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Enzymic and Nonenzymic Lipid Peroxidation: Inhibition by Substituted Phenoxazines

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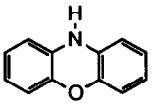
Abstract: A series of phenoxazines was evaluated as *in vitro* inhibitors of 5-lipoxygenase (5-LO) and iron-dependent lipid peroxidation. A linear relationship (r^2 = 0.91, n = 10) was found for the ester, hydroxamic acid and C-1 carboxylic acid representatives suggesting that hydroxamic acid mediated iron chelation may not contribute significantly to 5-LO inhibition by these compounds.

The *in vivo* oxidation of polyunsaturated fatty acids may occur through both enzymic and nonenzymic pathways.[1] Whereas enzymic lipid peroxidation is a stereoselective transformation that occurs within the active site of an enzyme (i.e. 5-lipoxygenase)[2] yielding endoperoxide and hydroperoxide products with important biological functions relevant to the inflammatory process,[3] nonenzymic lipid peroxidation is a nonstereoselective chain reaction that occurs under pathological conditions and may, in part, contribute to postischemic tissue damage by impairing cellular membrane structure and function. Consequently, lipid peroxidation inhibitors may find therapeutic utility in ischemic and inflammatory disorders.[4] We found that phenoxazine (**1**) potently inhibited both 5-lipoxygenase (5-LO) isolated from guinea pig PMNs[5] (IC_{50} = 20 nM) and iron-dependent lipid peroxidation of rabbit brain homogenate[6] (MIC = 55 nM).[7] Since the *in vitro* enzymatic oxidation of arachidonic acid by 5-LO from RBL-1 cells is inhibited by a variety of antioxidant agents,[8] the 5-LO inhibitory activity of phenoxazine may be related to its antioxidant properties. A recent report describes a linear relationship between nonenzymic lipid peroxidation and 5-LO inhibition (r^2 = 0.59, n= 11) for a structurally diverse series of inhibitors comprised of substituted phenols (e.g. BHT) and nitrogen heterocycles (e.g. BW 755c).[9] Although this data might suggest a common mechanism of inhibition, chemical inhibitors reportedly exhibit more specificity toward LO inhibition than might be expected based solely on antioxidant capacity.[8]



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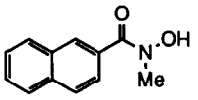
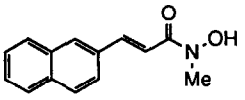


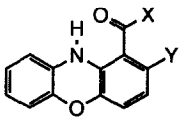
2a



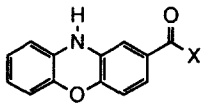
2b

A series of simple aryl hydroxamic acids (e.g. **2a** and **2b**) was reported by Summers, et al. to be *in vitro* inhibitors of RBL-1 5-LO.[10] The activity of these compounds was related to lipophilicity and required the presence of a hydroxamic acid functionality which was thought to bind in a position near the catalytically important iron atom.[11] The corresponding carboxylic acid of **2a** (2-naphthoic acid) was reported to be inactive. Since the experimental logP values for phenoxazine and naphthalene are similar[12] we proposed that a series of ester, carboxylic acid and hydroxamic acid substituents on the phenoxazine nucleus might allow us to establish a relationship between iron-dependent lipid peroxidation[13] and 5-LO for this class of compounds and to evaluate the contribution of functional groups to the redox inhibition of 5-LO by phenoxazine. The identification of outliers may therefore provide structural information regarding inhibitor-enzyme interactions such as chelation and steric

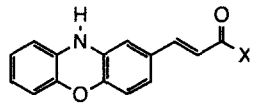
or electrostatic factors, parameters which may help delineate the mechanistic differences between enzymic and nonenzymic lipid peroxidation.



3: X = OH; Y = H
4: X = OH; Y = OMe
7: X = OMe; Y = H
8: X = H; Y = CO2Me
10: X = NHOH; Y = OMe



5: X = OH
8: X = OMe
11: X = NHOH
12: X = NMeOH



6: X = OH
9: X = OEt
13: X = NMeOH

The phenoxazines **3-13** were prepared by known methods.[14] A modified procedure of Olmsted, et al.[15] was used to prepare both 2-methoxyphenoxazine and phenoxazine-2-carboxylic acid methyl ester. The phenoxazine-1-carboxylic acid derivatives were prepared by direct lithiation and carboxylation of either phenoxazine[16] or 2-methoxyphenoxazine. Functional group modification then provided the additional carboxylic acid, ester and hydroxamic acid derivatives.

