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Antioxidant Activity of the Monoamine Oxidase B Inhibitor Lazabemide

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ABSTRACT. Free radical-induced damage to lipid and protein constituents of neuronal membranes contributes to the pathophysiology of neurodegenerative diseases, including Alzheimer's disease (AD). The development of an effective inhibitor of oxidative stress represents an important goal for the treatment of AD. In this study, the intrinsic antioxidant activity of lazabemide, a potent and reversible inhibitor of monoamine oxidase B (MAO-B), was tested in a membrane-based model of oxidative stress. Under physiologic-like conditions, lazabemide inhibited lipid peroxidation in a highly concentration-dependent manner. At low, pharmacologic levels of lazabemide (100.0 nM), there was a significant ($P < 0.001$) and catalytic reduction in lipid peroxide formation, as compared with control samples. The antioxidant activity of lazabemide was significantly more effective than that of either vitamin E or the MAO-B inhibitor, selegiline. The ability of lazabemide to inhibit oxidative damage is attributed to physico-chemical interactions with the membrane lipid bilayer, as determined by small angle x-ray diffraction methods. By partitioning into the membrane hydrocarbon core, lazabemide can inhibit the propagation of free radicals by electron-donating and resonance-stabilization mechanisms. These findings indicate that lazabemide is a potent and concentration-dependent inhibitor of membrane oxy-radical damage as a result of inhibiting membrane lipid peroxidation, independent of MAO-B interactions. BIOCHEM PHARMACOL **60**;5:709–716, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. lipid peroxidation; x-ray diffraction; membrane bilayer

Oxidative stress contributes significantly to mechanisms of irreversible cell injury associated with aging and age-related neurodegenerative disorders. Brain tissue is especially vulnerable to free radical injury, as it is high in lipid content but has a relatively weak antioxidant defense system [1]. Unsaturated fatty acids and oxidizable amino acids present in synaptic membranes are particularly susceptible to free radical damage. Increased membrane permeability caused by lipid peroxidation results in a disruption in transmembrane ion gradients and cellular metabolic processes, leading directly to a loss of membrane integrity and ultimately cell death [2, 3].

Several lines of evidence point to a role for free radical-mediated injury in the pathogenesis of AD§ [4]. An increased potential for lipid peroxidation has been demonstrated in AD brain homogenates when stimulated *in vitro* with Fe^{2+}-ascorbate or Fe^{2+}-H_2O_2, suggesting an increase in the amount of polyunsaturated fatty acids and/or decreased antioxidant levels [5–7]. In addition to an increase in vulnerability to lipid peroxidation, a significant elevation in TBARS, a product of oxidative damage, was observed in cortical brain regions associated with AD histopathologic alterations (e.g. hippocampus and pyriform cortex) [8]. In two other studies, an increase in TBARS was observed in various brain cortical regions, but not the cerebellum of AD patients when compared with control subjects [9]. Changes in membrane structure in an affected cortical region of the AD brain, as measured by x-ray diffraction approaches, are also consistent with increased levels of lipid peroxidation damage [10, 11]. In an earlier study, it was demonstrated that lipid peroxidation altered the intermolecular packing of phospholipid acyl chains, resulting in a marked reduction in membrane width and increased hydrocarbon core molecular volume [11]. An increase in oxidative stress with AD may be associated, in part, with an elevation in MAO-B activity and excessive deamination of dopamine and other amines [12]. An increase in MAO-B activity was correlated previously with plaque-associated astrocytes in AD brain tissue [12]. Thus, the development of effective inhibitors of MAO-B activity represents a logical approach to the treatment of this disease.

Based on the current evidence, new therapies developed for the treatment of AD will likely include powerful new

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§ *Abbreviations*: AD, Alzheimer's disease; TBARS, thiobarbituric acid-reactive substances; MAO, monoamine oxidase; DLPC, dilinoleoyl phosphatidylcholine; PBPC, porcine brain phosphatidylcholine; Aβ, amyloid β-peptide; and BHT, butylated hydroxytoluene.

