Probing the “Active Site” of Diamine Oxidase: Structure-Activity Relations for Histamine Potentiation by O-Alkylhydroxylamines on Colonic Epithelium

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ABSTRACT

The responses of the canine colonic epithelium to histamine are potentiated by O-alkylhydroxylamines. A study of a series of such compounds suggested that active compounds had the structure R-O-NH2, substitution of a nitrogen led to total loss of activity. The locus of the potentiation effect was traced to the inhibition of diamine oxidase. A new series of aliphatic and aromatic O-alkylhydroxylamines were synthesized to explore further the structure-activity relations of this effect. The potentiating effects of these compounds were determined by examining the changes in short circuit current (Isc) produced by histamine and from the activity of a soluble preparation of diamine oxidase. We found that 1) branched compounds are less active than their straight chain counterparts, 2) greater steric bulk of the aliphatic substituent decreased activity, 3) the presence of a double bond had no significant effect though a triple bond reduced activity, 4) longer straight chain compounds were less active than the shorter chain derivatives and 5) all benzylc compounds were less active than the straight chain aliphetics. O-1-benzyl was inactive however the meta or para oxygen substituted compounds as well as the O-(1-E-Cinnamyl) derivative were active. A current model for the action of diamine oxidase proposes a crucial role for a trihydroxyphenylalanine quinone cofactor as part of the active site together with a copper atom. Using molecular modeling based on our inhibition data we are able to define the region of space that is just beyond the reactive carbonyl of the trihydroxyphenylalanine residue at the active site of diamine oxidase. We suggest that a negatively charged species, such as an aspartate or a glutamate, resides in a trough about 7 to 8 Å from the trihydroxyphenylalanine carbonyl carbon and this species aids in the strong selective binding of substrates such as putrescine and histamine.

Histamine is an endogenous substance that has profound effects on a variety of cells and tissues (Falus, 1994; Hill, 1990). With specific reference to the intestinal lining, it has been shown to have marked stimulant effects in vitro that could result in diarrhea in vivo (Rangachari et al., 1992). This has been confirmed in cases of histamine poisoning where diarrhea is a prominent feature (Taylor, 1991). Under normal conditions, several mechanisms exist to rapidly inactivate the autacoid. Intereference with these inactivating mechanisms leads to potentiation of the effects of histamine. Among these, the rapid inactivation by the ubiquitous diamine oxidase is particularly significant (Kusche and Lorenz, 1983; Beaven, 1982; Jarisch and Wantke, 1996; Sessa and Perin, 1994). Inhibition of this enzyme as in instances of scombroid fish poisoning can have serious consequences (Taylor, 1991; Murray et al., 1982). Diamine oxidase can be inhibited by a variety of compounds including guanidine derivatives, aromatic diamidines, several antihistaminics, hydroxylamine and a variety of drugs used in clinical practice (Sattler et al., 1985; Beaven, 1982; Jarisch and Wantke, 1996).

Diamine oxidase, originally termed histaminase (Kusche and Lorenz, 1983), is a member of a family of amine oxidases (EC 1.4.4.6.) that are homodimers of 60 to 105 kDa subunits and contain tightly bound copper and a carbonyl cofactor (Janes et al., 1990). The enzyme, which is widely distributed, catalyses the oxidative deamination (RCH2NH2 + O2 + H2O \[\rightarrow\] RCHO + H2O2 + NH3) of diamines with three to six carbon atoms as well as histamine, which could be considered a cyclic diamine (Sessa and Perin, 1994). In a variety of mammals it is found in tissues such as the placenta, intestines, kidney and thymus. High activities of the enzyme are present in the mucosal layer of the intestine, its location suggesting a protective function, because endogenously released histamine can have profound pathological effects if degradation does not occur promptly (Beaven, 1982; Rangachari et al., 1992).
Earlier (Rangachari et al., 1992), we showed that a series of O-alkylhydroxylamines markedly potentiated the responses of the canine colonic epithelium to histamine. The underlying mechanism was the inhibition of diamine oxidase, because: 1) the potentiation was seen only with those agonists that possessed an imidazole nucleus (i.e., histamine, 2-methyl and 4-methylhistamine); 2) the compounds delayed the disappearance of histamine from the bathing solutions; 3) the compounds inhibited a preparation of colonic diamine oxidase; 4) selective inhibitors of diamine oxidase (aminoguanidine, semicarbazide) mimicked the effects; 5) putrescine, a substrate for the enzyme, also produced similar effects when added at a high concentration and 6) the effects were not seen with sodium nitroprusside, NaNO2 or derivatives of cGMP excluding NO or cGMP from a possible role.

Preliminary structure-activity relations suggested that whereas O-substituted aliphatic compounds were effective, N-substitution led to loss of activity. Thus O-methyl was fully active whereas N-methyl was totally inactive. These activities were defined using the responses of the intact tissue (Rangachari et al., 1992). However, it was possible that compounds labeled inactive may have been termed so merely because they were unable to penetrate the tissue to get access to the enzyme located in the epithelial cells.

In our study, we have conducted a more systematic study and explored these relations further. We compared and contrasted the effects of the synthesized compounds on the intact tissue as well as on an enzyme preparation from the same tissue. With the second generation of O-alkylhydroxylamines, we assessed the effects of increasing the length of the aliphatic chains, introducing double bonds and substituting bulky/charged aromatic groups.

