were administered iv 30 min after the predrug determination of VFT. Control studies show that 15-30 min after an episode of VF arterial pressure and ECG parameters recover to control values. A first determination of VFT was performed 15 min after dosing and the dog was promptly defibrillated; as soon as arterial pressure and ECG parameters had recovered, a second VFT determination was performed. This time defibrillation was withheld in order to observe an eventual drug-induced termination of fibrillation.

Acknowledgment. We express our appreciation to Dr. D. Cochran, Mr. A. Verwijs, and their associates for microanalytical and spectral data.

Supplementary Material Available: A listing of atomic coordinates, bond lengths and angles, isotropic and anisotropic thermal parameters, and H-atom coordinates for compound **5** (five pages). Ordering information is given on any current masthead page.

Phenothiazines as Lipid Peroxidation Inhibitors and Cytoprotective Agents

Melvin J. Yu,* Jefferson R. McCowan, K. Jeff Thrasher, Priscilla T. Keith, Charlotte A. Luttman, Peter P. K. Ho, Richard D. Towner, Barbara Bertsch, J. S. Horng, Suzane L. Um, Lee A. Phebus, and Royal D. Saunders

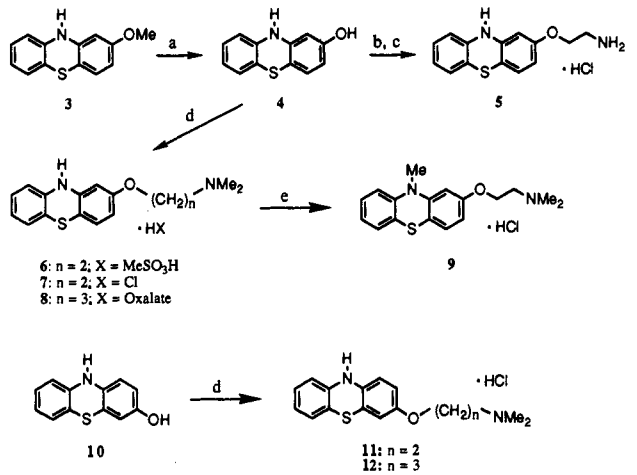
Lilly Research Laboratories, Eli Lilly and Company, Indianapolis, Indiana 46285. *Received August 12, 1991*

A series of phenothiazines was synthesized and evaluated as in vitro inhibitors of iron-dependent lipid peroxidation. The MIC (minimum tested concentration that gave ≥50% inhibition) for 2-(10*H*-phenothiazin-2-yloxy)-*N*,*N*-dimethylethanamine methanesulfonate (**6**) was 0.26 μM. Whereas methyl substitution at N-10 diminished activity nearly 100-fold, other structural modifications such as varying the amine group, the distance separating the amine substituent from the phenothiazine nucleus, and the linking group had little effect. Compound **6** was more effective than probucol, a known antioxidant, in blocking Cu^{2+} catalyzed oxidation of low-density lipoprotein (LDL) as measured by competitive scavenger receptor mediated degradation of ^{125}I-labeled acetyl-LDL by mouse peritoneal macrophage cells in vitro. At a concentration of 5 μM, compound **6** also protected primary cultures of rat hippocampal neurons exposed to hydrogen peroxide (50 μM) when assessed 18 h later by fluorescein diacetate and propidium iodide uptake.

Toxic oxygen metabolites such as oxygen-derived free radicals and hydrogen peroxide may play an important role in a number of human disease states.[1] For example, oxidants have been associated with postischemic tissue damage in the myocardium[2] and brain[3] and in the pathogenesis of atherosclerotic lesions through oxidative modification of native low-density lipoprotein.[4,5] Since cell membrane phospholipids are particularly vulnerable to free radical induced damage, lipid peroxidation may represent a potentially important mechanism of oxygen-mediated cellular injury.[6]

The phenothiazines chlorpromazine (**1**) and promethazine at relatively high concentrations have previously been

Scheme I[a]



[a] (a) Pyridine hydrochloride; (b) NaH, $ClCH_2CN$; (c) LAH; (d) NaH, $Br(CH_2)_nNMe_2$; (e) KH, MeI.

(1) For reviews, see: (a) Halliwell, B. Introduction to Free-Radicals in Human-Disease. *Saudi Med. J.* **1991**, *12*, 13-19. (b) Sinclair, A. J.; Barnett, A. H.; Lunec, J. Free-Radicals and Antioxidant Systems in Health and Disease. *Br. J. Hosp. Med.* **1990**, *43*, 334-344. (c) Lunec, J. Free-Radicals-Their Involvement in Disease Processes. *Ann. Clin. Biochem.* **1990**, *27*, 173-182. (d) Southorn, P. A. Free Radicals in Medicine. II. Involvement in Human Disease. *Mayo Clin. Proc.* **1988**, *63*, 390-408.

(2) For reviews see (a) Bolli, R. Oxygen-Derived Free Radicals and Myocardial Reperfusion Injury: An Overview. *Cardiovasc. Drug Therap.* **1991**, *5*, 249-268. (b) Lucchesi, B. R. Myocardial-Ischemia, Reperfusion and Free-Radical Injury. *Am. J. Cardiol.* **1990**, *65*, 14I-23I. (c) Downey, J. M. Free-Radicals and Their Involvement During Long-Term Myocardial-Ischemia and Reperfusion. *Ann. Rev. Physiol.* **1990**, *52*, 487-504. (d) Richard, V. J.; Murry, C. E.; Reimer, K. A. Oxygen-Derived Free-Radicals and Postischemic Myocardial Reperfusion-Therapeutic Implications. *Fund. Clin. Pharmacol.* **1990**, *4*, 85-103. (e) Ferrari, R. The Role of Free-Radicals in Ischemic Myocardium. *Br. J. Clin. Pract.* **1990**, *44*, 301-305. (f) Werns, S. W.; Lucchesi, B. R. Free-Radicals and Ischemic Tissue-Injury. *Trends Pharmacol. Sci.* **1990**, *11*, 161-166.

(3) For reviews, see: (a) Jesberger, J. A.; Richardson, J. S. Oxygen Free-Radicals and Brain-Dysfunction. *Int. J. Neurosci.* **1991**, *57*, 1-17. (b) Ikeda, Y.; Long, D. M. The Molecular Basis of Brain Injury and Brain Edema: The Role of Oxygen Free Radicals. *Neurosurgery* **1990**, *27*, 1-11. (c) Kontos, H. A. Oxygen Radicals in CNS Damage *Chem. Biol. Interact.* **1989**, *72*, 229-255.

(4) For reviews see: (a) Steinberg, D.; Parthasarathy, S.; Carew, T. E.; Khoo, J. C.; Witztum, J. L. Beyond Cholesterol. Modifications of Low-Density Lipoprotein that Increase its Atherogenicity. *N. Engl. J. Med.* **1989**, 915-924. (b) Steinbrecher, U. P.; Zhang, H.; Loughheed, M. Role of Oxidatively Modified LDL in Atherosclerosis. *Free Rad. Biol. Med.* **1990**, *9*, 155-168.

(5) Piotrowski, J. J.; Hunter, G. C.; Eskelson, C. D.; Dubick, M. A.; Bernhard, V. M. Evidence for Lipid Peroxidation in Atherosclerosis. *Life Sci.* **1990**, *46*, 715-721.

(6) See, for example: (a) Braughler, J. M.; Hall, E. D. Central Nervous System Trauma and Stroke. I. Biochemical Considerations for Oxygen Radical Formation and Lipid Peroxidation. *Free Rad. Biol. Med.* **1989**, *6*, 289-301. (b) Hall, E. D.; Braughler, J. M. Central Nervous System Trauma and Stroke. II. Physiological and Pharmacological Evidence for Involvement of Oxygen Radicals and Lipid Peroxidation. *Free Rad. Biol. Med.* **1989**, *6*, 303-313.

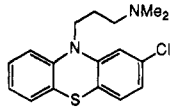
0022-2623/92/1835-0716$03.00/0

Table I. Lipid Peroxidation Inhibition[a]

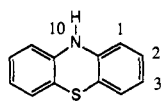
compound	lipid peroxidation inhibition (MIC, μM)[b]	compound	lipid peroxidation inhibition (MIC, μM)[b]
chlorpromazine	10 (n = 3)	11	0.056 (n = 5)
2	0.15 (n = 3)	12	0.089 (n = 3)
5	0.18 (n = 3)	14	2.6 (n = 3)
6	0.26 (n = 4)	17	2.5 (n = 3)
7	0.093 (n = 6)	23	0.068 (n = 3)
8	0.14 (n = 3)	24	0.17 (n = 3)
9	21 (n = 5)		

[a] Inhibition of iron-dependent peroxidation of rabbit brain vesicular membrane lipids in vitro. [b] Minimum tested concentration of agent that gave ≥50% inhibition.

reported to inhibit CCl_4-induced lipid peroxidation in rat liver microsomes.[7] Although relatively weak in potency,