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inhibitors of oxidative stress. In this study, the antioxidant activity of lazabemide, a potent and reversible inhibitor of MAO-B [12, 13], was tested in a well-defined membrane model of oxidative damage. The results of this study demonstrated that lazabemide had effective concentration-dependent antioxidant activity, independent of MAO-B interactions. The antioxidant activity was significantly ($P < 0.001$) greater than that of either the MAO-B inhibitor selegiline or vitamin E under identical experimental conditions. By intercalating into the membrane lipid bilayer, lazabemide can interfere with the efficient propagation of free radicals by various biochemical and biophysical mechanisms. The intrinsic antioxidant activity of lazabemide represents a new mechanism of action that has important implications for its use in the treatment of AD.

MATERIALS AND METHODS

Materials

DLPC and PBPC lipids were obtained from Avanti Polar Lipids, Inc. and stored at −80°. Lazabemide was provided by F. Hoffmann-LaRoche, Ltd.; selegiline and vitamin E were purchased from the Sigma Chemical Co. and stored at room temperature in a desiccator.

Lipid Peroxidation Analyses

For these experiments, DLPC multilamellar vesicles (500 μL, 1.0 mg/mL) were freshly prepared in buffer (0.5 mM HEPES, 154.0 mM NaCl, pH 7.3) in the absence and presence of freshly prepared lazabemide, vitamin E, or selegiline at various concentrations (10.0 nM through 10.0 μM). Multilamellar vesicles were prepared by the methods of Bangham *et al.* [14]. Briefly, lipids dissolved in chloroform (1.0 mg/mL) were dried down under a stream of nitrogen gas to a thin film in a test tube while vortexing. Residual solvent was removed by drying under a vacuum. Membrane vesicles were formed after adding buffer (0.5 mM HEPES, 154.0 mM NaCl, pH 7.3) and vortexing hard for 3 min at room temperature. Then the vesicles, prepared in the absence or presence of drug, were placed immediately in a shaking water bath at 37° [14]. After various incubation periods, 100-μL aliquots of the samples were removed, and the peroxidation reaction was terminated by the addition of 25 μL of 5.0 mM EDTA and 2 μL of 35.0 mM BHT. The extent of lipid peroxidation in the samples was measured by the CHOD-Iodide assay [15]. The concentration of triiodide (I_3^-) was measured spectrophotometrically based on the following reaction (L denotes a phospholipid molecule):

$$\mathrm{LOOH} + 2\mathrm{H}^+ + 2^- \rightarrow \mathrm{LOH} + \mathrm{H_2O} + \mathrm{I_2}$$

$$\mathrm{I_2} + \mathrm{I}^- \rightarrow \mathrm{I_3^-}$$

To the above aliquot, 1.0 mL of CHOD color reagent was added, and the sample was allowed to incubate in the absence of light for 4 hr. The absorbance of the solution was then measured at 365 nm ($\epsilon = 2.4 \times 10^4\ M^{-1}\ cm^{-1}$). Lipid peroxide formation was measured in triplicate, and values were expressed as means ± SD. The significance of differences between results from different experimental conditions was tested using the two-tailed Student's *t*-test.

Small Angle X-ray Diffraction Analysis of Membrane Samples

Porcine brain phospholipid membrane vesicles with a 0.6:1 cholesterol to phospholipid mole ratio were prepared in the presence and absence of lazabemide for small angle x-ray scattering analyses. The mole ratio selected was based on previous lipid analyses of neuronal plasma membrane preparations from human cortical tissue [10]. Lipids dissolved in chloroform (1.0 mg/mL) were dried down under a stream of nitrogen gas to a thin film in a test tube while vortexing. Residual solvent was removed by drying under a vacuum. The membrane vesicles (5.0 mg/mL) were freshly prepared in buffer (0.5 mM HEPES, 154.0 mM NaCl, pH 7.3) in the absence and presence of freshly prepared lazabemide at a mole ratio of 1:30 by vortexing [14]. Oriented membrane samples for x-ray diffraction were prepared by centrifugation in a Sorvall AH-629 swinging bucket ultracentrifuge rotor (Dupont Corp.) at 35,000 *g* for 90 min at 5° in Lucite sedimentation cells, each containing an aluminum foil substrate [16]. For these experiments, 250 μg phospholipid was used for each sample. Following centrifugation, the supernatants were removed, and each membrane pellet was mounted on a curved glass support and suspended overnight in a humidity chamber containing a saturated salt solution. Then the oriented membrane samples were placed in sealed brass canisters with thin aluminum foil windows in which temperature and relative humidity were controlled. The samples were aligned at near-grazing incidence with respect to a collimated x-ray beam. The radiation source was a monochromatic x-ray (CuK_α radiation, $\lambda = 1.54$ Å) from a Rigaku RU-200 high brilliance rotating anode x-ray generator (Rigaku USA). The diffraction data were collected on both a one-dimensional position-sensitive electronic detector (Innovative Technologies, Inc.) and a two-dimensional PhosphorImager plate. The sample-to-detector distance was 150 mm.