The strategy we adopted was as follows: 1) all compounds were tested for their abilities to inhibit diamine oxidase activity in a preparation obtained from the canine colon. 2) The same compounds were screened rapidly using the "secondary rise" screening procedure we developed in the previous study (Rangachari et al., 1992). Serosal addition of histamine elicited a rapid increase in short-circuit current across the tissue. This response attained a peak and faded within a matter of minutes. The addition of an "active" hydroxylamine produced a sharp secondary increase in Isc. Compounds that produced this response also potentiated responses to histamine when added prior to the addition of the agonist. 3) Following this rapid screen, selected compounds were used to obtain a quantitative estimate of potentiation by estimating changes in pD2 to exogenous histamine.

Based on the data obtained, we attempted to develop a structure-activity relationship for the derivatives synthesized. Using this information we have also used molecular modeling techniques in an effort to define the nature of the active site of diamine oxidase.

Methods and Materials

Tissue Preparation

Many of the procedures used in our study were described in detail in our earlier publications (Rangachari et al., 1992), so only the salient points will be emphasized. Briefly, adult dogs of either sex were killed rapidly by i.v. pentobarbitone sodium (100 mg/kg) and the proximal colon quickly excised and immersed in warm, oxygenated modified Krebs solution. An initial dissection was carried out to remove the circular and longitudinal muscles, followed by a finer dissection to remove the muscularis mucosa and attendant submucosal plexuses as well. The resulting preparation was set up in conventional Ussing chambers for recording short-circuit currents. The tissues were bathed on both sides with warm, oxygenated modified Krebs’s solution having the following composition (in mM): 116 NaCl, 4.6 KCl, 1.2 MgCl2, 1.5 CaCl2, 22 NaHCO3, 1.2 NaH2PO4 and 10 glucose. Short-circuit currents (Isc) were measured in μA using a World Precision Instruments (Sarasota, FL) dual voltage clamp. The data were collected and analyzed using the Acknowledge MP100 data acquisition system (Biopac, Santa Barbara, CA).

Diamine Oxidase Preparation

Colonic tissues were collected as described above. To prepare the enzyme, all the muscle layers were removed to provide sheets of the epithelial tissue. The epithelial tissues were weighed, homogenized in 9 volumes of phosphate buffer (v:w:w) and centrifuged for 30 min (48,000 × g, 4°C). The supernatant was collected and used as the source of the enzyme. The supernatants from five dogs were pooled and frozen in small aliquots for use as the test solution in these experiments. The enzyme was found to be stable for at least 6 wk.

Ussing Chamber Experiments

Two sets of experiments were carried out in Ussing chambers. In both cases, changes in Isc were measured as indices of tissue responsiveness. All tissues were allowed to stabilize for 45 min to 1 h before experiments were begun.

“Secondary Rise” Protocol. The secondary-rise screening protocol was adopted from an earlier study (Rangachari et al., 1992) to identify active hydroxylamine compounds. Briefly, the tissues were exposed to a single concentration of histamine (10-4 M) and when the initial response to the agonist had begun to fade, the hydroxylamine compound was added at a concentration of 10-4 M. The presence or absence of a secondary increase in Isc in response to the addition of hydroxylamine was noted.

Concentration-Response Studies. Multiple pieces of tissue from the same dog were set up for each experiment. Cumulative concentration response curves were constructed using histamine. Control tissues were treated with histamine alone. Test tissues were pre-treated with selected hydroxylamines (at a fixed concentration of 10-4 M) for 15 min before addition of histamine. On each day all the five test compounds and the two reference compounds N-methyl and O-methyl) were studied.

Determination of Diamine Oxidase Activity

The procedure used was the method of Okuyama and Kobayashi (1961) as modified by Kusche et al. (1975). The assay mixture for the test consisted of 1) 0.6 ml of 0.1 M sodium phosphate buffer (pH 7.2), 2) 0.1 ml of test sample solution (diamine oxidase ± inhibitors), 3) 0.05 ml of substrate solution (containing 4.5 mM putrescine dihydrochloride and 1.0 μCi/ml of [1,4-14C]putrescine dihydrochloride). Based on our previous experience, we used an incubation period of 10 min at 37°C at which time the reaction was stopped by adding 1.0 ml of alkaline buffer (800 mM NaOH, 600 mM NaHCO3, pH 12.2). The labeled reaction product ([C14]1-pyrroline) was directly extracted into 6 ml of scintillation fluid (toluene containing 3.5 g/liter of 2,5-diphenyloxazole). The radioactivity present in 5 ml of the scintillation fluid was measured using a Beckman LS-5801 liquid scintillation counter. Three blanks were routinely used. These were 1) a sample blank—perchloric acid added before substrate solution (zero time incubation), 2) an enzyme blank—0.1 mM aminoguanidine added to inactivate the enzyme and a 3) reagent blank—where buffer was used instead of the enzyme. The data from these experiments were expressed as percentages of the control diamine oxidase activity (dpm of [14C]·min-1·g-1·protein-1) (see Rangachari et al., 1992).
General Experimental Methods for Preparation of O-Alkylhydroxylamines

Melting points were measured on a Gallenkamp capillary tube melting point apparatus and are uncorrected. Proton and $^1$C nuclear magnetic resonance spectra were recorded on a Bruker AC-200 (200 MHz) or Bruker AC-300 (300 MHz) spectrometer. Solvents used were chloroform-$d$, DMSO-$d_6$ or deuterium oxide with TMS as an internal standard. The abbreviations (s) = singlet, (d) = doublet, (t) = triplet, (q) = quartet, (p) = pentet, (s) = sextet, (s) = septet and (m) = multiplet are used to describe spin-spin coupling patterns. Proton spectra were collected in sixteen scans in 16K data points. The FID patterns were processed using exponential multiplication (line broadening = 0.3) and were zero-filled to 32K before Fourier transformation. The $^1$C spectra were collected in adequate scans to determine all carbon signals in 16K data points. The FID patterns were processed as with the proton spectra (line broadening = 3.0) and zero-filled to 32K before Fourier transformation.