1: Chlorpromazine



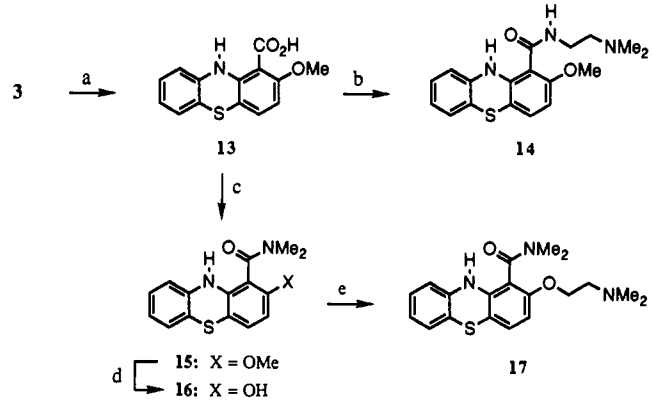
2

earlier polarographic studies by Kabasakalian and McGlotten demonstrated that the half-wave potential of substituted phenothiazines is dependent on the N-10 substituent and that N-10 unsubstituted analogues have lower oxidation potentials than those bearing an alkylamine group.[8] This effect was also observed in more recent electrochemical studies of phenothiazines.[9,10] If redox potential and lipophilicity are important parameters in defining nonenzymic lipid peroxidation inhibitory activity, then lowering the oxidation potential of chlorpromazine through appropriate structural modifications might result in lipid peroxidation inhibitors with substantially improved potency. To test our hypothesis, we prepared a series of N-10 unsubstituted phenothiazines and electrochemically determined the oxidation potential (cyclic voltammetry) for a representative number of analogues with reference to their activity as inhibitors of iron-dependent lipid peroxidation. We then explored the potential utility of these compounds in two in vitro models of oxidant damage, a model of oxidatively modified LDL degradation by mouse peritoneal macrophage and a model of oxidant-induced degeneration of rat hippocampal neurons.[11]

Chemistry

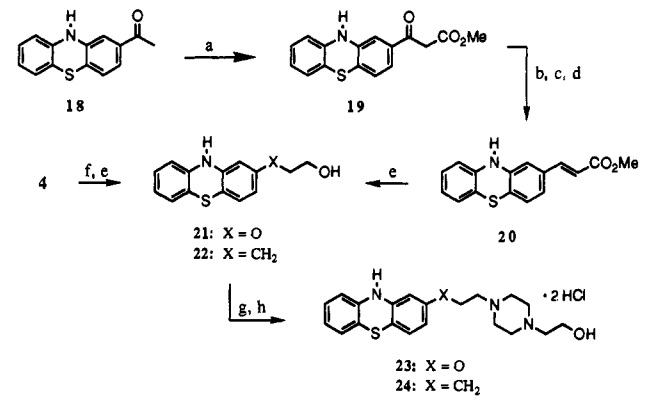
Based upon the oxidation potential data reported by

Scheme II[a]



[a] (a) n-BuLi, CO_2; (b) CDI, $Me_2N(CH_2)_2NH_2$; (c) CDI, $NHMe_2$; (d) pyridine hydrochloride; (e) NaH, $Br(CH_2)_2NMe_2$.

Scheme III[a]



[a] (a) NaH, MeOCOOMe; (b) $NaBH_4$; (c) Ac_2O, pyridine; (d) t-BuOK; (e) LAH; (f) NaH, $BrCH_2CO_2Et$; (g) Ph_3P, CBr_4; (h) 1-(2-hydroxyethyl)piperazine.

Kabasakalian and McGlotten,[8] a series of phenothiazines unsubstituted at N-10 and bearing an alkylamine substituent at C-1, C-2, and C-3 were synthesized. Commercially available 2-methoxyphenothiazine was conveniently demethylated[12] with pyridine hydrochloride and directly alkylated with (dimethylamino)ethyl or -propyl bromide to give products **6–8** (Scheme I). Methylation at N-10 was achieved by alkylation of compound **6** free base to give **9**, and the C-3 substituted isomers **11** and **12** were similarly prepared from 3-hydroxyphenothiazine.[13] Analogues with a C-1 carboxamide group were obtained from 2-methoxyphenothiazine by lithiation and carboxylation to ultimately furnish compounds **14** and **17** (Scheme II). To address problems associated with limited saline[14] solubility of these agents, analogues with a 1-(2-hydroxyethyl)piperazine in place of the dimethylamine were prepared to allow dihydrochloride salt formation (Scheme III). This particular group was chosen based upon the structure of fluphenazine and related phenothiazine antipsychotic drugs.[15] Compound **23** was ob-

(7) Slater, T. F. The Inhibitory Effects in Vitro of Phenothiazines and Other Drugs on Lipid-Peroxidation Systems in Rat Liver Microsomes, and their Relationship to the Liver Necrosis Produced by Carbon Tetrachloride. *Biochem. J.* **1968**, *106*, 155–160.
(8) Kabasakalian, P.; McGlotten, J. Polarographic Oxidation of Phenothiazine Tranquilizers. *Anal. Chem.* **1959**, *31*, 431–433.
(9) Kauffmann, J.-M.; Patriarche, G. J.; Vire, J.-C. Unusual Electrochemical Behaviour of a New Phenothiazine. *Analyst* **1985**, *110*, 349–350.
(10) Zimova, N.; Nemec, I.; Ehlova, M.; Waisser, K. The Effect of the Structure of the Substituent in Position Ten on the Voltammetric Behaviour of Phenothiazine Derivatives. *Collect. Czech. Chem. Commun.* **1990**, *55*, 63–71.
(11) Portions of this work have been presented previously: (a) McCowan, J. R.; Yu, M. J.; Um, S. L.; Bertsch, B.; Towner, R. D.; Phebus, L. A.; Clemens, J. A.; Horng, J. S.; Ho, P. P. K. In Vitro Inhibitors of Lipid Peroxidation and Oxidative LDL Modification. 200th National American Chemical Society Meeting in Washington D.C., August 1990, MEDI 129. (b) Horng, J. S.; Um, S. L.; Towner, R. D.; Ho, P. P. K. Effects of Probucol on the Oxidation and Receptor-Mediated Degradation of LDL and Ac-LDL. *FASEB J.* **1990**, *4*, A1155.
(12) Kadaba, M.; Massie, S. P. Ring Derivatives of Phenothiazine. IV. Further Studies on the Thionation Reaction, and the Synthesis of Phenothiazinols. *J. Org. Chem.* **1959**, *24*, 986–987.
(13) Nodiff, E.; Hausman, M. J. A Simple Preparation for Some Hydroxyphenothiazines. *J. Org. Chem.* **1966**, *31*, 625–626.
(14) Solubility was examined in 0.9% aqueous sodium chloride.
(15) See for example: Baldessarini, R. J. Drugs and the Treatment of Psychiatric Disorders. In *The Pharmacological Basis of Therapeutics*, 7th ed.; Gilman, A. G., Goodman, L. S., Rall, T. W., Murad, F., Eds.; Macmillan Publishing Co.: New York, 1985; pp 387–445.

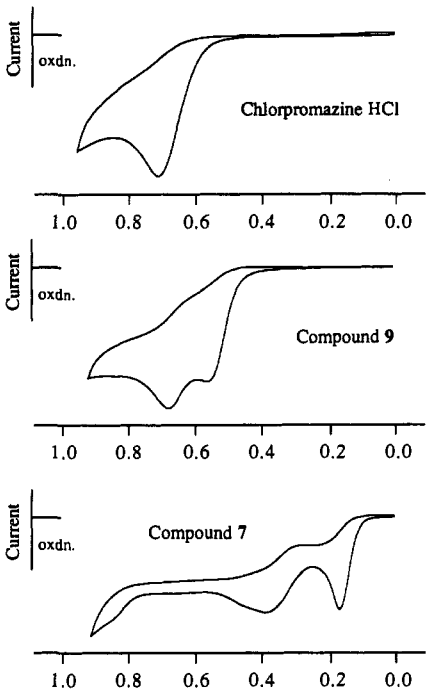


Figure 1. Cyclic voltammogram of chlorpromazine hydrochloride and compounds **7** and **9**. Measurements were conducted using a carbon disk electrode against a Ag/AgCl reference electrode in pH 7.4 phosphate buffered saline at 37 °C. The initial potential was 0.0 V vs the Ag/AgCl reference with a scan rate of 10 mV/s in a positive direction. At approximately 950 mV, the scan was reversed. A new electrode surface was used for each compound.

tained in four steps from 2-hydroxyphenothiazine, and compound **24** was most conveniently prepared by a one-carbon extension of commercially available 2-acetylphenothiazine followed by functional group modification in a fashion analogous to compound **23** (Scheme III).

Results

The phenothiazines were tested as in vitro inhibitors of iron-dependent peroxidation of rabbit brain vesicular membrane lipids (Table I). Several of these derivatives exhibited potent inhibitory activity in this assay with MIC's under 1 μM.[16] The oxidation potentials for representative compounds were determined by cyclic voltammetry in pH 7.4 phosphate buffered saline using a carbon paste disk electrode[17] against a Ag/AgCl reference electrode (Figure 1). Under our voltammetric conditions, all of the examined phenothiazines exhibited irreversible behavior and consistent with expectations, the N-10 unsubstituted analogue **7** had a lower oxidation potential than both the methylated derivative **9** and chlorpromazine hydrochloride.[18] This difference in redox potential was

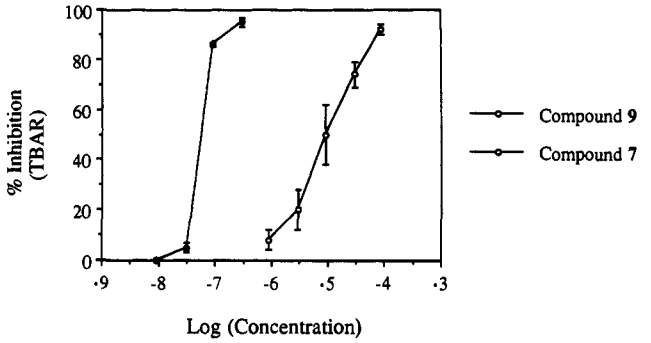


Figure 2. Effect of compounds **7** and **9** on iron-dependent peroxidation of rabbit brain vesicular membrane lipids in vitro. The test agent dissolved in DMSO (5 μL) was added to the supernatant fraction (0.5 mL) prepared by low-speed centrifugation of homogenized rabbit brain and then incubated with ADP (220 μM) and $FeCl_3$ (2 μM) at 37 °C for 1 h. The formation of lipid peroxide decomposition products were measured by the TBAR method. Each point represents the mean ± SEM (n = 3–6).