Table 1: 5-LO and Lipid Peroxidation Inhibition

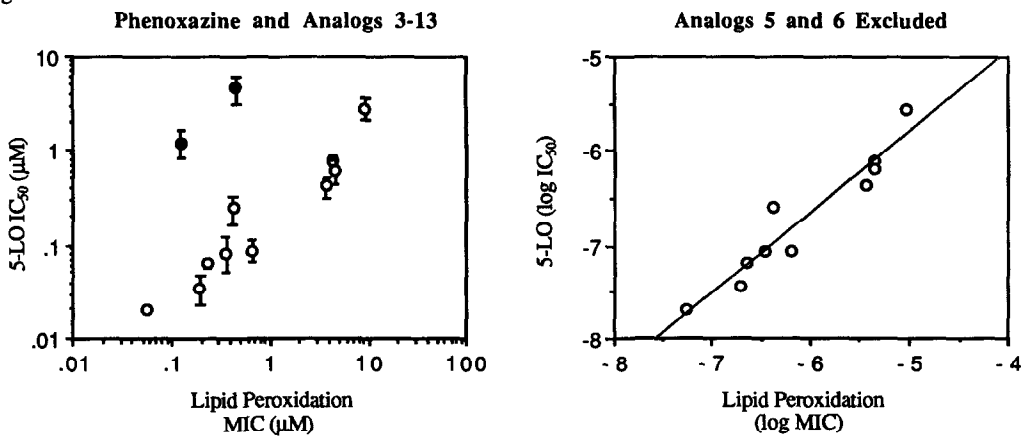
Compound	5-LO IC_{50} (μM)[a]	Lipid Peroxidation MIC (μM)[b]
1	0.020 ± 0.001	0.055
3	0.61 ± 0.17	4.4
4	2.8 ± 0.76	9.1
5	4.6 ± 1.5	0.44
6	1.2 ± 0.38	0.12
7	0.77 ± 0.092	4.2
8	0.065 ± 0.008	0.22
9	0.035 ± 0.012	0.19
10	0.42 ± 0.11	3.7
11	0.25 ± 0.078	0.41
12	0.087 ± 0.022	0.65
13	0.085 ± 0.035	0.35

[a]Inhibition of [^{14}C]-arachidonic acid conversion to 5-HETE by broken cell 5-LO *in vitro* (guinea pig PMN). Mean ± SEM (n = 3-5).

[b]Inhibition of iron-dependent lipid peroxidation of rabbit brain homogenate *in vitro*. Minimum tested conc. of agent that gave ≥ 50% inhibition (n = 3).

With respect to lipid peroxidation, there is little activity differences between the ester, carboxylic acid and hydroxamic acid groups (Table 1). The position of the substituent on the heterocyclic ring appears to have the greater influence on inhibitory activity in this system which spans greater than two orders of magnitude. Analogs substituted at C-2 were generally more potent than those substituted at C-1. With respect to 5-LO inhibition, the activity is dependent upon both the substituent and its position on the phenoxazine ring. The data for the compounds in Table 1 are plotted in Figure 1. Although taken as a whole the data is poorly correlated ($r^2 = 0.33$), two of the points, compounds **5** and **6**, appear to be outliers. If these two compounds are considered separately, then the correlation increases dramatically for the remaining ten compounds ($r^2 = 0.91$). Since the points for compounds **12** and **13** fall on the line (Figure 1) 5-LO inhibition by these two analogs is probably not significantly influenced by possible hydroxamic acid chelation to the catalytic iron atom.

Figure 1:



Since conformational differences with respect to the phenoxazine nucleus might account for the poorly correlated activity of compounds **5** and **6**, we performed AM1[17] calculations with complete geometry optimization for compounds **3** and **5**. In contrast to the phenothiazine ring which is known to be non-planar,[18] the parent phenoxazine nucleus has either a rigid or a time-weighted average planar conformation with little or no folding along its short axis.[19] Consequently, the phenoxazine congeners might be predicted to conformationally resemble the anthracene analogs of **2a** and **2b**.[20] The SYBYL (version 3.5)[21] molecular modeling software package was used to generate the structures and the MOPAC (version 5.0) program on the CRAY-2 using the AM1 method (version 5.0) was then run with complete geometry optimization. A nearly planar conformation[22] is predicted for both isomers suggesting that simple geometrical alterations of the heterocyclic nucleus by a carboxylic acid substituent at C-2 may not account for the poorly correlated 5-LO activity of compound **5**.