DMF was dried by distilling a known volume of benzene to remove the water as an azeotrope. The DMF was then distilled under reduced pressure and stored over molecular sieves under a nitrogen atmosphere. ACN was distilled from CaH$_2$ under dry nitrogen immediately before use. All other solvents used were of reagent grade. NMR solvents (chloroform-$d$, DMSO-$d_6$) were stored over molecular sieves (4 Å) before use.

The general method used for synthesis of O-alkyl and O-benzylhydroxylamines (compounds 1–16, Fig. 1) was a modification of the Gabriel procedure for synthesis of primary amines (Gabriel, 1887) using N-hydroxyphthalimide followed by removal of the phthaloyl group by hydrazinolysis (Ing and Manske, 1926; Drain, 1965; Rangachari et al., 1992). The major changes in the present work were the use of dimethylformamide as solvent and sodium hydride to generate the N-hydroxyphthalimide anion. Product hydroxylamines were normally converted to their hydrochloride salts, lyophilized and stored as the dry solid before use. The dry hydrochlorides were found to be quite stable over long time periods.

The most difficult hydroxylamines to prepare were the O-p-hydroxybenzyls (compounds 11 and 12) because of the tendency for the intermediates, and the final product, to undergo elimination and form quinonemethides. The procedure of Drain et al. (1965) proved acceptable for these two compounds, although the yield of purified 11 from the hydrazinolysis and hydrolysis steps was only 15%.

The intermediate O-alkyl-N-hydroxyphthalimides were decomposed to give the free O-alkylhydroxylamines with hydrazine in slight excess (1.1–1.34 mole equivalents). Because hydrazine and substituted hydrazides (e.g., semicarbazide) are inhibitors of diamine oxidase it was important to remove as much hydrazine as possible from the prepared compounds. This was accomplished by taking advantage of the different pK$_a$s of hydrazine (8.23) and hydroxylamine (6.03) and extraction of a dichloromethane solution of the reaction products with equal volumes of a buffer of pH 7.4. Routinely two or three such extractions were carried out, depending on the excess of hydrazine used, leaving the residual hydrazine at a maximum in the parts per thousand range. For hydrazine at this level to have an effect on our experiments, the concentrations of O-alkylhydroxylamines would need to be in the 10$^{-3}$ M range, a

![Fig. 1. Shown are the structures and numbering system for all of the O-alkylhydroxylamines synthesized for this study. Details regarding the preparation and chemical properties for each of these compounds are given in the Appendix.](image-url)
concentration that is two orders of magnitude above the $10^{-5} \text{ M}$ cut-off that we accept for a compound to be active.

The synthesis of O-2-buty1 (3) is described in detail in the Appendix as a typical example. Spectroscopic data, as well as any variations in the general method, for the remaining compounds listed in figure 1 are also included in the Appendix.

**Data Analysis and Statistics**

All data obtained were analyzed using distribution-free methods (analysis of variance-Kruskal Wallis). Mean effective dose values (pD$_2$) were determined by non-linear regression analysis (Fig P, ver. 6.0; Biosoft, MA). A four-parameter logistic equation of the following form was used:

$$Y = [(A-D)/1 + (x/C)^{-B}] + D$$

$Y$ represents the magnitude of the response to the agonist at a particular concentration, $x$. A-D are computer-generated parameters reflecting maximal responses (A), slope factor (B), pD$_2$ (C) and minimal response (D), respectively. Based on this expression, pD$_2$ values were estimated from the values of $C$ obtained for each concentration-response curve. pD$_2$ values from treated tissues were compared to those from the control tissues to evaluate statistical significance. Molecular modeling was performed using PC-Model (Serena Software, Bloomington, IN).

**Drugs and Reagents**

All chemical reagents used for making physiological salt solutions were of analar grade and purchased from either Sigma Chemical Co. (St. Louis, MO) or Aldrich (Milwaukee, WI). Histamine dihydrochloride, aminoguanidine hemisulfate and semicarbazide dihydrochloride were obtained from Sigma. [14C]Putrescine dihydrochloride was obtained from New England Nuclear (Wilmington, DE). Commercially available hydroxylamine derivatives were bought from Sigma or Aldrich. All other hydroxylamines were synthesized as described above (see Appendix for details). All of the chemicals used to synthesize these compounds were also purchased from either Sigma or Aldrich.

**Results**

**Effect on Diamine Oxidase Activity.** All the compounds synthesized were tested for their abilities to inhibit diamine oxidase. We also tested a known inhibitor of diamine oxidase (aminoguanidine) as well as the reference compounds, O-methyl and N-methyl. The data for the aliphatic and the benzylic hydroxylamines are shown separately (Fig. 2, A and B). In figure 2A we have highlighted (in black) the inhibitions produced by all compounds at a concentration of $10^{-6} \text{ M}$. At this concentration, the standard inhibitor, aminoguanidine, produces a complete inhibition of diamine oxidase activity.