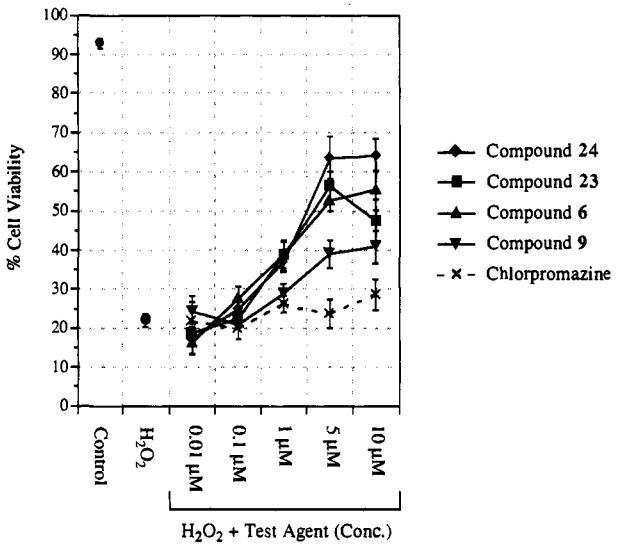


Figure 3. Effect of chlorpromazine and compounds **6**, **9**, **23**, and **24** on in vitro rat hippocampal neuronal cell survival following a 15-min exposure to 50 μM H_2O_2. Oxidant induced toxicity studies with mature (10–14 days in vitro) primary cultures were conducted as follows: The conditioned media was removed, and the cultures were rinsed with a Hepes-buffered saline (HBS) solution and then exposed to 50 μM H_2O_2 in HBS for 15 min at 37 °C. The test compounds were added concurrently with H_2O_2 during the 15-min exposure. After 15 min, the test solutions were removed, the cultures were rinsed three times with HBS, and the conditioned media was returned to the cultures. Viability was assessed the next day with the vital stains fluorescein diacetate and propidium iodide. Each compound was tested over the indicated concentration range at n = 3 with cultures prepared on different days. The two control values represent cultures that were incubated in the absence and presence of H_2O_2 without the test agent. Each point represents the mean ± SE.

clearly manifested in their ability to block iron-dependent lipid peroxidation in vitro (Figure 2). On the other hand, the inhibitory activity of this series in vitro appears to be insensitive to many of the structural variations examined such as the nature of the amine group (e.g. compounds **7** and **23**), the chain length separating the amine substituent from the phenothiazine nucleus (e.g. compounds **11** and **12**) and the nature of the linking group (e.g. compounds **23** and **24**). Even the parent phenothiazine nucleus, compound **2**, exhibited good inhibitory activity. Although adding an electron-withdrawing substituent at C-1 de-

(16) The concentration-response curve for lipid peroxidation inhibition is extremely steep. Consequently, we report the inhibitory activity as an MIC (minimum tested concentration of inhibitor that gave ≥50% inhibition).

(17) (a) Engstrom, R. C.; Johnson, K. W.; DesJarlais, S. Characterization of Electrode Heterogeneity with Electrogenerated Chemiluminescence. *Anal. Chem.* **1987**, *59*, 670–673 and references cited therein. (b) Adams, R. N. Carbon Paste Electrodes. *Anal. Chem.* **1958**, *30*, 1576.

(18) The electrochemical behavior of chlorpromazine hydrochloride using a ruthenium wire electrode in aqueous acid was recently reported: Biryol, I.; Dermis, S. Electrochemical Analysis of Some Phenothiazine Derivatives-I. Chlorpromazine HCl. *J. Pharm. Biomed. Analysis* **1988**, *6*, 725–735.

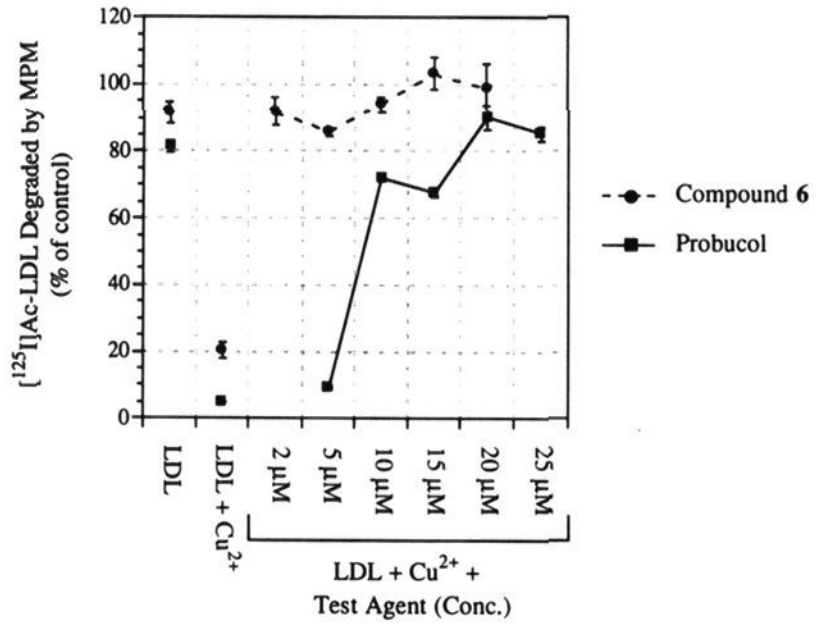


Figure 4. Effect of probucol and compound **6** on Cu^{2+}-catalyzed oxidative modification of native unlabeled LDL as measured by the competitive scavenger receptor mediated degradation of [^{125}I]Ac-LDL by MPM cells. Unlabeled LDL at 200 μg/mL was incubated with Cu^{2+} (5 μM) in the presence and absence of test agent at 37 °C for 20 h, and the degree of oxidation (final LDL concentration = 50 μg/mL) was determined by measuring its ability to inhibit MPM mediated [^{125}I]Ac-LDL degradation. The control degradation rates with [^{125}I]Ac-LDL (1.3 μg/mL) were 130.2 ± 4.4 ng/10^6 cells/21 h (n = 6) for probucol and 131.3 ± 5.9 ng/10^6 cells/19 h (n = 6) for compound **6**. Each point (mean ± SE, n = 3) represents the total amount of [^{125}I]Ac-LDL degraded minus the amount degraded under cell-free conditions. The differences between all of the drug treated groups and the corresponding untreated control are significant using Student's t test ($p < 0.001$).

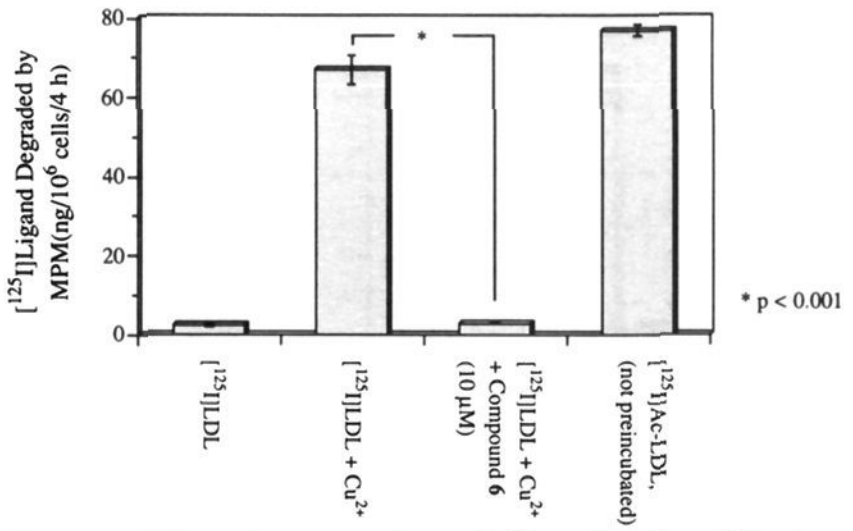


Figure 5. Effect of compound **6** on Cu^{2+}-catalyzed modifications of [^{125}I]LDL as measured by direct scavenger receptor mediated degradation by MPM cells. [^{125}I]LDL (100 μg/mL) and Cu^{2+} (5 μM) were incubated in the presence or absence of compound **6** (10 μM) in HBSS-Tris HCl (pH 8.3) at 37 °C for 20 h. At the end of the incubation period, the [^{125}I]LDL was immediately diluted to 10 μg/mL with DMEM and incubated with freshly isolated MPM (4 h, 37 °C). The final concentration of [^{125}I]LDL was 3.33 μg/mL. Each point (mean ± SE, n = 3) represents the total amount of [^{125}I]LDL degraded minus the amount degraded under cell-free conditions. Significance between the [^{125}I]LDL + Cu^{2+} groups in the presence and absence of compound **6** was determined using Student's t test ($p < 0.001$).

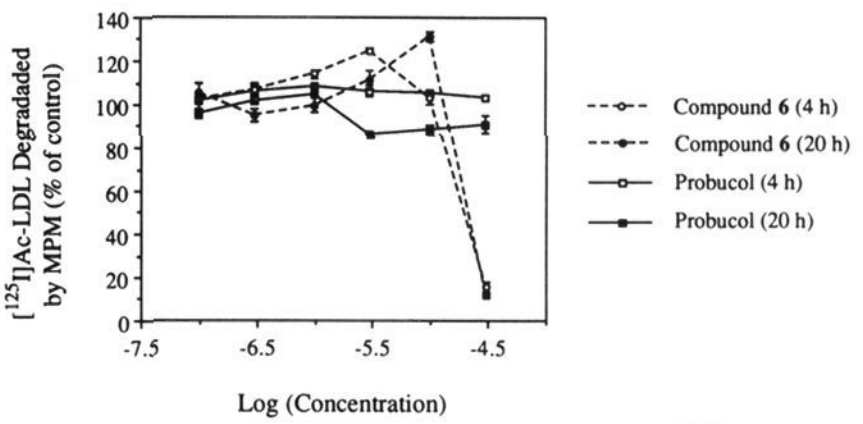


Figure 6. Effect of probucol and compound **6** [^{125}I]Ac-LDL degradation by MPM cells. Open and closed symbols represent values determined after a 4- and 24-h incubation period, respectively. [^{125}I]Ac-LDL (1.3 μg/mL) was incubated with freshly isolated MPM in the presence and absence of test agent at 37 °C. The control degradation rates were 66.8 ± 1.0 ng/10^6 cells and 91.1 ± 2.2 ng/10^6 cells for the 4- and 20-h incubation periods, respectively. Each point (mean ± SE, n = 3) represents the total amount of [^{125}I]Ac-LDL degraded minus the amount degraded under cell-free conditions.

creased potency, this reduction was less striking than adding an N-10 methyl group. Thus, the most important determinant of in vitro lipid peroxidation inhibitory activity within this structural class appears to be the absence of substitution at N-10 of the phenothiazine ring.