Each individual diffraction peak was background-corrected using a linear subtraction routine that averaged the noise. The lamellar intensity functions from the oriented membrane samples were corrected by a factor of $s = 2 \sin \theta/\lambda$, the Lorentz correction, in which λ is the wavelength of the x-ray radiation (1.54 Å) and θ is the Bragg angle equal to one-half of the angle between the incident beam and the scattered beam. A swelling analysis was used to assign unambiguous phases to the experimental structure factors [17].

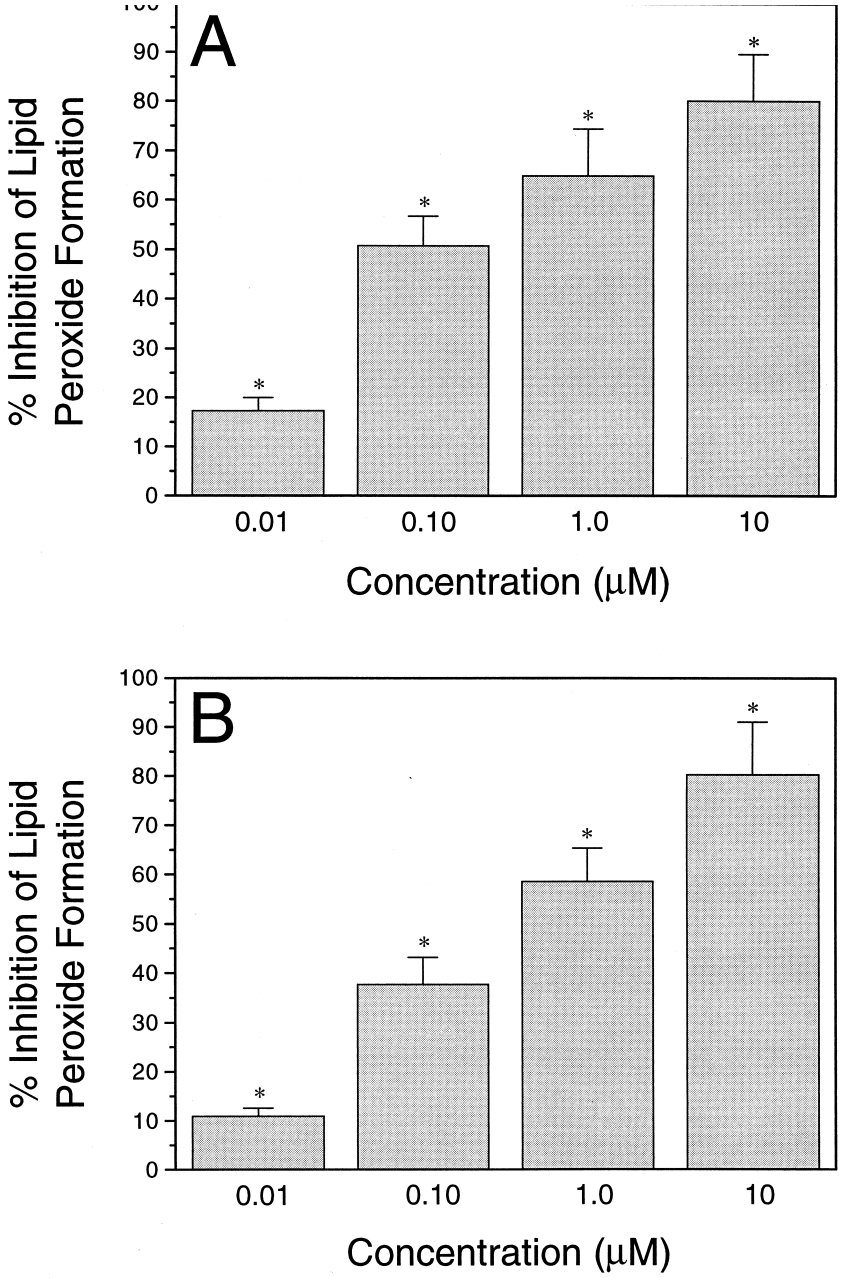

FIG. 1. Antioxidant effects of lazabemide in membrane vesicles enriched with polyunsaturated fatty acids as a function of drug concentration (10.0 nM through 10.0 μM) at 37° following 48-hr (A) and 72-hr (B) incubation periods. Values are expressed as means ± SD, N = 3 (*P < 0.001 vs control). The control levels of lipid peroxide formation were 640 μM and 1.4 mM following 48- and 72-hr incubation periods, respectively.

RESULTS

Effects of Lazabemide, Selegiline, and Vitamin E on Lipid Peroxidation

The concentration-dependent antioxidant effects of lazabemide, selegiline, and vitamin E were evaluated in membrane vesicles reconstituted from phospholipids enriched with the polyunsaturated fatty acid DLPC. The DLPC-enriched membrane vesicles were used to analyze the antioxidant activities