Since phenoxazine is known to be easily oxidized[23] and presumably inhibits lipid peroxidation and 5-LO through a redox mechanism, AM1 calculations with complete geometry optimization for the radical cations were similarly performed. Using a planar starting geometry, the radical cations of **3** and **5** are both predicted to possess a planar optimized geometry with a dihedral angle greater than 179°.[24]

The linear relationship between iron-dependent lipid peroxidation and 5-LO is consistent with a common mechanism of inhibition for the phenoxazine series. However two compounds, **5** and **6**, appear to be outliers suggesting that additional factors such as unfavorable electrostatic interactions with the 5-LO enzyme and the C-2 carboxylic acid substituent may be involved. Since semiempirical calculations predict no gross geometrical differences between the carboxylic acid analogs **3** and **5**, the position of the acid substituent rather than the conformation of the phenoxazine ring may be the governing feature.

Antioxidants could inhibit 5-LO by at least two mechanisms. Since low concentrations of hydroperoxides are believed to initiate the enzymatic reaction, reduction of these intermediates might be expected to depress 5-LO activity.[8] Alternatively, antioxidants could impair enzyme function by reducing active site functionality.[25] The described compounds, however, appear to exhibit a greater structural specificity toward 5-LO inhibition than might be predicted based solely on free radical scavenging capacity. The poorly correlated inhibitory activities of the C-2 carboxylic acids **5** and **6** might therefore suggest 5-LO active site involvement and may provide a handle for elucidating enzyme-redox inhibitor interactions.

Acknowledgment. We thank Dr. Donald Boyd for assistance with the semiempirical calculations and for locating the experimental logP values.

References and Notes

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5. 5-Lipoxygenase activity was assayed as follows: 10 mL of a 2% casein solution was injected intraperitoneally to guinea pigs weighing 250-300 gm. After 16-18 hrs, the guinea pigs were sacrificed by suffocation in a CO_2 chamber. The peritoneal cavity was infused with 70 mL of saline and 40-50 mL of the fluid were recovered from the cavity. After centrifugation, cell pellets were washed twice in Hank's balanced salt solution (HBSS) without Ca^{2+} ion. The cells were then suspended in sodium phosphate buffer (5 mL), pH 7.1 containing 1 mM EDTA and 0.1% gelatin. About 2-3 x 10^8 cells were obtained from one guinea pig. Analysis for cell composition indicated that more than 95% of the cells were PMNL. The PMNL suspension was disrupted by five 0.5 second sonic pulses at a setting of 3 in a Branson Sonifier, Model 350, equipped with a microtip. The sonicates were combined and centrifuged at 30,000 x g for 10 min. The supernatant was kept frozen at -70° until use. Enzyme activity was determined by assaying for 5-HETE formation by incubating 0.2 mL of the supernatant obtained above with 1-^{14}C-arachidonic acid (specific activity = 58.4 mCi/mmol, Amersham, Illinois) (5 μM), $CaCl_2$ (1 mM), ATP (2 mM), and GSH (1 mM) for 15 minutes at 37°C. The enzyme reaction was stopped by adding 1 M citric acid (10 μL) and 10 μL of an alcohol solution containing 20 mg/mL each of indomethacin and butylated hydroxyanisole (BHA). The reaction mixture was spotted (50 μL) on a silica gel plate (Baker TLC plate S1250-PA-19C) and eluted with a mixture of ethyl acetate: 2,2,4-trimethylpentane: glacial acetic acid: H_2O (90:95:20:100). The positions of arachidonic acid and its metabolites (5-HETE and LTB_4) were determined from a developed x-ray film that had been exposed to the TLC plate or 1-2 days. The amount of 5-HETE formed was quantitated by scraping the silica gel area corresponding to the spot on the x-ray film and the radioactivity was measured in a Beckman LS 5800 liquid scintillation counter.
6. Lipid peroxidation of rabbit brain homogenate was assayed as follows: Frozen mature stripped rabbit brain (Pell Freez) was thawed, minced and homogenized in 25 mM Tris-HCl containing 0.15 M KCl, pH 7.5 (10% w/v). The test agent dissolved in DMSO (5 μL) was added to the supernatant fraction (0.5 mL) which was then incubated with ADP (220 μM) and $FeCl_3$ (2 μM) at 37°C for 1 h. The control supernatant was not incubated. The reaction was terminated by adding 0.4 mL TBARS reagent (0.02% TCA and 0.8% thiobarbituric acid) and boiled for 15 min. The sample was acidified with 2.3 N HCl (0.5 mL), extracted with n-butanol (1 mL) and centrifuged at 3,000 x g for 5 min. The absorbancy of the butanol phase was determined at 532 nm in a Spectronic-20 spectrophotometer and the amount of MDA present was determined by linear regression analysis of a standard curve.
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