The data shown reveals that of the aliphatic hydroxylamines, the straight chain compounds up to O-hexyl produced virtually complete inhibition of diamine oxidase activity. The presence of the double bond in O-1-allyl did not reduce activity. However, the presence of a triple bond as in O-1-pent-4-ynyl or branching as in O-1-(2-methyl)propyl reduced activity. Straight chain compounds where the chain length exceeds eight carbons were much less effective as inhibitors of the enzyme. Of the second generation of aliphatic compounds, O-1-(3-methyl)butyl was found to be the least effective, its inhibitory potential being only marginally greater than N-methyl, the negative control at a concentration of $10^{-5} \text{ M}$.

All of the benzylic compounds were less effective inhibitors of diamine oxidase activity than the straight chain aliphatic hydroxylamines at a concentration of $10^{-5} \text{ M}$ (Fig. 2B). At a 10-fold higher concentration ($10^{-4} \text{ M}$), O-1-benzyl, the parent compound was still only slightly more effective than N-methyl. However, substitutions on the benzene ring resulted in a dramatic increase in the inhibitory effects of these compounds at $10^{-5} \text{ M}$. O-p-hydroxybenzyl produced a complete inhibition of diamine oxidase activity. The other substituted benzylic compounds also demonstrated increased inhibitory effects, producing inhibitions of diamine oxidase activity ranging from 70 to 91%. Interestingly, even O-(1-E-Cinnamyl) which has a large bulky substituent produced a complete inhibition of enzyme activity at $10^{-5} \text{ M}$.

**“Secondary-Rise” Experiments.** These experiments were carried out using the protocol described earlier. Serosal addition of histamine ($10^{-4} \text{ M}$) produced a sharp transient increase in $I_{sc}$. The addition of an active compound to the serosal solutions after the fade in the response produced a
sharp increase in $I_{sc}$ that exceeded the original peak. Those compounds that produced no change in the slope were termed inactive. There was an interim category that had less definitive effects. To this class belonged compounds that either produced a flattening of the curve or a slow secondary increase in $I_{sc}$. We termed these compounds marginally active.

**Potency Experiments.** Changes in $pD_2$ to histamine produced by selected compounds were estimated from cumulative concentration-response curves. The compounds selected for detailed study were two aliphatic compounds, O-1-pentyl and O-1-octyl, and three of the aromatic derivatives, O-1-benzyl, O-p-methoxybenzyl and O-p-hydroxybenzyl. In addition, we tested two reference compounds. These were the contrasting methylhydroxylamines, where the O-substituted compound is fully active whereas the N-substituted compound is inactive. All compounds were tested at $10^{-4}$ M. In these experiments, the protocol described in “Materials and Methods” was followed. For illustrative purposes, the results obtained with O-p-hydroxybenzyl hydroxylation are shown (see Fig. 4). As can be seen, there is a clear shift to the left with no significant change in the maximal responses obtained. The data obtained with the hydroxylamines tested are tabulated (see Table 2). A one-way analysis of variance was performed on the changes in maximal responses and the $pD_2$ values. The maxima did not show any significant changes; however, significant differences were found among the compounds.

**Fig. 3.** Typical response patterns observed in the “secondary rise” experiments. A, In the first panel, we show an example of a compound that produced an active response. The addition of O-1-allyl hydroxyamine at $10^{-4}$ M produces a sharp increase that is rapid and is significantly larger than the original response to histamine. B, The addition of these compounds produces either a mere flattening of the fade (O-1-E-Cinnamyl) or a slow weak secondary increase (O-1-octyl) in $I_{sc}$. Compounds eliciting this type of response were grouped together and termed marginally active. C, the addition of the O-1-decyl produced no secondary increase in $I_{sc}$ after the initial histamine response. Such compounds were termed negative.

### Table 1

Summary of biological activity based on secondary increases in $I_{sc}$

<table>
<thead>
<tr>
<th>Compound</th>
<th>$RI^a$</th>
<th>$IC_{50} (\mu M)$</th>
<th>Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reference compounds</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aminoguanidine</td>
<td>1.0</td>
<td>0.11</td>
<td>Active</td>
</tr>
<tr>
<td>N-methyl hydroxyamine</td>
<td>2100.1</td>
<td>221.56</td>
<td>Inactive</td>
</tr>
<tr>
<td>Aliphatic hydroxylamines</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>O-1-allyl, 1</td>
<td>2.2</td>
<td>0.23</td>
<td>Active</td>
</tr>
<tr>
<td>O-1-pentyl, 2</td>
<td>3.3</td>
<td>0.35</td>
<td>Active</td>
</tr>
<tr>
<td>O-2-butyl, 3</td>
<td>6.6</td>
<td>0.70</td>
<td>Active</td>
</tr>
<tr>
<td>O-1-benzyl, 4</td>
<td>11.1</td>
<td>1.17</td>
<td>Active</td>
</tr>
<tr>
<td>O-1-pent-4-ynyl, 5</td>
<td>23.3</td>
<td>2.46</td>
<td>Active</td>
</tr>
<tr>
<td>O-1-(2-methyl)propyl, 6</td>
<td>35.6</td>
<td>3.76</td>
<td>Active</td>
</tr>
<tr>
<td>O-1-octyl, 7</td>
<td>64.6</td>
<td>6.82</td>
<td>Marginal</td>
</tr>
<tr>
<td>O-1-decyl, 8</td>
<td>115.6</td>
<td>12.20</td>
<td>Negative</td>
</tr>
<tr>
<td>O-1-(3-methyl)butyl, 9</td>
<td>147.5</td>
<td>15.56</td>
<td>Marginal</td>
</tr>
<tr>
<td>Benzyl hydroxylamines</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>O-1-E-Cinnamyl, 10</td>
<td>10.8</td>
<td>1.14</td>
<td>Marginal</td>
</tr>
<tr>
<td>O-p-hydroxybenzyl, 11</td>
<td>12.6</td>
<td>1.33</td>
<td>Active</td>
</tr>
<tr>
<td>O-p-hydroxy-m-bromobenzyl, 12</td>
<td>14.8</td>
<td>1.56</td>
<td>Marginal</td>
</tr>
<tr>
<td>O-m-methoxybenzyl, 13</td>
<td>20.6</td>
<td>2.17</td>
<td>Marginal</td>
</tr>
<tr>
<td>O-p-methoxybenzyl, 14</td>
<td>36.2</td>
<td>3.82</td>
<td>Marginal</td>
</tr>
<tr>
<td>O-p-bromobenzyl, 15</td>
<td>52.6</td>
<td>5.55</td>
<td>Marginal</td>
</tr>
<tr>
<td>O-benzyl, 16</td>
<td>161.7</td>
<td>17.06</td>
<td>Negative</td>
</tr>
</tbody>
</table>