of these agents, as this system has well-defined rates of lipid peroxidation that are highly reproducible under physiologic-like conditions of low metal ions. In addition, linoleic acid is an abundant polyunsaturated fatty acid found in neuronal synaptic membranes. In these experiments, the lipid peroxidation reaction (autoxidation) occurred gradually at 37° in the absence of exogenous chemical initiators. These experiments were designed to test the intrinsic antioxidant activity of these compounds, independent of MAO-B interactions.

Following incubation periods of 48 and 72 hr, the control membrane vesicles had lipid peroxide levels of 640 μM and 1.4 mM, respectively. As demonstrated in Fig. 1, lazabemide inhibited free radical-induced lipid peroxidation in a highly concentration-dependent manner at 37° over the 72-hr incubation period (10.0 nM through 10.0 μM). The antioxidant activity of lazabemide was highly catalytic: the addition of 1.0 μM lazabemide inhibited lipid peroxide

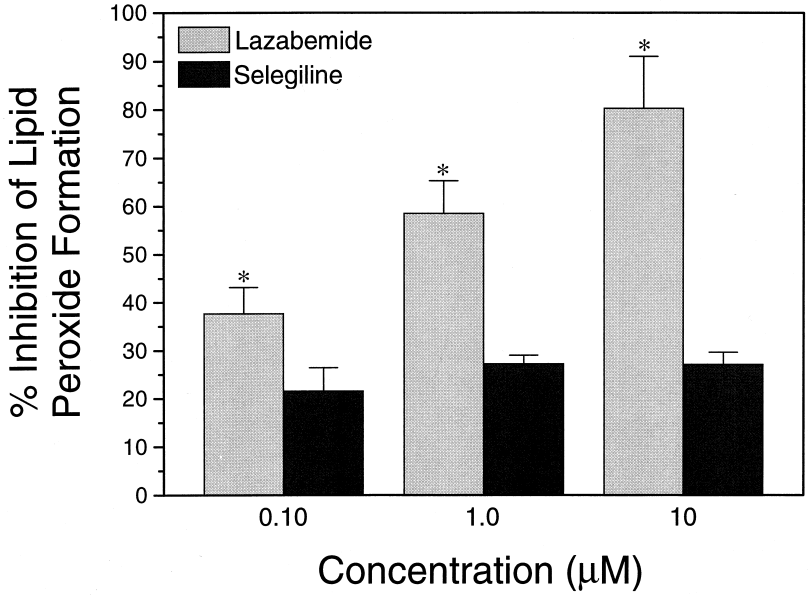

FIG. 2. Comparative concentration-dependent effects (100.0 nM through 10.0 μM) of lazabemide and selegiline on lipid peroxide formation in membrane vesicles at 37°. Values are expressed as means ± SD, N = 3 (*$P < 0.001$ vs selegiline). The control level of lipid peroxide formation was 1.4 mM after a 72-hr incubation period (37°).