Chlorpromazine and compounds **6**, **9**, **23**, and **24** were evaluated as cytoprotective agents using primary cultures of neuronal cells exposed to 50 μM H_2O_2 for 15 min.[19] At test agent concentrations of 5 μM or greater, compounds **6**, **23**, and **24** increased cell survival as assessed by vital dye staining 18 h later (Figure 3). On the other hand, the N-10 methyl derivative, **9**, was less effective while chlorpromazine was totally ineffective over the concentration range of 0.01–10 μM. Although multiple mechanisms may contribute toward H_2O_2-induced toxicity, these results are in the rank order of potency predicted by the compound's antioxidant capacity as measured electrochemically and by their ability to inhibit iron-dependent lipid peroxidation in vitro.

Since oxidative modification of LDL has been proposed as an important early step in the atherosclerotic process,[4] the activity of compound **6** in a model of oxidatively modified LDL uptake and degradation by macrophage cells was investigated.[20] Probucol (10 μM), an hypolipidemic antioxidant previously documented to inhibit oxidative modification of LDL[21] and to reduce the progression of atherosclerosis in Watanabe heritable hyperlipidemic rabbits,[22,23] blocked Cu^{2+}-catalyzed oxidation of native unlabeled LDL as measured by competitive scavenger receptor mediated degradation of ^{125}I-labeled acetyl-LDL ([^{125}I]Ac-LDL) by mouse peritoneal macrophage (MPM)

(19) The time-course for neuronal cell death and the morphological changes associated with H_2O_2 exposure under these assay conditions were presented at the American Society for Neurochemistry Meeting in Charleston, SC March 10–15, 1991. Saunders, R. D., Keith, P. T.; Luttman, C. A. Role of Oxidants in the Delayed Cell Death of Neurons In Vitro.

(20) Um, S. L.; Horng, J. S. Scavenger Receptor-Mediated Degradation of [^{125}I]-Ac-LDL by Macrophages: A Simplified Method Using Cell Suspensions. *FASEB J.* **1990**, *4*, A1153.

(21) Parthasaraty, S.; Young, S. G.; Witzum, J. L.; Pittman, R. C. Steinberg, D. Probucol Inhibits Oxidative Modification of Low Density Lipoprotein. *Proc. Natl. Acad. Sci. U.S.A.* **1986**, *77*, 641–644.

(22) Kita, T.; Nagano, Y.; Yokode, M.; Ishii, K.; Kume, N.; Ooshima, A.; Yoshida, H.; Kawai, C. Probucol Prevents the Progression of Atherosclerosis in Watanabe Heritable Hyperlipidemic Rabbit, an Animal Model for Familial Hypercholesterolemia. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *84*, 5928–5931.

(23) Carew, T. E.; Schwenke, D. C.; Steinberg, D. Antiatherogenic Effect of Probucol Unrelated to its Hypocholesterolemic Effect: Evidence that Antioxidants in Vivo Can Selectively Inhibit Low Density Lipoprotein Degradation in Macrophage-rich Fatty Streaks and Slow the Progression of Atherosclerosis in the Watanabe Heritable Hyperlipidemic Rabbit. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *84*, 7725–7729.

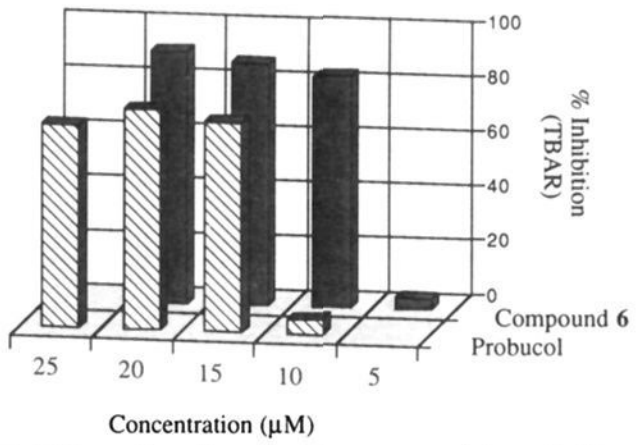


Figure 7. Effect of probucol and compound **6** on Cu^{2+}-catalyzed LDL oxidation as measured by TBAR formation. HBSS-Tris buffer (pH 8.5, 1.0 mL) containing LDL (200 μg/mL) and Cu^{2+} (5 μM) in the presence/absence of test agent (dissolved in EtOH, final concentration of EtOH was 1%) were incubated for 24 h at 37 °C and the TBAR content determined. Each bar represents the mean for two determinations.

cells in vitro (Figure 4). At concentrations above 10 μM good protection against Cu^{2+}-induced oxidative modification was observed with this agent. At a concentration of 5 μM, the protective effect was weak but still statistically significant. Compound **6**, however, was completely protective at a concentration of 2 μM and thus appears to be more potent than probucol in this model. Compound **6** (10 μM) similarly blocked Cu^{2+}-catalyzed oxidation of ^{125}I-labeled LDL and its subsequent scavenger receptor mediated recognition and degradation (Figure 5). In addition, compound **6** alone had no statistically significant effect on the degradation of [^{125}I]Ac-LDL at concentrations up to 10 μM (Figure 6), suggesting that the inhibitory activity of this agent is most likely related to protection of the lipoprotein rather than disruption of MPM scavenger receptor function. Consistent with these observations, both compound **6** and probucol reduced Cu^{2+}-catalyzed oxidation of native LDL as measured by thiobarbituric acid reactive (TBAR) product formation (Figure 7).[24]

Discussion

Lipid peroxidation is a radical chain reaction that occurs under pathophysiological conditions to produce a complex mixture of products and may ultimately lead to loss of membrane structure and function. Chlorpromazine can accumulate in the lipid bilayer of biological membranes[25,26] and reportedly inhibits lipid peroxidation in rat brain and liver[27] as well as reduces oxidative injury to cardiac phospholipid by membrane stabilization.[28] Consequently, we hypothesized that modifications of the chlorpromazine structure that results in increased antioxidant activity while approximately maintaining molecular lipophilicity may furnish agents with enhanced cytoprotective properties. By simple structural modifications of the phenothiazine chlorpromazine, potent in vitro inhibitors of iron-dependent lipid peroxidation were identified.[29]

Electrochemically, the described N-10 unsubstituted phenothiazines exhibit low oxidation potentials, and this property is manifest functionally by an enhanced ability to inhibit peroxidation of rabbit brain vesicular membrane lipids. Since oxygen-derived free radicals are implicated in a number of disease states, lipid peroxidation inhibitors may find utility in a variety of therapeutic areas. For example, antioxidants may be useful in treating atherosclerosis by slowing the conversion of native LDL to a more atherogenic modified form.[4] Modified forms of LDL recognized and degraded by the scavenger receptors of MPM in vitro include oxidatively modified LDL and acetyl-LDL (Ac-LDL).[30] Since native LDL is not recognized and degraded by the scavenger receptor, it cannot competitively block degradation of [^{125}I]Ac-LDL by MPM. However, Cu^{2+}-catalyzed oxidation converts native LDL to a form that is recognized and can compete with [^{125}I]-Ac-LDL for scavenger receptor mediated uptake and degradation. A representative from this series, compound **6**, appears to inhibit oxidative modification of radiolabeled and unlabeled LDL in vitro as assessed by MPM scavenger receptor mediated degradation. This activity is limited to the oxidative modification step since scavenger receptor mediated degradation of [^{125}I]Ac-LDL is unaffected by the test agent at concentrations as high as 10 μM.

A recent study reported that chlorpromazine and trifluoperazine were as effective as probucol in inhibiting Cu^{2+}-catalyzed LDL oxidation and its subsequent recognition and catabolism by MPM.[31] Although the mechanism of protection in this system is presently not well defined, Breugnot and co-workers suggested that lipophilic agents such as probucol and phenothiazines, in addition to antioxidant activity, may alter phospholipid physical properties and thereby render the lipoprotein more resistant to oxidative attack.[31] Amphiphiles such as β-blockers have also been reported to reduce iron-dependent sarcolemmal lipid peroxidation in vitro possibly by lipid membrane interactions rather than direct free-radical scavenging.[32] In addition, lidocaine, an antiarrhythmic and local anesthetic, has been reported to reduce coronary sinus levels of conjugated dienes (a potential marker of lipid peroxidation) during reperfusion of postischemic myocardium, possibly by membrane stabilization.[33] Al-

(24) The TBAR assay was run using the same lot of Cu^{2+}-catalyzed oxidatively modified LDL that was employed in the MPM degradation studies. For a discussion on limitations of the TBAR method, see: Janero, D. R. Malondialdehyde and Thiobarbituric Acid-Reactivity as Diagnostic Indices of Lipid Peroxidation and Peroxidative Tissue Injury. *Free Rad. Biol. Med.* **1990**, *9*, 515–540.

(25) Seeman, P.; Lee, T. Enhanced Binding of Chlorpromazine to Cholesterol-Depleted Synaptosome Fractions. *Can. J. Physiol. Pharmacol.* **1974**, *52*, 522–525.