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$pD_2$ values. A post hoc analysis of the mean $pD_2$ values was carried out.

As expected, the data show that the compounds identified as active from the screening experiments (i.e., O-1-pentyl and O-p-hydroxybenzyl) produced significant shifts in the $pD_2$ to histamine. By contrast the clearly negative compound, O-1-benzyl produced no changes in $pD_2$. Interestingly, the three compounds that we had classified as marginally active based on our screening experiments (O-1-octyl, O-p-methoxybenzyl and O-p-hydroxybenzyl) also produced significant shifts in the $pD_2$ to histamine. The reference compounds behaved as expected. Thus O-methyl produced a significant increase in $pD_2$ whereas N-methyl did not.

**Discussion**

The responses of the canine colonic epithelium to histamine are markedly potentiated by O-alkylhydroxylamines. Our earlier studies suggested strongly that the underlying mechanism was the inhibition of diamine oxidase (Rangachari et al., 1992).

In this study, we sought to explore further the structure-activity relations involved. We synthesized a second generation of hydroxylamines and assessed their biological activity using an enzyme preparation of diamine oxidase from the canine colonic epithelium as well as an in vitro preparation from the same tissue.

All compounds were also tested using a preparation of soluble DAO. Here it was assumed that access to the enzyme would not be a significant factor. Using DAO activity as an index, a difference appeared between the “active” aliphatic compounds and the benzylic hydroxylamines (Fig. 2, A and B). The former produced inhibitory effects at concentrations tenfold lower ($10^{-6}$ M) than the “active” benzylic ones. This may reflect the greater steric bulk associated with the added benzyl group and its substituents. At $10^{-5}$ M several of the benzylhydroxylamines became active, and the trends seen with the aliphatic compounds at $10^{-6}$ M became more pronounced. At the highest concentration tested ($10^{-4}$ M) selec-
Further experiments were carried out to determine the potency (shift in $pD_2$) of compounds selected (at least two from each category) to added histamine in tissues that had been pretreated with selected hydroxylamines. These data indicated that compounds that were initially classified as either active or inactive retained their identity; however, under these conditions where the tissue received a prolonged exposure (pretreatment) to the hydroxylamine, marginally active compounds exhibited potentiating effects. This suggests that access to the enzyme may be a limiting factor for these compounds.

The generally accepted mechanism for the deamination carried out by diamine oxidase (Hartman and Klinman, 1991; Hartman et al., 1993) is summarized in figure 5 with putrescine (17) acting as substrate. Amine oxidases contain a modified amino acid residue 6-hydroxydopa, or TOPA quinone, which is an active site prosthetic group that plays a crucial role in deamination. A series of quinone imine and quinol-amine intermediates are produced before putrescine is converted to 4-aminobutanal which cyclises to form $\Delta^1$-pyrroline.

The intermediate aminal (18) has been included to point out firstly the curious partial reversibility of many carbonyl reagents that affect diamine oxidases (Pec et al., 1992). We have modified slightly the accepted scheme, shown in detail in figure 5, by representing the enzyme bound base [B-Enz, now known to be a histidine (Shah and Ali, 1994)] as initially protonated. We have done so, because it can act as a general acid catalyst for the quinone oxygen as the amine begins its nucleophilic attack on the carbonyl group. The quinone-amine equilibrium is fast in both directions but the aminoliminium ion equilibrium can be very slow in the reverse direction and in addition pH dependent (Tamura et al., 1989). And second, the change in geometry from tetrahedral in (18) to planar in (19) is basis of the spatial model of the TOPA portion of the active site of diamine oxidase we present below.

The reaction mechanism rationale for the inhibition of diamine oxidases by hydroxylamines, and other carbonyl reagents such as semicarbazides and aminoguanidine, is illustrated in figure 6. In general, when a hydroxylamine like $O$-1-pentyl reacts with a carbonyl group an oxime is formed. As an example, the oxygen of $O$-1-pentylxime (24) occupies the site of the CH$_2$ alpha to the nitrogen in (19) and the enzyme histidine base has no protons to remove, halting the whole oxidation process at this point (step C, Fig. 6). A number of other compounds such as polyamines, aryl diamines and amiloride are inhibitors of diamine oxidase but they are noncompetitive and generally require higher concentrations ($>10^{-5}$ M) to be effective.