formation by > 0.8 mM after 72 hr. In parallel experiments, the MAO-B inhibitor selegiline did not exhibit concentration-dependent antioxidant activity over the same range of concentrations; total inhibition of lipid peroxide formation by selegiline did not exceed 30% (Fig. 2). At a 1.0 μM level, the antioxidant activity of lazabemide was twice as effective ($P < 0.001$) as that of either vitamin E or selegiline under identical conditions (Fig. 3).

X-ray Diffraction Analysis of the Membrane Interactions of Lazabemide

To understand the physico-chemical basis for the antioxidant activity of lazabemide, the structure of membranes containing this amphiphilic molecule was evaluated by using small angle x-ray diffraction approaches. X-ray scattering from oriented brain membrane lipid bilayers yielded strong, reproducible diffraction orders at 20° (Fig. 4). The unit cell periodicity or *d*-space (the measured distance from the center of one membrane to the next, including surface hydration) for the control brain membrane bilayer sample was 56.5 ± 0.3 Å, while the intrabilayer headgroup separation was 44 Å. In the presence of lazabemide at a 1:30 drug:lipid mole ratio, the brain membrane bilayer *d*-space value was unchanged at 57.8 ± 0.3 Å. The addition of lazabemide to the brain membrane bilayer preparations did not alter the phospholipid headgroup separation.

One-dimensional electron density profiles (Å vs electrons/Å^3) generated from the phased x-ray diffraction data indicated a centrosymmetric membrane bilayer structure (Fig. 5). The two peaks of electron density on either side of the figure correspond to phospholipid headgroups, while the minimum of electron density at the center of the membrane is associated with terminal methylene segments. The effects of lazabemide on membrane structure were demonstrated by directly subtracting the electron density

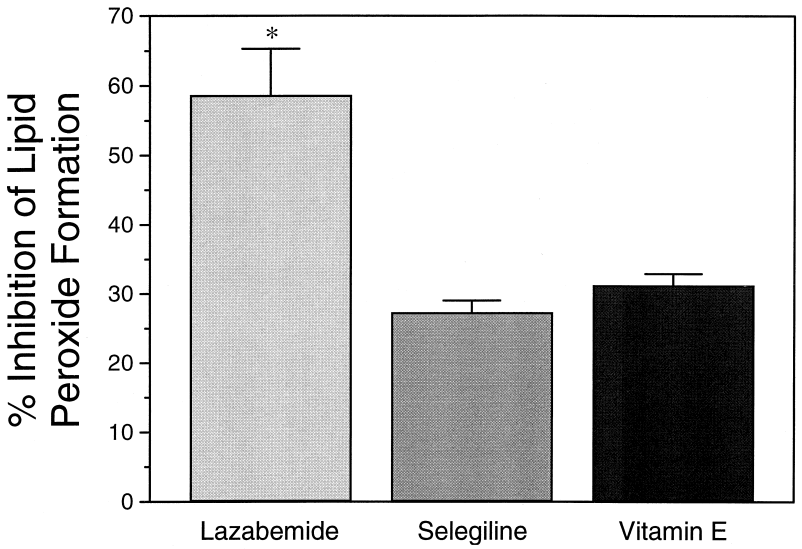

FIG. 3. Comparative effects of lazabemide, selegiline, and vitamin E on lipid peroxide formation in membrane vesicles at 37° at a concentration of 1.0 μM. Values are expressed as means ± SD, N = 3 (*$P < 0.001$ vs selegiline and vitamin E). The control level of lipid peroxide formation was 1.4 mM after a 72-hr incubation period (37°).