(26) Kanaho, Y.; Sato, T.; Fujii, T. The Affinity of Various Phenothiazine Drugs for Membranes of Intact Human Erythrocytes and Their Membrane-Transforming Activity. *Mol. Pharmacol.* **1981**, *20*, 704–708.

(27) Hoshi, K.; Yoshida, K.; Yonaha, M.; Fujina, S. Effects of Phenobarbital, Amitriptyline, Chlorpromazine or Perphenazine on Lipid Peroxidation in Rat Liver and Brain. *Res. Commun. Psychol. Psychiatry* **1990**, *15*, 129–140.

(28) Janero, D. R.; Burghardt, B. Prevention of Oxidative Injury to Cardiac Phospholipid by Membrane-Active "Stabilizing Agents". *Res. Commun. Chem. Pathol. Pharmacol.* **1989**, *63*, 163–173.

(29) The calculated log *P* values for chlorpromazine, promethazine and compound **6** free base are 5.36, 4.33, and 4.26, respectively.

(30) Goldstein, J. L.; Ho, J. K.; Basu, S. K.; Brown, M. S. Binding Site on Macrophages that Mediate Uptake and Degradation of Acetylated Low Density Lipoprotein, Producing Massive Cholesterol Deposition. *Proc. Natl. Acad. Sci. U.S.A.* **1979**, *76*, 333–337.

(31) Breugnot, C.; Maziere, C.; Salmon, S.; Auclair, M. Santus, R.; Morliere, P. Lenaers, A.; Maziere, J. C. Phenothiazines Inhibit Copper and Endothelial Cell-Induced Peroxidation of Low Density Lipoprotein. *Biochem. Pharmacol.* **1990**, *40*, 1975–1980.

(32) Mak, J. T.; Weglicki, W. B. Protection by Beta-Blocking-Agents Against Free Radical-Mediated Sarcolemmal Lipid-Peroxidation. *Circ. Res.* **1988**, *63*, 262–266.

(33) Lesnefsky, E. J.; Van Benthuysen, K. M.; McMurtry, I. F.; Shikes, R. H.; Johnston Jr., R. B.; Horwitz, L. D. Lidocaine Reduces Canine Infarct Size and Decreases Release of a Lipid Peroxidation Product. *J. Cardiovasc. Pharmacol.* **1989**, *13*, 895–901.

though many classes of anesthetics may elicit anesthetic activity through nonspecific membrane interactions,[34] lipid membrane perturbations by certain lipophilic agents are highly structure dependent despite similar octanol/water partition coefficients (e.g. anesthetic steroids).[35] Consequently, the possibility remains that the described phenothiazines may exert their protective actions, in part, through structure-dependent lipid interactions in addition to antioxidant activity. Delineating the relative importance of these two potential mechanisms for the described series, however, will require additional investigation.

The N-10 unsubstituted analogues were more efficacious than the corresponding N-10 methyl congener in protecting primary cultures of rat hippocampal neurons against hydrogen peroxide induced toxicity in vitro. Since the latter compound exhibited a higher oxidation potential and was less effective in blocking iron-dependent lipid peroxidation, the mechanism by which these phenothiazines exert their cytoprotective action may relate to their free-radical scavenging capacity. However, multiple independent mechanisms such as lipid membrane stabilization as discussed above or enzyme inhibition (e.g. calmodulin[36]) in addition to antioxidant activity may contribute significantly to cellular protection.[37] For example, phenothiazines reportedly help to maintain membrane integrity of cultured neonatal heart cells as measured by antimyosin antibody and propidium iodide uptake[38] and to preserve normal ventricular compliance in isolated buffer-perfused rabbit hearts following global ischemia,[39] protective actions believed to be calmodulin dependent. Calmodulin inhibition has also been suggested to underly the salutary effects observed in vivo with chlorpromazine administration in canine models of regional myocardial ischemia.[40,41] However, calmodulin inhibition, per se, may not be primarily responsible for the observed cytoprotection of cultured neuronal cells under our assay conditions since chlorpromazine was ineffective over the tested concentration range. Finally, in addition to chlorpromazine which reportedly inhibits cyclooxygenase and soybean lipoxidase activity in vitro,[42] a large number of substituted phenothiazines have been claimed in the patent literature to inhibit leukotriene biosynthesis.[43] Thus, although the described agents were designed and evaluated from an antioxidant perspective, other mechanisms may, at least in part, account for the observed cytoprotection.

Experimental Section

Methods. Melting points were determined on a Thomas-Hoover capillary melting point apparatus and are uncorrected. The ^1H NMR spectra were recorded with a GE QE-300 spectrometer and were consistent with the assigned structure. Mass spectra were recorded with a CEC 21-110 (EI) or with a Varian-MAT 731 (FD) spectrometer. Microanalytical data were provided by the Physical Chemistry Department of Lilly Research Laboratories. Where analyses are indicated only by symbols of the elements, results obtained were within ±0.4% of the theoretical values.

10*H*-Phenothiazin-2-ol (4). A mixture of 2-methoxyphenothiazine (20 g, 87 mmol) and pyridine hydrochloride (50 g, 430 mmol) was heated to 170 °C for 12 h under N_2. The reaction mixture was cooled and dissolved in EtOAc with heating. The solution was washed with H_2O, dried over $MgSO_4$, and concentrated under reduced pressure to furnish 19.6 g of the desired crude product. Recrystallization from toluene provided 13 g (69%) of 2-hydroxyphenothiazine, mp 202–210 °C (lit. mp 207–209 °C,[44] 205–206 °C[45]).

2-(10*H*-Phenothiazin-2-yloxy)ethanamine Hydrochloride (5). NaH (0.44 g, 9.3 mmol, 50% oil dispersion) was washed with hexanes under N_2 and suspended in DMF (10 mL). A solution of 2-hydroxyphenothiazine (2 g, 9.3 mmol) in DMF (10 mL) was added dropwise, and after evolution of H_2 ceased, neat chloroacetonitrile (0.7 g, 9.3 mmol) was added. The reaction mixture was stirred at room temperature for 30 min and then partitioned between EtOAc and H_2O. The organic layer was washed twice with brine, dried over $MgSO_4$, and concentrated under reduced pressure. Recrystallization from EtOAc provided 0.75 g of 2-(10*H*-phenothiazin-2-yloxy)acetonitrile (32%), mp 177–178 °C. Anal. ($C_{14}H_{10}N_2OS$) C, H, N.

The product obtained above (0.5 g, 2 mmol) was dissolved in THF (15 mL) and added to a suspension of LAH (0.22 g, 5.8 mmol) in THF (15 mL) under N_2. The reaction mixture was stirred at reflux for 1 h, cooled, and sequentially quenched with H_2O, 15% NaOH, and H_2O. Filtration and solvent removal furnished the desired free base. The hydrochloride salt was formed and precipitated from EtOH to give 0.35 g of **5** (60%), mp 307–308 °C. Anal. ($C_{14}H_{15}N_2OSCl$) C, H, N.

2-(10*H*-Phenothiazin-2-yloxy)-*N*,*N*-dimethylethanamine Methanesulfonate (6). NaH (5.8 g, 120 mmol, 50% oil dispersion) was washed with hexanes under N_2, suspended in DMF (100 mL), and cooled to 0 °C. A solution of 2-hydroxyphenothiazine (13 g, 60 mmol) in DMF (50 mL) was added, and the reaction mixture was stirred at room temperature for 1 h. Upon

(34) Seeman, P. The Membrane Actions of Anesthetics and Tranquilizers. *Pharmacol. Rev.* **1972**, *24*, 583–655.

(35) See for example Makriyannis, A.; DiMeglio, C. M.; Fesik, S. W. Anesthetic Steroid Mobility in Model Membrane Preparations as Examined by High-Resolution ^1H and ^2H NMR Spectroscopy. *J. Med. Chem.* **1991**, *34*, 1700–1703 and references cited therein.

(36) For a general discussion on calmodulin and biological membrane binding by phenothiazines, see Bondy, B. Binding of Phenothiazines to Non-Neuronal Constituents. In *Phenothiazines and 1,4-Benzothiazines, Bioactive Molecules*; Gupta, R. R., Ed.; Elsevier: New York, 1988, Vol. 4, pp 791–808.

(37) (a) Schaffer, S. W.; Roy, R. S.; McCord, J. M. Possible Role for Calmodulin in Calcium-Paradox-Induced Heart Failure, *Eur. Heart J.* **1983**, *4(H)*, 81–87. (b) Chien, K. R.; Reeves, J. P.; Buja, L. M.; Bonte, F.; Parkey, R. W.; Willerson, J. T. Phospholipid Alterations in Canine Ischemic Myocardium. *Circ. Res.* **1981**, *48*, 711–719. (c) Anaise, D.; Bachvaroff, R.; Sato, K.; Waltzer, W.; Oster, Z.; Atkins, H., Pollack, W.; Rapoport, F. T. A Membrane Stabilization Approach to Long-Term Renal Preservation. *Transplant Proc.* **1985**, *17*, 14457–1460. (d) Okumura, K.; Ogawa, K.; Satake, T. Effects of Trifluoperazine and Chlorpromazine on Calcium Repleted Injury in Isolated Ventricular Strips. *Basic Res. Cardiol.* **1985**, *80*, 556–563.

(38) Scott, J. A.; Khaw, B.-A.; Fallon, J. T.; Locke, E.; Rabito, C. A.; Peto, C. A.; Homcy, C. J. The Effect of Phenothiazines Upon Maintenance of Membrane Integrity in the Cultured Myocardial Cell. *J. Mol. Cell. Cardiol.* **1986**, *18*, 1243–1254.

(39) Thomas, G. E.; Levitsky, S.; Feinberg, H. Chlorpromazine Inhibits Loss of Contractile Function, Compliance and ATP in Ischemic Rabbit Heart. *J. Mol. Cell Cardiol.* **1983**, *15*, 621–628.

