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Phenothiazine and Phenoxazine Derivatives of Nafazatrom. In Vitro Evaluation as 5-Lipoxygenase and Iron-Dependent Lipid Peroxidation Inhibitors.

Melvin J. Yu,* Jefferson R. McCowan, Barbara Bertsch, Peter P.K. Ho, Lee A. Phebus, Kenneth J. Ruterbories, Terry D. Lindstrom, Jeffrey K. Smallwood and Paul J. Simpson
Lilly Research Laboratories, Eli Lilly and Company
Indianapolis, IN 46285

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Abstract: A series of phenothiazine and phenoxazine analogs of nafazatrom were prepared and evaluated as 5-lipoxygenase and iron-dependent lipid peroxidation inhibitors (rabbit brain homogenate) *in vitro*. Nafazatrom and two representatives from the described series were also evaluated as peroxyl radical and superoxide anion scavengers.

Nafazatrom (**1**) has been shown to contain infarction in a canine model of ischemia/reperfusion injury[1] and reportedly exhibits a number of *in vitro* properties including 5-lipoxygenase (5-LO)[2] inhibition and antioxidant activity. Although the precise mechanism by which this agent exerts its beneficial effect in animal models of myocardial infarction is unclear, inhibition of neutrophil functional responses *in vitro* and leukocyte infiltration *in vivo* have been proposed to underly its myocardial protective effects.[3] Since mediators such as toxic oxygen metabolites released by activated neutrophils may contribute to postischemic tissue damage, an important component of nafazatrom's activity in reducing myocardial reperfusion injury may relate to its antioxidant capacity.

Since the pyrazolidinone ring of nafazatrom is responsible for its antioxidant activity and is an efficient scavenger of oxygen radicals generated by pulse radiolysis,[4] we hypothesized that structural manipulations that increase the overall ability of this agent to act as an antioxidant may consequently enhance its beneficial properties. To test this hypothesis, we replaced the naphthalene ring of nafazatrom with a phenothiazine or phenoxazine nucleus, two heterocycles known to readily undergo one-electron oxidation.[5,6]

Figure 1

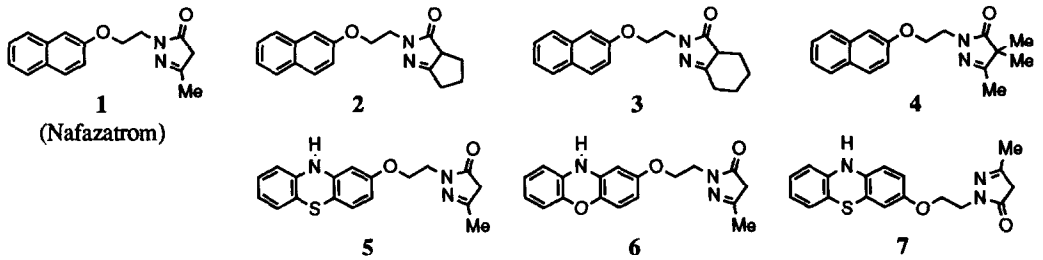

Figure 1 lists the compounds that were prepared and investigated. In addition to the phenothiazine and phenoxazine derivatives, we also examined two bicyclic pyrazolidinone analogs, compounds **2** and **3**, to evaluate the effect of ring fusion on 5-LO and lipid peroxidation inhibitory activity. Compound **4** cannot tautomerize to an enol form and thus was expected to be devoid of antioxidant activity. These compounds were prepared by standard methods starting with either 2-hydroxyphenothiazine[7] or 2-(naphthoxy)acetic acid.[8]

The oxidation potential for these pyrazolidinones was determined by cyclic voltammetry using a carbon

paste disc electrode against a Ag/AgCl reference electrode in pH 7.4 phosphate buffered saline at 37°C (Figure 2).[9] As anticipated, the phenothiazine and phenoxazine congeners **5** - **7** exhibited the lowest oxidation potential of the series while the gem-dimethyl derivative did not oxidize at a potential as high as +600 mV. The fused bicyclic analogs **2** and **3** also exhibited a half-wave potential lower than that for nafazatrom. In all cases, irreversible behavior was observed.