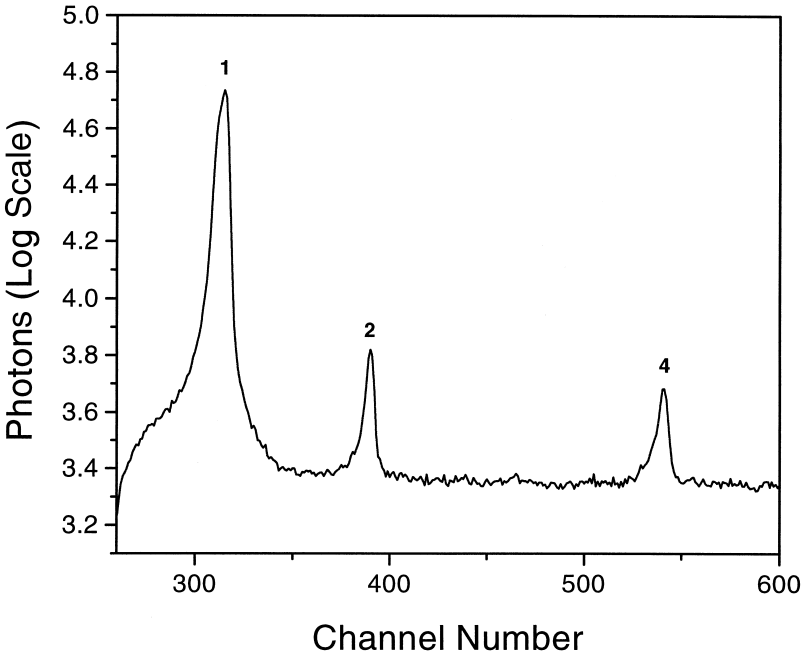

FIG. 4. Representative x-ray diffraction pattern for brain lipid bilayers prepared in the presence of lazabemide at a 1:30 drug to phospholipid mole ratio. Strong first-, second-, and fourth-order diffraction peaks were obtained as labeled.

profiles, as shown in Fig. 5. In the presence of lazabemide, there was a discrete increase in electron density 5–15 Å from the center of the membrane. As a further control, the membrane interactions of lazabemide were also examined in DLPC vesicles prepared in an identical manner as for the brain lipid membranes. Similar membrane interactions were observed for lazabemide in this synthetic lipid system (data not shown).

The extent of the increase in membrane electron density in this region of the membrane (Fig. 6) is consistent with a molecular model that places the long axis of lazabemide in an orientation that lies parallel to the phospholipid acyl chains: the drug extends from the upper acyl chains and glycerol backbone to the phosphate moiety of the phospholipid headgroups (Fig. 6). The increase in electron density may be attributed, in part, to the electron-dense chlorine atom that serves as a strong x-ray scattering source. At this location in the membrane, lazabemide would have both electrostatic and hydrophobic interactions with neighboring phospholipid molecules. The charged, tertiary amine of lazabemide can bind to anionic oxygen associated with phosphate moieties in the phospholipid headgroups, while the hydrophobic ring structure intercalates into the upper hydrocarbon core (Fig. 6).

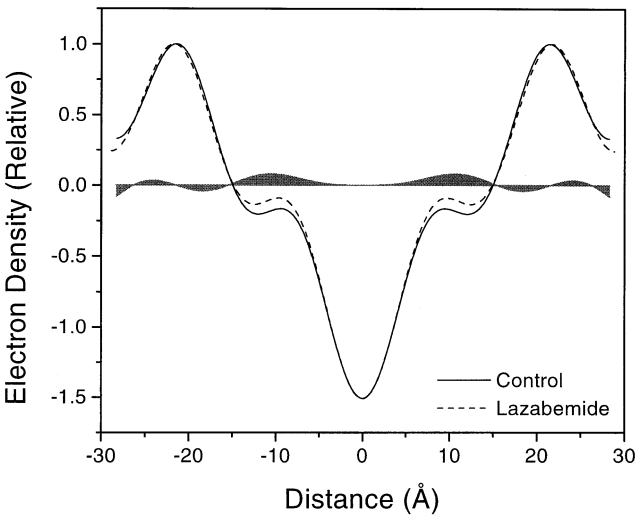

FIG. 5. One-dimensional electron density profiles (Å vs electrons/Å^3) for brain membrane lipid bilayers prepared in the absence and presence of lazabemide at a 1:30 mole ratio. The shaded areas in the center of the figures indicate positive differences between the control (solid line) and drug-containing (dashed line) samples due to the presence of the drug.