(40) Soufer, R.; Heiman, D.; Zohgi, S.; Melnick, D.; Zaret, B. L. Chlorpromazine Limits Extent of Myocardial Reperfusion Injury in Dogs. *Circulation* **1987**, *76* (*Suppl. II*), IV–229.

(41) Slezak, J.; Tribulova, N.; Gabauer, I.; Ziegelhoffer, Holec, V.; Slezak, J., Jr. Diminution of "Reperfusion Injury" in Reperfused Ischemic Myocardium by Phenothiazines. A Quantitative Morphological Study. *Gen. Physiol. Biophys.* **1987**, *6*, 491–512.

(42) Duniec, Z.; Robak, J.; Gryglewski, R. Antioxidant Properties of Some Chemicals vs Their Influence on Cyclooxygenase and Lipoxidase Activities. *Biochem. Pharmacol.* **1983**, *32*, 2283–2286.

(43) See, for example: Fortin, R.; Lau, C. K.; Guindon, Y.; Rokach, J.; Yoakim, C. Phenothiazine and Derivatives and Analogs and Use as Leukotriene Biosynthesis Inhibitors. U.S. Patent 4,666,907, 1987.

(44) Bourquin, J.-P.; Schwarb, G.; Gamboni, G.; Fischer, R.; Ruesch, L.; Guldimann, S.; Theus, V.; Schenker, E.; Renz, J. 23. Syntheses in the phenothiazine family. III. New phenothiazine derivatives. *Helv. Chem. Acta* **1959**, *42*, 259–281.

(45) Buu-Hoi, N. P.; Saint-Ruf, G.; Lobert, B. Pyridine hydrochloride and hydrobromide, convenient and selective N-dealkylation agents. *Bull. Soc. Chem. Fr.* **1969**, *5*, 1768.

recooling to 0 °C, a solution of (dimethylamino)ethyl bromide hydrobromide (14 g, 60 mmol) in DMF (150 mL) was added. The resulting mixture was stirred at room temperature overnight, concentrated under reduced pressure, and then partitioned between EtOAc and H_2O. The separated organic layer was washed with H_2O and brine and dried over Na_2SO_4. After solvent removal in vacuo, chromatography (Prep 500, SiO_2, gradient elution: $CHCl_3$ to 10% MeOH/$CHCl_3$) provided 3.48 g of the free base as an oil that solidified upon standing. The mesylate salt was prepared and recrystallized from MeOH/i-PrOH to yield 2.4 g (9%) of **6**, mp 174–175 °C. Anal. ($C_{17}H_{22}N_2O_4S_2$) C, H, N.

2-(10*H*-Phenothiazin-2-yloxy)-*N*,*N*-dimethylethanamine Hydrochloride (7). The hydrochloride salt was similarly prepared from the free base in EtOH and recrystallized from H_2O, mp 112–113 °C. Anal. ($C_{16}H_{19}N_2OSCl·H_2O$) C, H, N.

2-(10*H*-Phenothiazin-2-yloxy)-*N*,*N*-dimethylpropanamine Oxalate (8). In a fashion analogous to compound **6**, 2-hydroxyphenothiazine (13 g, 60 mmol) was alkylated with (dimethylamino)propyl chloride hydrochloride (10 g, 63 mmol) and chromatographed (Prep 500, SiO_2, gradient elution: $CHCl_3$ to 20% MeOH/$CHCl_3$) to provide 9.6 g of the free base as an oil. Attempts to crystallize the corresponding hydrochloride salt were unsuccessful. The oxalate salt was prepared from 3.62 g of the above free base in warm EtOH to provide 3.1 g of **8**, mp 158–164 °C. Anal. ($C_{19}H_{22}N_2O_5S$) C, H, N.

2-[(10-Methyl-10*H*-phenothiazin-2-yl)oxy]-*N*,*N*-dimethylethanamine Hydrochloride (9). A solution of 2-(10*H*-phenothiazin-2-yloxy)-*N*,*N*-dimethylethanamine (1.5 g, 5.2 mmol) in THF (10 mL) was added dropwise at 0 °C to a THF (20 mL) suspension of KH (0.22 g, 5.5 mmol) that had been washed with hexanes. The reaction mixture was stirred until evolution of H_2 ceased, at which time CH_3I (0.78 g, 5.5 mmol) was added. After being stirred at room temperature for 1 h, the mixture was diluted with EtOAc, washed with brine, dried over Na_2SO_4 and $MgSO_4$, concentrated in vacuo, and column chromatographed (SiO_2, 1% MeOH/$CHCl_3$) to furnish 1.3 g of the desired product as an oil. The corresponding hydrochloride salt was prepared and recrystallized from EtOH to give 0.77 g of **9** (44%) mp 183–185 °C. Anal. ($C_{17}H_{21}N_2OSCl$) C, H, N.

2-(10*H*-Phenothiazin-3-yloxy)-*N*,*N*-dimethylethanamine Hydrochloride (11). NaH (1.3 g, 28 mmol, 50% oil dispersion) was washed with hexanes under N_2 and suspended in THF (20 mL). A solution of 3-hydroxyphenothiazine[13] (3.0 g, 14 mmol) in THF (20 mL) was added, and the reaction mixture was stirred at room temperature until evolution of H_2 ceased. Upon cooling to 0 °C, a solution of (dimethylamino)ethyl bromide hydrobromide (3.25 g, 14 mmol) in DMF (25 mL) was added. The resulting mixture was stirred at room temperature overnight, concentrated under reduced pressure, and then partitioned between EtOAc and brine. The separated organic layer was washed with H_2O and brine and dried over Na_2SO_4 and $MgSO_4$. The reaction product was combined with that obtained from an identical reaction starting with 7.1 g of 3-hydroxyphenothiazine. The combined crude products were chromatographed (Prep 500, SiO_2, gradient elution: $CHCl_3$ to 20% MeOH/$CHCl_3$) to provide the free base as a dark solid. The corresponding hydrochloride salt was prepared and recrystallized from i-PrOH/H_2O to furnish 1.79 g of **11** (13%) as a purple-gray solid, mp 196–197 °C. Anal. ($C_{16}H_{19}N_2OSCl$) C, H, N.

2-(10*H*-Phenothiazin-3-yloxy)-*N*,*N*-dimethylpropanamine Hydrochloride (12). In a fashion analogous to compound **6**, 3-hydroxyphenothiazine (8.3 g, 39 mmol) was alkylated with (dimethylamino)propyl chloride hydrochloride (6.1 g, 39 mmol) and chromatographed (Prep 500, SiO_2, gradient elution: $CHCl_3$ to 20% MeOH/$CHCl_3$) to provide 4.76 g of the desired free base. The corresponding hydrochloride salt was prepared and recrystallized twice from i-PrOH/MeOH to afford 2.9 g of **12** (22%), mp 215–217 °C. Anal. ($C_{17}H_{21}N_2OSCl$) C, H, N.

2-Methoxy-10*H*-phenothiazine-1-carboxylic Acid (13). A solution of 2-methoxyphenothiazine (13 g, 57 mmol) in Et_2O (400 mL) was stirred under N_2 in a flask equipped with a dry ice condenser. A solution of *n*-BuLi (89 mL, 140 mmol, 1.6 M in hexane) was added. After the mixture was stirred at reflux for 3 h, a steady stream of CO_2 gas was blown over the surface of the reaction mixture for 40 min, and the resulting mixture was extracted with 1 N NaOH (250 mL). The separated aqueous phase was washed once with Et_2O, and the organic layer was back-extracted once with 1 N NaOH. The combined aqueous layers were acidified with 5 N HCl (150 mL) and extracted twice with EtOAc. The combined organic layers were washed with brine, dried over Na_2SO_4, and concentrated under reduced pressure to yield 12.63 g of the crude acid. Recrystallization from EtOAc afforded 6.34 g of **13**, mp 155–157 °C.

***N*-[2-(Dimethylamino)ethyl]-2-methoxy-10*H*-phenothiazine-1-carboxamide Hydrochloride (14).** A mixture of 2-methoxy-10*H*-phenothiazine-1-carboxylic acid (1.00 g, 3.6 mmol) and 1,1′-carbonyldiimidazole (0.59 g, 3.6 mmol) in THF (20 mL) was stirred at room temperature for 30 min. *N*,*N*-Dimethylethylenediamine (0.32 g, 3.6 mmol) was then added. After being stirred at reflux for 21 h, the reaction mixture was cooled, diluted with EtOAc, washed with H_2O, 1 N NaOH, and brine, dried over Na_2SO_4, and concentrated under reduced pressure. The hydrochloride was formed in H_2O/MeOH, lyophilized, and then crystallized from Et_2O/MeOH in the refrigerator to furnish 864 mg (62%) of **14** as a yellow-green solid, mp 184–186 °C. Anal. ($C_{18}H_{22}N_3O_2SCl$) C, H, N.

2-[2-(Dimethylamino)ethoxy]-*N*,*N*-dimethyl-10*H*-phenothiazine-1-carboxamide Hydrochloride (17). A mixture of 2-methoxy-10*H*-phenothiazine-1-carboxylic acid (8.5 g, 31 mmol) and 1,1′-carbonyldiimidazole (5.1 g, 31 mmol) in THF (100 mL) was stirred at room temperature for 1 h. A solution of Me_2NH (15 mL, 40% aqueous solution) was added, and the reaction mixture was stirred for 24 h. After solvent removal in vacuo, the residue was taken up in EtOAc, washed with 1 N HCl, H_2O, 1 N NaOH, and brine, dried over $MgSO_4$, and concentrated under reduced pressure to a brown oil. Column chromatography (SiO_2, 50% EtOAc/hexanes) and crystallization furnished 7.63 g (82%) of **15**, mp 147–150 °C. Anal. ($C_{16}H_{16}N_2O_2S$) C, H, N.