For our proposed model for the TOPA portion of diamine oxidase’s active site, we have taken the simple view that the TOPA quinone fragment is somewhat restricted in its rotation about the C$\beta$-C$\gamma$(ring) bond by the coordinating copper (or via any ligand between it and the copper). An alternative, induced fit view, where bulky groups cause enzyme conformational changes which either prevent access of the –ONH$_2$ or slow the rate of the elimination step (step B in Fig. 6), is however, equally plausible. We consider our model to be consistent with recent x-ray crystallographic studies on pea seedling DAO (Kumar et al., 1996) where the copper atom is found to be in a solvent-inaccessible site; i.e., the enzyme.

**Fig. 4.** Pretreatment of epithelial tissues with $10^{-4}$ M $O$-p-hydroxybenzylhydroxylamine produces a significant leftward shift in the cumulative dose-response to histamine ($EC_{50} = 2.6 \times 10^{-6}$ vs. $1.4 \times 10^{-5}$ for the control), with no significant change in maximal responses (292.8 vs. 247.9 $\mu$A/cm$^2$). Values shown are the means of five animals. Tissues were set up in pairs, with one member of the pair used as a control. Differences were determined using the Wilcoxon paired-sample test with a significance level of 0.05.

**TABLE 2**

Summary of the potency effects of $O$-alkylhydroxylamines

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>$pD_2$ (histamine)</th>
<th>Tissue Maximum (m$A$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>4.87 ± 0.25</td>
<td>485 ± 32</td>
</tr>
<tr>
<td>$O$-methylhydroxylamine</td>
<td>5.71 ± 0.13</td>
<td>509 ± 67</td>
</tr>
<tr>
<td>$N$-methylhydroxylamine</td>
<td>5.34 ± 0.14</td>
<td>450 ± 69</td>
</tr>
<tr>
<td>$O$-pentylhydroxylamine</td>
<td>5.72 ± 0.16</td>
<td>548 ± 86</td>
</tr>
<tr>
<td>$O$-octylhydroxylamine</td>
<td>5.52 ± 0.07</td>
<td>417 ± 68</td>
</tr>
<tr>
<td>$O$-benzylhydroxylamine</td>
<td>5.14 ± 0.18</td>
<td>503 ± 46</td>
</tr>
<tr>
<td>$O$-($p$-methoxybenzyl) hydroxylamine</td>
<td>5.73 ± 0.07</td>
<td>367 ± 29</td>
</tr>
<tr>
<td>$O$-($p$-hydroxybenzyl) hydroxylamine</td>
<td>5.68 ± 0.06</td>
<td>518 ± 68</td>
</tr>
</tbody>
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Values are means ± S.E. of five animals.

$^a$ Values are means ± S.E. of five animals.

$^b$ Significant difference in $pD_2$ value between $O$-alkylhydroxylamine-treated and control histamine responses (ANOVA with post hoc Newman Keuls analysis, $P < 0.05$).
must undergo conformational changes before an amine can interact with the active TOPA carbonyl. The results from our study of the hydroxylamines, together with use of molecular modeling and energy minimization of the conformations of the $O$-alkylhydroxylamine inhibitors, form the basis of the model. Because putrescine is believed to be the endogenous substrate for this enzyme, and it was used to determine diamine oxidase activity, we have chosen it rather than histamine for the model shown in figure 7.

At the point when the aminol is eliminating toward the

Fig. 5. The generally accepted mechanism for the catalyzed deamination of putrescine (17) into $\Delta^1$-pyrroline by the TOPA-Cu(II) active site of diamine oxidase is shown. A schematic representation of this process is given with all relevant details being illustrated in the box shown immediately below. The free carbonyl group of enzyme bound TOPA quinone reacts with one nitrogen of putrescine (17) giving the intermediate aminol (18), which eliminates water to form the intermediate quinone-imine (19) (arbitrarily drawn here in the anticonformation to the TOPA-OH group) for convenience. Intermediate 19 aromatizes by loss of a proton to an enzyme bound base to give an aliphatic iminium species (20) which then hydrolyses via attack by water to the amino-quinol (21) and 4-aminobutanal (22). Aldehyde 22 cyclises separately to form $\Delta^1$-pyrroline (not shown) and the amino-quinol 21 proceeds through the Cu(II)-O$_2$ redox cycle to regenerate TOPA quinone and ammonium ion. In the presence of excess substrate, the intermediate quinone-imine (23) can react alternatively with putrescine to form the diamino analogue of 18 which eliminates NH$_3$ instead of H$_2$O giving imine 19 (Mure and Klinman, 1993). All compounds and intermediates have been numbered to facilitate their discussion in the text.
The active site may be visualized as a trough-like region extending away from the TOPA carbonyl. Figure 7a represents a side view from within the enzyme and perpendicular to the TOPA ring. Figure 7b is a view from above the enzyme and in the plane of the TOPA ring. Figure 7c shows the same top view as figure 7b but with the atoms represented as solid spheres. The illustrated trough is in the same plane as the TOPA ring and wide enough to accommodate an extended, straight hydrocarbon chain; that is of the order of 4 to 4.5 Å in width and a depth of 4.8 to 5 Å if the chain were to be fully enfolded. From about 4.2 to 6.5 to 7 Å out from the TOPA carbonyl the trough becomes increasingly resistant to accommodating any branches in the hydrocarbon chain (e.g., methyl groups), but from about 8 Å and further, larger groups or atoms have minimal effects. The trough walls out to about 6.5 to 7 Å would most likely be composed of hydrophobic residues. In the region of 7 to 8 Å the enzyme contains a charged basic site, e.g., an aspartate or a glutamate residue, which acts to bind ionically the cationic terminus of a putrescine, a cadaverine or histamine. The presence of such a charged base, suggested in general terms by Bardsley et al. (1971), helps rationalize the strong selective binding of putrescine to diamine oxidase and inhibition of the enzyme by positively charged species such as guanidines, amidines and isothioureas. The immediate region around the TOPA carbonyl is probably relatively flexible, because the initial nucleophilic attack of the amine must take place perpendicular to the TOPA ring.