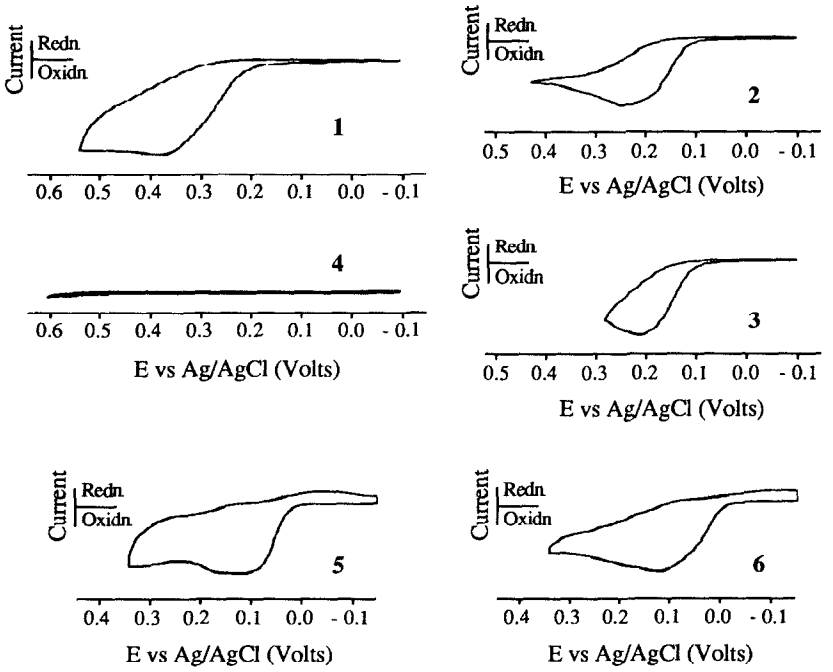

Figure 2. Cyclic voltammograms of compounds **1** - **6**. Measurements were conducted using a carbon paste disc electrode against a Ag/AgCl reference electrode in pH 7.4 phosphate buffered saline at 37°C. The initial potentials were -0.1 volts for compounds **1** - **4** and -0.15 volts for compounds **5** - **6** with a scan rate of 10 mV/second in a positive direction. A new electrode surface was used for each compound.

The ability of these agents to inhibit iron-dependent lipid peroxidation of rabbit brain homogenate and broken cell 5-LO isolated from guinea pig PMN was measured (Table 1).[10] Replacing the naphthalene ring of nafazatrom with a phenothiazine or phenoxazine nucleus substantially increased iron-dependent lipid peroxidation and 5-LO inhibitory activity *in vitro* (>100-fold and >15-fold, respectively). Although the range of IC_{50}'s in the 5-LO assay is relatively narrow (20-fold), the activity in these two test systems appear to parallel one another. In addition, the relative potencies of these analogs to inhibit iron-dependent lipid peroxidation are consistent with their electrochemical behavior in that the more potent compounds exhibit a lower oxidation potential.

Two representative analogs, compounds **5** and **6** were examined in a fluorescence based assay using 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH) and R-phycoerythrin as described by Glazer.[11] Under these assay conditions, both congeners are approximately equipotent with nafazatrom in scavenging peroxyl radicals

generated by AAPH in an aqueous environment (Figure 3). This is in contrast to the marked differences observed in the iron-dependent lipid peroxidation assay using rabbit brain homogenate.

Table 1: Inhibition of Iron-Dependent Lipid Peroxidation and 5-LO *in Vitro*

Compound	Lipid Peroxidation Inhib. (MIC, μM)[a]	5-LO Inhib. (IC_{50}, μM)[b]
1 (nafazatrom)	110	1.1 ± 0.2
2	34	0.48 ± 0.07
3	32	0.31 ± 0.07
4	NA[c]	NA[d]
5	0.69	0.057 ± 0.022
6	0.72	0.11 ± 0.03
7	0.49	0.061 ± 0.016

[a]Minimum tested concentration of agent that gave ≥ 50% inhibition of iron-dependent lipid peroxidation (rabbit brain homogenate, n = 3). [b]Inhibition of ^{14}C-arachidonic acid conversion to 5-HETE by broken cell 5-LO isolated from guinea pig PMN (mean ± SEM, n = 3). [c]Not active at highest concentration tested (30 μg/mL). [d]Not active at highest concentration tested (3 μg/mL)