A mixture of the above amide (3.00 g, 10 mmol) and pyridine hydrochloride (10 g) was heated to 165 °C under N_2 for 24 h with occasional shaking. The reaction mixture was cooled and partitioned between H_2O and EtOAc. The organic layer was washed twice with 5% $Na_2S_2O_4$ and brine, dried over Na_2SO_4, and concentrated under reduced pressure. The black tarry residue was dissolved in EtOAc and filtered through SiO_2 with EtOAc. The filtrate was concentrated in vacuo and crystallized from CH_2Cl_2/hexanes to provide 1.30 g (45%) of **16** as a light-green solid, mp 165–167 °C. Anal. ($C_{15}H_{14}N_2O_2S$) C, H, N.

The above hydroxyphenothiazine (6.26 g, 22 mmol) was alkylated with (dimethylamino)ethyl bromide hydrobromide (5.1 g, 22 mmol) in a fashion analogous to compound **6** to provide 3.4 g of the crude product after chromatography (Prep 500, gradient elution: $CHCl_3$ to 15% MeOH/$CHCl_3$); 2 g of the free base was converted to its hydrochloride salt and crystallized from i-PrOH to yield 1.49 g of **17**, mp 236–240 °C. Recrystallization from MeOH/i-PrOH furnished 1.14 g of analytically pure material, mp 242–243 °C. Anal. ($C_{19}H_{24}N_3O_2SCl$) C, H, N.

4-[2-(10*H*-Phenothiazin-2-yloxy)ethyl]-1-piperazineethanol Dihydrochloride (23). A solution of potassium *tert*-butoxide (11.27 g, 100 mmol) in THF (150 mL) was added dropwise to a solution of 2-hydroxyphenothiazine (21.5 g, 100 mmol) in THF (250 mL) under N_2 at 0 °C. After the mixture was stirred for 45 min, ethyl bromoacetate (16.74 g, 100 mmol) was added, and the resulting mixture was stirred at room temperature for 4 h. The reaction was quenched with saturated aqueous NH_4Cl and extracted with EtOAc. The organic layer was washed with H_2O, dried over Na_2SO_4, and concentrated under reduced pressure to give 32.70 g of ethyl 2-(10*H*-phenothiazin-2-yloxy)acetate as a dark solid that was used without further purification.

A solution of the above crude ester (30.5 g, ~100 mmol) in THF (200 mL) was added dropwise to a suspension of LAH (4.25 g, 110 mmol) in THF (300 mL) at 0 °C under N_2. After being stirred for 3 h at room temperature, the reaction mixture was sequentially quenched with H_2O (5 mL), 15% NaOH (5 mL), and H_2O (25 mL). Filtration and solvent removal provided 25.13 g (96%) of **21** as a brown solid, mp 142–146 °C.

In a fashion analogous to **24**, the above alcohol was converted to compound **23** as a white plates, mp 212–215 °C after recrystallization from MeOH. Anal. ($C_{20}H_{24}N_3O_2SCl_2$) C, H, N.

4-[3-(10*H*-Phenothiazin-2-yl)propyl]-1-piperazineethanol Dihydrochloride (24). NaH (15 g, 370 mmol, 60% oil dispersion)

was washed with hexanes under N_2 and suspended in THF (300 mL). Solid 2-acetylphenothiazine (30.0 g, 124 mmol), MeOH (0.4 mL), and dimethyl carbonate (16.8 g, 186 mmol) were added. After being stirred for 2.5 h, the reaction mixture was quenched with H_2O, concentrated under reduced pressure, diluted with EtOAc, washed with H_2O and brine, dried over Na_2SO_4, and concentrated under reduced pressure to furnish 35.26 g (95%) of **19** as a brick-red solid that was sufficiently pure for the next reaction. An analytical sample was obtained by recrystallization from EtOAc, mp 153–156 °C. Anal. ($C_{16}H_{13}NO_3S$) C, H, N.

$NaBH_4$ (2.22 g, 60 mmol) was added to a stirred solution of the above product (18 g, 60 mmol) in THF (750 mL) and MeOH (750 mL) at 0 °C under N_2. After being stirred for 2.5 h, the reaction mixture was carefully quenched with H_2O, concentrated under reduced pressure and partitioned between EtOAc and 1 N HCl. The organic layer was washed with H_2O and brine, dried over $MgSO_4$, and concentrated under reduced pressure. The resulting oil was triturated with CH_2Cl_2 and filtered to provide 4.9 g of desired material. The filtrate was column chromatographed (Prep 500, gradient elution: 30% EtOAc/hexanes to 50% EtOAc/hexanes) to provide an additional 6.32 g of desired alcohol (total yield 62%). An analytical sample was obtained by recrystallization from MeOH, mp 149–150 °C. Anal. ($C_{16}H_{15}NO_3S$) C, H, N.

The above alcohol (11.22 g) was dissolved in Ac_2O (25 mL) and pyridine (50 mL). After standing at room temperature for 15 h, the volatiles were removed under reduced pressure. After being concentrated in vacuo twice from toluene, the residue was mixed with CH_2Cl_2 and filtered. The filtrate was chromatographed (Prep 500, gradient elution: 10% EtOAc/hexanes to 20% EtOAc/hexanes) to provide 9.34 g (73%) of the desired acetate as a tan solid. An analytical sample was obtained by recrystallization from EtOAc/hexanes, mp 114–115 °C. Anal. ($C_{18}H_{17}NO_4S$) C, H, N.

Solid potassium *tert*-butoxide (3 g, 27 mmol) was added to a solution of the above acetate (9.14 g, 27 mmol) in THF (200 mL) at 0 °C under N_2. After being stirred for 1 h, the reaction mixture was poured into EtOAc and cracked ice. The separated aqueous layer was acidified with 1 N HCl (27 mL) and extracted with EtOAc. The combined organic layers were washed with brine, dried over $MgSO_4$, and concentrated under reduced pressure to leave 7.9 g of a yellow solid. Trituration with CH_2Cl_2 and filtration provided 5.06 g (66%) of **20** as a yellow solid. An analytical sample was obtained by recrystallization from EtOAc, mp 205–207 °C. Anal. ($C_{16}H_{13}NO_2S$) C, H, N.

A solution of the above ester (5.06 g, 18 mmol) in THF (100 mL) was added to a suspension of LAH (3.4 g, 89 mmol) in THF (150 mL) at room temperature under N_2. After being stirred at reflux for 2 days, the reaction mixture was cooled to 0 °C and quenched sequentially with H_2O (3.4 mL), 15% NaOH (3.4 mL), and H_2O (14 mL). The aluminum salts were removed by filtration and suspended in a mixture of EtOAc and 1 N HCl. The organic layer was separated, dried over $MgSO_4$, and combined with the filtrate. Solvent removal under reduced pressure gave 4.6 g (99%) of **22**. An analytical sample was obtained by recrystallization from MeOH, mp 143–144 °C. Anal. ($C_{15}H_{15}NOS$) C, H, N.

A solution of triphenylphosphine (6.4 g, 24.5 mmol) in THF (50 mL) was added to a solution of the above alcohol (4.5 g, 17.5 mmol), CBr_4 (8.7 g, 26.3 mmol), and THF (100 mL) at room temperature under N_2. The reaction mixture was stirred at reflux for 15 min, cooled, and concentrated under reduced pressure. Column chromatography (SiO_2, 10% EtOAc/hexanes) provided 2.38 g of the desired bromide. An analytical sample was obtained by recrystallization from EtOAc/hexanes, mp 148–149 °C. Anal. ($C_{15}H_{14}NBrS$) C, H, N.

A mixture of the above bromide (3.2 g, 10 mmol), 1-(2-hydroxyethyl)piperazine (6.5 g, 50 mmol), and EtOH (35 mL) was heated to 80 °C for 3 h. The solvent was removed under reduced pressure, and the resulting oil was partitioned between EtOAc and H_2O. The separated aqueous phase was extracted once with EtOAc. The combined organic layers were washed with H_2O and brine, dried over $MgSO_4$, and concentrated under reduced pressure. Chromatography (Prep 500, gradient elution: $CHCl_3$ to 10% MeOH/$CHCl_3$) furnished 3.46 g (93%) of an oil that crystallized upon standing. The dihydrochloride salt was formed and recrystallized from EtOH/H_2O to afford 3.14 g (71%) of **24** as a tan solid, mp 250–252 °C. Anal. ($C_{21}H_{29}N_3OSCl_2$) C, H, N.

Cyclic Voltammetry. Cyclic voltammetry was carried out using a carbon paste disk electrode packed with carbon paste made from 3 parts 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 potential was 0.0 V vs the Ag/AgCl reference with a scan rate of 10 mV/s in a positive direction. At about 950 mV, the scan direction was reversed.

Lipid Peroxidation. Peroxidation of rabbit brain vesicular membrane lipids was assayed as previously described.[46] Frozen mature stripped rabbit brain (Pell Freez) was thawed, minced, homogenized in 25 mM Tris-HCl containing 0.15 M KCl, pH 7.5 (10% w/v), and centrifuged at low speed (10000*g* for 15 min). 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 of 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 1-butanol (1 mL), and centrifuged at 3000*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.