The assumption of hydroxylamine side chain extension and coplanarity may seem arbitrary but the concept of a “binding trough” extending from the TOPA carbonyl fits well with the natural substrates, for on modeling the putrescine iminium cation (19) (see Fig. 8). The NH$_3^+$ nitrogen was found to be 7.4 Å from the carbon of the quinone carbonyl as illustrated and, as noted above, an enzyme base at ca. 8 Å from the carbonyl carbon would ionically anchor the NH$_3^+$ group and increase enzyme specificity. Such a suggestion is supported by the observation that shorter (e.g., 1,2-diaminoethane) and longer diamines (e.g., 1,6-diaminohexane) are less active substrates than putrescine (Pec and Frebort, 1992). Most likely the base has some flexibility in accommodating intermediate chain lengths and compounds with no charges along the chain should offer few problems, but compounds with negative charges such as 2-(O-oxyamino)ethanoic acid should be, and in fact are, very poor substrates (Rangachari et al., 1992). It is noteworthy that histamine, which is a good substrate for diamine oxidase, has in a planar extended conformation the most distant N of which is 6.7 Å from the TOPA carbonyl carbon and if the N is protonated, it should also be anchored by the enzyme-bound base.

The idea of the trough having increased resistance to large groups comes from the reduction in inhibition of DAO activity produced by branched chain O-alkylhydroxylamines. In
initial screening tests $O$-1-propyl and $O$-1-butylhydroxylamines were effective inhibitors, with $O$-2-butyl being less effective, and tert-butyl having no effect on DAO activity (Rangachari et al., 1992). In the studies reported here on DAO inhibition, the straight chain $O$-1-pentylhydroxylamine (2) produced greater inhibition at $10^{-6}$ M than the branched compounds $O$-2-butyl (3), $O$-1-(2-methyl)propyl (6) and $O$-1-(3-methyl)butyl (9).

To illustrate how methyl substitution increases steric bulk, models of $TOPA-O$-1-pentyloxime (24) and $TOPA-O$-2-butyloxime, together with geometric data, are shown in figure 8. When both $\beta$-Hs of $O$-1-butyl are replaced by CH$_3$s, as in tert-butylhydroxylamine, the steric effects become so large the oxime will not form at all. A model of $O$-1-(2-methyl)propyl (6), illustrated in figure 7c, shows the size of a methyl group on the $\gamma$ carbon and how the enzyme resists steric bulk in this region. Moving large groups further away beyond the $\varepsilon$ carbon of a chain (7.33 Å away from the $TOPA$ carbonyl carbon) results in a decrease in enzyme resistance to steric bulk and a consequence increase in inhibitory activity (see the discussion on $O$-(1-E-Cinnamyl) below).

The $O$-benzyl group of compounds contributes further to the “binding trough” concept. As a group they are appreciably less effective than the aliphatic hydroxylamines and they are poor inhibitors of the enzyme at $10^{-6}$ M, but at $10^{-5}$ M they show a clear distinction of activities as noted above. The parent compound, $O$-1-benzyl (16) shows only marginal inhibition at $10^{-6}$ M. However, a para hydroxyl substituent on the ring causes a remarkable increase in activity. The flat, aromatic ring leads to it having large steric effects in the ring plane but small effects perpendicular to the ring. As a consequence the conformations of the ring about adjacent bonds are important and molecular modeling of $O$-1-benzyl gave a twisted conformation as the lowest energy conformer, where the phenyl ring is turned by 45° from the $TOPA-N$-O plane. This conformer is illustrated as a top view of a solid sphere structure in figure 8d (and should be compared with $TOPA-O$-1-(E-Cinnamyl). Panel a shows a side view of the trough from within the enzyme and perpendicular to the $TOPA$ ring, b shows a view from above the enzyme surface and in the plane of the $TOPA$ ring and c shows the same top view as for (b) but with the atoms represented as solid spheres.

Fig. 7. A diagrammatic representation of the proposed substrate trough of the $TOPA$ quinone portion of diamin oxidase is shown. The trough extends away from the $TOPA$ and is in the same plane as the $TOPA$ ring. The syn (to the OH of $TOPA$) form of the iminium ion is represented here. Our data offer no information on whether the putrescine iminium ion is syn or anti (molecular modeling showed the two forms to have similar energies). What our data does suggest is that if the proposed trough lies on the same surface side as the enzyme bound $TOPA$, then our oximes are probably syn because of the data from $O$-(1-E-Cinnamyl). Panel a shows a side view of the trough from within the enzyme and perpendicular to the $TOPA$ ring, b shows a view from above the enzyme surface and in the plane of the $TOPA$ ring and c shows the same top view as for (b) but with the atoms represented as solid spheres.

Fig. 8. Top views of selected $TOPA$ $O$-alkyl and $O$-benzylhydroxylamines illustrating how atoms protruding sideways into the proposed trough wall result in decreased inhibitory effects (see Fig. 6c). Atoms that tend to lie above the trough, as in $O$-(1-E-Cinnamyl), enable retained activity. a, $TOPA$ $O$-1-pentyloxime; b, $TOPA$ $O$-2-butyloxime; c, $TOPA$ $O$-1-(2-methyl)propyl; d, $TOPA$ $O$-benzyloxime; e, $TOPA$ $O$-1-Cinnamyloxime."
O-(1-E-Cinnamyl) (10) was at first sight surprising because it showed inhibitory activity at 10⁻⁴ M. However, molecular modeling illustrated by the solid sphere structure shown in figure 8e, showed the phenyl ring adopts conformations where it is approximately perpendicular to the TOPA-N-O plane and beyond the position where an e carbon of a straight chain would be (7.33 Å away). That is, steric bulk this far away from the TOPA carbonyl carbon seems to have fewer enzyme interactions.

