α-[^{1}H]-*p*-CF_3-benzylamine	7.5±0.1	7.8±0.1	9.3±0.1
α, α-[^{2}H]-*p*-CF_3-benzylamine	7.6±0.1	8.1±0.2	9.1±0.2

Table 3

Steady-state kinetic constants for rat MAO A catalyzed oxidation of *para*-substituted benzylamine analogues at pH 7.5.

***para* substituent**	**k_{cat}(H) (min^{-1})**	**K_m(H) (μM)**	**$^{D}k_{cat}$**	**k_{cat}(D) (min^{-1})**	**K_m(D) (μM)**	**^{D}(V/K)**
p-H	20±0.2	211.1±18.7	6.7±0.2	2.9±0.1	423±51.9	14±2.1
p-CF_3	334±5.4	194±9.9	8.4±0.2	40±0.7	273±13.7	12±0.9
p-Br	62±1.0	34±3.2	13±0.2	4.7±0.1	30±1.6	12±1.3
p-Cl	44±1.0	54±6.2	14±0.4	3.1±0.1	32±3.2	8.2±1.3
p-F	14±0.4	174±13.6	11±0.4	1.2±0.1	109±8.7	7.1±0.8
p-Me	16±0.1	55±2.2	11±0.2	1.5±0.1	42±3.9	8.1±0.8
p-MeO	9.2±0.2	80±9.5	11±0.5	0.8±0.1	60±10.2	8.4±1.8

Table 4

Steady-state kinetic constants for rat and human MAO A catalyzed oxidation of α, α-[^{1}H]-*para*-substituted benzylamine analogues at pH 9.0.

	Rat MAO A		Human MAO A	
***para* substituent**	**k_{cat} (min^{-1})**	**k_{cat}/K_m ($min^{-1}mM^{-1}$)**	**k_{cat} (min^{-1})**	**k_{cat}/K_m ($min^{-1}mM^{-1}$)**
p-H	33±0.16	137±2.5	9.6±0.091	13±0.29
p-CF_3	811±11	2161±87	283±5.7	381±18
p-Br	171±1.4	3527±142	61±0.79	326±17
p-Cl	120±1.5	2089±123	39±0.49	223±11
p-F	59±0.84	386±23	18±0.33	38±2.2
p-Me	48±0.30	1074±39	25±0.24	347±15
p-MeO	28±0.22	296±12	11±0.081	100±3.2

Table 5

Comparative Correlations of k_{cat} values for the rat and human MAO A with *para*-substituted benzylamine analogues at pH 9.0 and at 7.5 with steric, electronic and hydrophobic substituent parameters.

Parameter	Rat MAO A log k_{cat}, pH 9.0				Human MAO A log k_{cat}, pH 9.0				Rat MAO A log k_{cat}, pH 7.5				Human MAO A log k_{cat}, pH 7.5[a]			
	Slope	R^2	F[b]	Significance	Slope	R^2	F	Significance	Slope	R^2	F	Significance	Slope	R^2	F	Significance
σ	1.8±0.3	0.94	**38**	0.002	1.6±0.4	0.88	17	0.0096	1.8±0.3	0.94	**38**	0.002	1.9±0.4	0.90	**23**	0.005
E_s	−0.6±0.1	0.88	18	0.008	−0.6±0.1	0.94	**41**	0.0014	−0.6±0.2	0.84	12	0.019	−0.7±0.2	0.88	18	0.009
V_w	0.5±0.3	0.61	3	0.145	0.6±0.3	0.69	4.6	0.084	0.5±0.3	0.56	2.3	0.192	0.5±0.3	0.53	2	0.218
π	1.1±0.3	0.84	12	0.019	1.2±0.3	0.88	18	0.0085	1.1±0.4	0.80	9.2	0.029	1.3 ±0.3	0.88	17	0.009
σ + E_s	1.2±0.1, −0.3±0.1	0.99	**335**	<0.0001	0.8±0.1, −0.4± 0.1	0.99	**336**	<0.0001	1.3±0.3, −0.3±0.1	0.98	**45**	0.002	1.2±0.3, −0.4±0.1	0.98	**38**	0.002
σ + π	1.3±0.4, 0.4±0.2	0.97	28	0.004	0.9±0.4,0.7±0.3	0.95	18	0.01	1.5±0.4, 0.4±0.3	0.96	22	0.007	1.2±0.4, 0.7±0.3	0.96	**25**	0.006
σ + V_w	1.6±0.1, 0.3±0.1	0.99	**129**	0.0002	1.4±0.2, 0.4±0.1	0.98	**40**	0.0023	1.7±0.2,0.2±0.1	0.98	**41**	0.002	1.7±0.4, 0.2±0.2	0.94	14	0.015

[a] Taken from data in Miller & Edmondson.[12]

[b] The F value is a statistical term relating the residuals of each point to the fitted line to the residuals of each point to the mean value. F is weighted for the number of variables in the correlation and the number of data points. A higher value of F indicates a better fit. The significance is calculated from the F value and represents the fractional chance that the correlation is meaningless.

Table 6

Correction of *para*-substituted benzylamine rat MAO A binding constants for selective binding of the deprotonated forms.

para substituent	Amine pKa [a]	Calculated K_d (μM)	pKa corrected K_d (μM)[b]	logK_d (M)
p-H	9.3	460	6.7	−5.2
p-CF_3	8.8	284	15	−4.8
p-Br	9.1	30	0.76	−6.1
p-Cl	9.1	29	0.75	−6.1
p-F	9.3	103	1.7	−5.8
p-Me	9.5	41	0.39	−6.4
p-MeO	9.6	58	0.43	−6.4

[a] Benzylamine analogue pKa values were taken from Miller and Edmondson.[12]

[b] pKa corrected binding constants were calculated using Equation 2.

Table 7

Correlations of binding affinities (K_d) of deprotonated para-substituted benzylamine analogues to rat MAO A with steric, electronic and hydrophobic substituent parameters.

parameter	correlation (slope)	*y*-intercept	correlation coefficient	F value	significance
σ	0.60±1.10	−6.00±0.20	0.26	0.3	0.613
E_s	0.78±0.29	−5.42±0.24	0.81	7.4	0.053
V_w	−0.77±0.15	−5.15±0.18	0.93	25	0.007
π	−0.54±0.54	−5.79±0.28	0.45	1.0	0.373
π + V_w	−0.04±0.29 −0.76±0.20	−5.15±0.21	0.93	9.6	0.050
π + E_s	1.21±0.44 1.62±0.36	−5.26±0.18	0.95	13	0.032
σ + V_w	0.18±0.48 −0.76±0.18	−5.16±0.21	0.93	10	0.047
σ + π	2.06±0.86 −1.29±0.47	−5.53±0.22	0.86	4.1	0.139