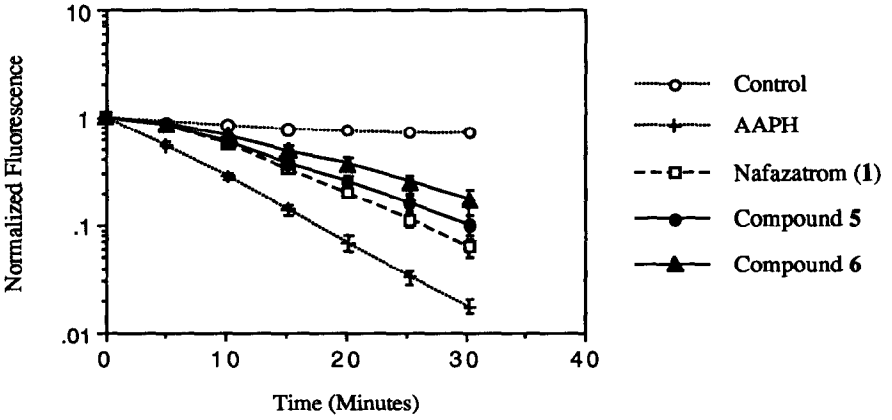

Figure 3. Effect of nafazatrom and compounds **5** and **6** (1 μM) on the decay of R-phycoerythrin (R-PE) fluorescence (λ_{ex} = 544 nm, λ_{em} = 590 nm) induced by 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH) in phosphate buffer (pH 7.0) at 37°C. The control values represent the fluorescence of R-PE in the absence of drug and AAPH. The AAPH values represent R-PE fluorescence in the presence of AAPH without drug. Each point represents the mean ± SE (n = 4). Differences between compounds **1**, **5** and **6** were not statistically significant at the 30 minute time point (student's unpaired t-test).

Using a modified procedure of Fantone and Kinnes,[12] compounds **5** and **6** were also tested for their ability to scavenge superoxide anion generated by fMLP (10^{-7} M) activated human neutrophils *in vitro* (Figure 4).

Although the tricyclic derivatives of nafazatrom were more potent in both the lipid peroxidation and 5-LO assays, they do not appear to be significantly more potent as peroxyl radical or superoxide anion scavengers as measured by the AAPH and neutrophil systems, respectively.

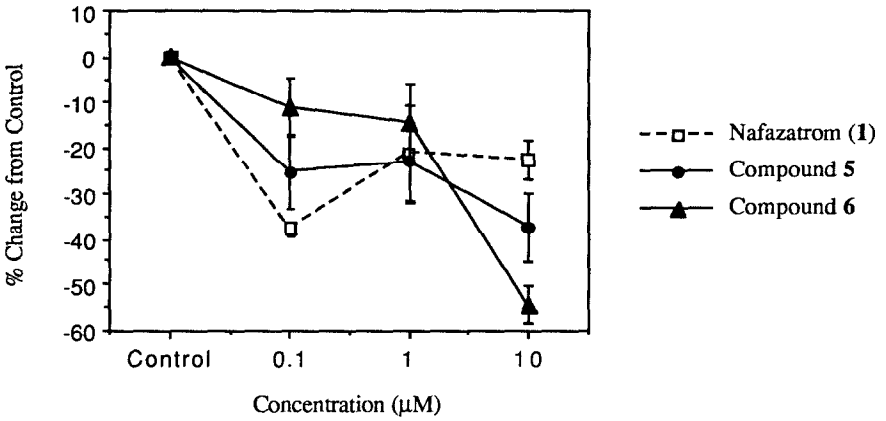

Figure 4. Effect of nafazatrom and compounds **5** and **6** on superoxide generated by human neutrophils stimulated by fMLP (10^{-7} M) in Hank's buffer at 37°C. Superoxide production was defined as the superoxide dismutase (SOD) inhibitable (50 μg/mL SOD in reference wells) reduction of ferricytochrome c to ferrocytochrome c (absorbance measured at 550 nm). The amount of superoxide produced in the absence of test agent was as follows (nmols/10^6 cells/10 min, mean ± SE, n = 4 - 5): Nafazatrom, 38.0 ± 3.3; compound **5**, 39.9 ± 4.3; compound **6**, 39.3 ± 4.1. Each point represents the mean ± SE (n = 3 - 5).

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8. Satisfactory spectral and analytical data were obtained for all final compounds.
9. Cyclic voltammetry was carried out using a carbon paste disk electrode packed with carbon paste made from 3 parts UCP1-M graphite and 2 parts light paraffin oil and polished on a smooth piece of paper. A fresh carbon paste surface was used for each compound. A Ag/AgCl electrode (BAS MF-2020) and a stainless steel rod served as reference and auxiliary (counter) electrodes, respectively. Experiments were conducted at 37°C in pH 7.4 phosphate buffered saline. The initial potentials were -0.1 volts for compounds **1** - **4** and -0.15 volts for compounds **5** - **6** with a scan rate of 10 mV/second in a positive direction.
10. The 5-LO and lipid peroxidation assays were conducted as previously described. Yu, M.J.; McCowan, J.R.; Bertsch, B.; Ho, P.P.K. Enzymic and Nonenzymic Lipid Peroxidation: Inhibition by Substituted Phenoxazines. *Biomed. Chem. Lett.* **1991**, *1*, 107-110.
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