**Summary**

O-Alkylhydroxylamines inhibit diamine oxidase by reacting with the carbonyl of the TOPA quinone at the active site to form an oxime of diamine oxidase. The ability of each member of the series to inhibit the enzyme is determined by their ability to access the TOPA quinone a "trough-like" region of the enzyme. However, in the tissue another determinant appears to be the access to the location of the enzyme. Thus the pharmacological responses observed in canine colon (histamine potentiation) reflect both the ability of the O-alkylhydroxylamine to penetrate the tissue and the ability to interact with the trough-like region of the enzyme and the TOPA-carbonyl of the active site.

**Appendix**

**Synthesis of O-(2-Butyl)-N-Hydroxyphthalimide**

A sample of N-hydroxyphthalimide (3.099 g, 18.4 mmol) was dissolved in dry dimethylformamide (DMF) under a nitrogen atmosphere. Sodium hydride in a 60% mineral oil suspension (0.778 g (20.9%) of the purified hydroxylamine hydrochloride 3 as a yellow-orange crystalline solid. The compound showed: mp 60 to 70°C; 1H NMR (200 MHz); (DMSO-d₆) δ 8.91 (C₄), 17.70 (C₁), 21.61 (-CH₂CH₃), 26.70 (-CHCH₂CH₃), 27.18 (-CH₂CH₂OH), 73.95 (-CH₂OH). 1H NMR (200 MHz; DMSO-d₆): δ 8.91 (C₄), 17.70 (C₁), 21.61 (-CH₂CH₃), 26.70 (-CHCH₂CH₃), 27.18 (-CH₂CH₂OH), 73.95 (-CH₂OH).

1-O-hexyl-N-hydroxyphthalimide: Brown-yellow liquid, (94.0%). 1H NMR (200 MHz; CDCl₃): δ 0.85 (t, 3H, J₅,₆ 6.50 Hz, -CH₃), 1.72 (m, 4H, J₆,₇ 6.50 Hz, -CH₂CH₂CH₃), 1.72 (m, 4H, J₆,₇ 6.50 Hz, -CH₂CH₂CH₃), 1.72 (m, 4H, J₆,₇ 6.50 Hz, -CH₂CH₂CH₃), 1.72 (m, 4H, J₆,₇ 6.50 Hz, -CH₂CH₂CH₃).

**Derivatives**

1-O-allylhydroxphthalimide. Brown crystalline solid (88.8%) yield; melting point [m.p.], 45 to 50°C; thin-layer chromatography [tlc], silica plate (0.25 mm), mobile phase: hexanes:ethyl acetate: methanol 74:25:1, Rf 0.94. 1H nuclear magnetic resonance (NMR 200 MHz; DMSO-d₆): δ 8.91 (C₄), 17.70 (C₁), 21.61 (-CH₂CH₃), 26.70 (-CHCH₂CH₃), 27.18 (-CH₂CH₂OH), 73.95 (-CH₂OH). 1H NMR (200 MHz; CDCl₃): δ 8.91 (C₄), 17.70 (C₁), 21.61 (-CH₂CH₃), 26.70 (-CHCH₂CH₃), 27.18 (-CH₂CH₂OH), 73.95 (-CH₂OH).
O-(1-pent-4-yl)hydroxylamine hydrochloride (5). Yellow-white solid (82.8%), mp 126–131°C. 1H NMR (200 MHz; DMSO-d6): δ 1.76 (pn, 2H, J2,3 6.90 Hz, J1,2 6.88 Hz, -OCH2CH2-), 2.22 (t/d, 2H, J1,2 6.88 Hz, -OCH2CH2-), 4.06 (t, 2H, J1,2 6.64 Hz, -OCH2-). 13C NMR (50 MHz; DMSO-d6): δ 14.24 (-CH2-CH2-), 26.28 (-OCH2CH2-), 71.84 (-OCH2-), 72.78 (-CH-), 83.29 (-CH3).

O-1-(1-methyl)propyl-N-hydroxyphthalimide (10). Colorless solid (87%), mp 164–166°C. 1H NMR (300 MHz; DMSO-d6): δ 4.68 (d/d, 2H, JCH-CH 6.7 Hz, JCH2-CH 6.7 Hz, -OCH3). 6.55 (adj. 5H, phenyl), 7.75 (4H, phenyl).

O-1-(1-ethyl)N-hydroxyphthalimide (11). Yellow crystal solid (94%), mp 94–102°C. 1H NMR (300 MHz; CDCl3): δ 3.81 (3H, -OCH3), 4.98 (2H, -OCH2-), 7.12 (2H, Jortho 8.33 Hz, Hortho), 7.26 (2H, Jortho 8.52 Hz, Hortho), 7.74 (2H, Jpara 2.33 Hz, Hmeta), 7.75 (4H, phenyl).
NMR (50 MHz; DMSO-d$_6$): $\delta$ 75.71 (–OCH$_2$–), 128.58 (phenyl ortho C), 129.07 (phenyl ipso C–CH$_2$O), 129.18 (phenyl meta C).

References


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