Cytoprotection. Primary cultures of rat hippocampal neurons were prepared by modifications of the methods described by Banker and Cowan[47] and Novelli et al.[48] Brains from 18-day gestation rat fetuses were removed and the hippocampi dissected under sterile conditions. The dissected hippocampi were placed in 29 mL of "solution 1" (124 mM NaCl, 5.37 mM KCl, 1 mM NaH_2PO_4, 14.5 mM glucose, 25 mM Hepes, 1.2 mM $MgSO_4$, 3 mg/mL BSA, 20 drops/L phenol red, and 1 mL/L penicillin-streptomycin 5000 units/mL, pH 7.4); 0.75% trypsin (1 mL) was added, and the tissue was passed several times through a sterile pipette. After incubation at 37 °C for 15 min, the tissue was centrifuged for 5 min, the supernatant was removed, and solution 1 (15 mL) containing DNase I (26.6 μg/mL) and soybean trypsin inhibitor (STI) (166.4 μg/mL) were added to stop trypsinization. The mixture was centrifuged for 3 min, the supernatant was decanted, and solution 1 (5 mL) containing DNase/STI was added. The tissue was triturated through a Pasteur pipette 15 times and then through a fire-polished pipette 10 times. Solution 1 (12.5 mL) containing $MgSO_4$ (2.5 mM) and $CaCl_2$ (0.1 mM) was added, and the tissue was vortexed and then allowed to stand for 5 min. The solution was filtered through sterilized Spectra Mesh (209μ from Fisher) and then centrifuged for 5 min. Cells were assessed for viability using trypan blue and then plated on Gibco 6-well plates coated with poly-D-lysine at a density of 30000 cells/cm^2. Plating media consisted of Dulbecco's Modified Eagles Media containing 10% fetal calf serum, glucose (4.0 g/L), KCl (1.46 g/L), and penicillin-streptomycin (5000 U/mL, 1 mL), pH 7.4. Cells were maintained in an incubator at 37 °C in a mixture of 5% CO_2/95% room air.

H_2O_2 toxicity studies were performed on mature (10–14 days in vitro) cultures as follows: The conditioned media was removed and the cultures rinsed with a Hepes-buffered saline (HBS) solution (136 mM NaCl, 5.4 mM KCl, 0.62 mM $MgSO_4$, 1.0 mM $CaCl_2$, 1.1 mM KH_2PO_4, 1.1 mM Na_2HPO_4, 25 mM Hepes, and 5000 units/mL penicillin-streptomycin, pH 7.4). The cultures were exposed to 50 μM H_2O_2 in HBS for 15 min at 37 °C. The test compounds were added concurrently with H_2O_2 during the 15-min exposure. After 15 min, the test solutions were removed, the cultures were rinsed three times with HBS, and the condi-

(46) 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*, 107–111.

(47) Banker, G. A.; Cowan, W. M. Rat Hippocampal Neurons in Dispersed Cell Culture. *Brain Res.* **1977**, *126*, 397–425.

(48) Novelli, A.; Reilly, J. A.; Lysko, P. G.; Henneberry, R. C. Glutamate Becomes Neurotoxic via the N-Methyl-D-Aspartate Receptor when Intracellular Energy Levels are Reduced. *Brain Res.* **1988**, *451*, 205–212.

tioned media was returned to the cultures. Viability was assessed the next day with the vital stains fluorescein diacetate and propidium iodide.[49] At least 100 cells in randomly chosen fields were counted per culture to determine percent viability. Each compound was tested at $n = 3$ with cultures prepared on different days.

Modified LDL Studies. Noninduced resident peritoneal macrophage (MPM) cells were isolated from BALB/c mice as described by Schultz and co-workers,[50] resuspended in Dulbecco's modified Eagle's medium (DMEM) at a concentration of (2–5) $\times 10^6$ cells/mL, and stored in ice until needed. The macrophage cell counts were estimated using a hemacytometer.

Degradation of [^{125}I]lipoprotein (BTI, MA) was measured according to the modified methods of Henriksen et al.[51] and Drevon et al.[52] Aliquots of freshly prepared MPM were incubated with [^{125}I]lipoprotein (1–5 μg/mL) in triplicate at 37 °C for 4 h in DMEM (1.5 mL) containing 10% FCS and gentamicin (50 μg/mL) in polystyrene tubes.[20] The degradation was terminated by adding bovine serum albumin (final concentration, 10 mg/mL) and TCA (final concentration, 10%). After centrifugation, the supernatant was filtered through a 0.45-μm Millipore (type HAWP) filter. $AgNO_3$ (final concentration, 1.5%) was added to precipitate free iodide, and the radioactivity of the supernatant was measured in a Micromedic System gamma spectrometer. Specific degradation was defined as the total radioactivity in the medium minus the radioactivity not inhibited by 200 μg/mL Ac-LDL or Cu^{2+}-oxidized LDL. The specific degradation of [^{125}I]Ac-LDL was typically about 95% at an [^{125}I]Ac-LDL concentration of 1.5 μg/mL. The level of significance between groups was calculated using Student's t test.

Cu^{2+}-catalyzed oxidative LDL modification was performed in the following manner: radiolabeled or unlabeled LDL (100–300 μg/mL) in the presence or absence of test agent was incubated with Cu^{2+} (5 μM) in HBSS-Tris HCl medium (pH 8.3) in polystyrene tubes at 37 °C for 20 h. After incubation, aliquots of unlabeled samples were used immediately for an estimate of LDL oxidation by measuring its ability to competitively inhibit scavenger receptor mediated degradation of [^{125}I]Ac-LDL by MPM. Aliquots of ^{125}I-radiolabeled LDL, on the other hand, were first diluted and the degree of oxidation assessed by direct MPM scavenger receptor mediated degradation as described above.

Acknowledgment. We thank Kathleen Cloutier and Michael Pozgai for their expert assistance in conducting the literature searches to support this project.

Registry No. 1, 50-53-3; 2, 92-84-2; 3, 1771-18-2; 4, 24316-35-6; 5, 137966-36-0; 6, 137966-38-2; 7, 137966-39-3; 8, 138008-49-8; 9, 137966-40-6; 10, 1927-44-2; 11, 137966-41-7; 12, 137966-42-8; 13, 137966-43-9; 14, 137966-44-0; 15, 137966-45-1; 16, 137966-46-2; 17, 137966-47-3; 18, 6631-94-3; 19, 137966-48-4; 19 alcohol derivative, 137966-49-5; 19 acetate derivative, 137966-50-8; 20, 137966-51-9; 21, 137966-52-0; 22, 137966-53-1; 22 bromide derivative, 137966-54-2; 23, 137966-55-3; 24, 137966-56-4; ethyl 2-(10*H*-phenothiazin-2-yloxy)acetate, 137966-57-5.

(49) Jones, K. H.; Senft, J. A. An Improved Method to Determine Cell Viability by Simultaneous Staining with Fluorescein Diacetate-Propidium Iodide. *J. Histochem. Cytochem.* **1985**, *33*, 77–79.
(50) Schulz, R. M.; Papamatheakis, J. D.; Luetzeler, J.; Ruiz, P.; Chirigos, M. A. Macrophage Involvement in the Protective Effect of Pyran Copolymer against the Madison Lung Carcinoma (M109). *Cancer Res.* **1977**, *37*, 358–364.
(51) Henriksen, T.; Mahoney, E. M.; Steinberg, D. Enhanced Macrophage Degradation of Low Density Lipoprotein Previously Incubated with Cultured Endothelial Cells: Recognition by Receptors for Acetylated Low Density Lipoproteins. *Proc Natl. Acad. Sci. U.S.A.* **1981**, *78*, 6499–6503.
(52) Drevon, C. A.; Attie, A. D.; Pangburn, S. H.; Steinberg, D. Metabolism of Homologous and Heterologous Lipoproteins by Cultured Rat and Human Skin Fibroblasts. *J. Lipid Res.* **1981**, *22*, 37–46.

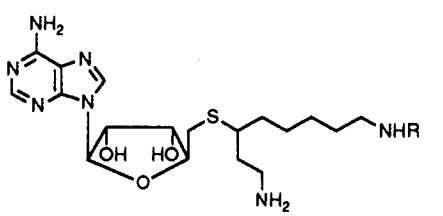
α-Methyl Polyamines: Metabolically Stable Spermidine and Spermine Mimics Capable of Supporting Growth in Cells Depleted of Polyamines

John R. Lakanen, James K. Coward,* and Anthony E. Pegg†

Departments of Chemistry and Medicinal Chemistry, University of Michigan, Ann Arbor, Michigan 48109-1055, and Department of Physiology, Pennsylvania State University, Hershey, Pennsylvania 17033. *Received August 5, 1991*

In order to assess the tolerance of the target enzyme spermine synthase for α-substituents on the aminopropyl moiety of the substrate spermidine, 1-methylspermidine (MeSpd, **2**) was synthesized. It was determined that MeSpd is a poor substrate for spermine synthase and is not a substrate for spermidine N^1-acetyltransferase, suggesting that α-methylated polyamines might be metabolically stable and therefore useful tools for studying polyamine effects in intact cells. On the basis of initial cellular results with **2**, 1-methylspermine (MeSpm, **3**) and 1,12-dimethylspermine (Me_2Spm, **4**) were also synthesized. When added to cells (L1210, SV-3T3, or HT29) depleted of both putrescine and spermidine by prior treatment with α-(difluoromethyl)ornithine (DFMO), these α-methylated polyamines were able to restore cell growth to that observed in the absence of DFMO. In accord with the enzyme data noted above, metabolic studies indicated a slow conversion of **2** to **3**, but no metabolism of 4 in these cells. It was concluded from these results that the α-methylated polyamines are able to substitute for the natural polyamines spermidine and spermine in critical biochemical processes which involve polyamines for continued cell growth. In accord with the hypothesis, preliminary data indicate that MeSpd and Me_2Spm are as effective as spermidine and spermine, respectively, in promoting the conversion of B-DNA to Z-DNA.

The polyamines putrescine, spermidine, and spermine are synthesized and degraded via a series of enzyme-catalyzed reactions which are now well-established[1] (Scheme I). We have described the synthesis[2,3] and biological activity[4,5] of two highly specific multisubstrate adduct inhibitors[6] of the aminopropyltransferases. These compounds, *S*-adenosyl-1,8-diamino-3-mercaptooctane (AdoDATO, **1a**) and *S*-adenosyl-1,12-diamino-3-mercapto-9-azadodecane (AdoDATAD, **1b**), are very potent inhibitors of spermidine synthase and spermine synthase, respectively. In the course of investigating the biological activity of these inhibitors, we observed that AdoDATAD was



1a: R = H
b: R = $(CH_2)_3NH_2$

† Pennsylvania State University.

0022-2623/92/1835-0724$03.00/0