DISCUSSION

The key result of the present study was the finding that the MAO-B inhibitor lazabemide has potent membrane lipid antioxidant activity. The antioxidant effects of lazabemide are attributed to its chemical structure and direct physicochemical interactions with the membrane lipid bilayer, as evidenced by changes in membrane electron density profiles. The location determined for lazabemide would provide a biophysical basis for the ability of this compound to interfere with the intermolecular propagation of unstable free radicals through the membrane hydrocarbon core. Specifically, results of the x-ray diffraction analyses support a model that places the amphiphilic lazabemide molecule in the phospholipid bilayer hydrocarbon core, near the glycerol backbone. Such a model would predict that the time-averaged location of the ring structure is in close proximity to polyunsaturated fatty acids associated with phospholipid acyl chains, an important target for peroxidation. As previously shown for other amphiphilic agents, the location of lazabemide at this region of the membrane is

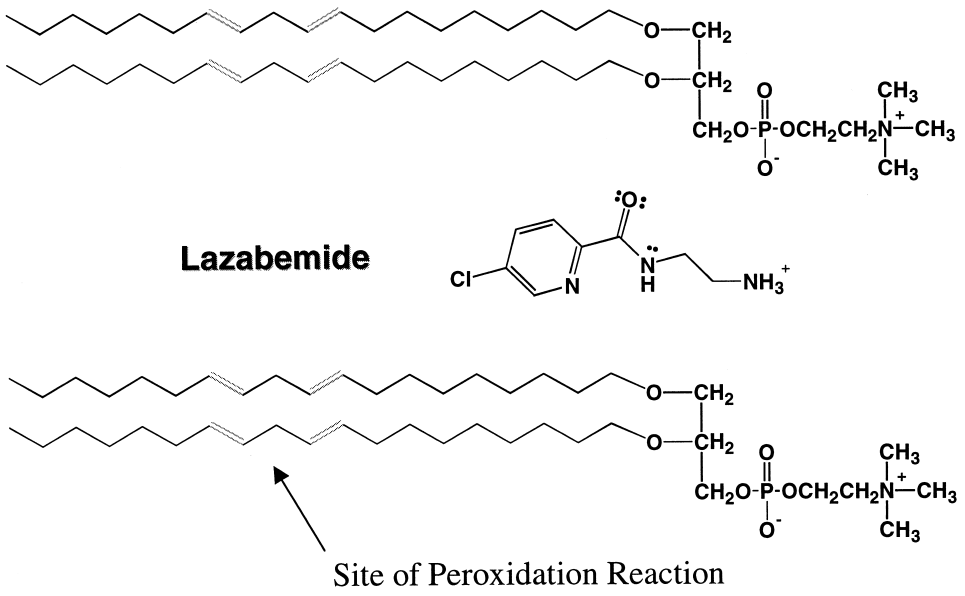

FIG. 6. Schematic molecular model for the interactions of lazabemide with neighboring phospholipid molecules based on the results of the x-ray diffraction data. At this location in the membrane, lazabemide can inhibit the propagation of free radicals by electron-donating and resonance-stabilization mechanisms.

highly favorable for interfering with free radical damage by breaking the lipid peroxidation chain reaction in the membrane [18–20].

In addition to a favorable location for lazabemide in the membrane, this molecule has specific chemical features that would serve to inhibit free radical-mediated cell damage. Free radicals are small molecules, typically oxygen-derived, that have lost an electron, producing a highly unstable state. Antioxidants can work by donating electrons to these free radical species, thereby neutralizing their deleterious effects on the membrane. Consistent with this mechanism, it is clear that lazabemide has a chemical structure that would contribute to an antioxidant effect. Specifically, the proton associated with the amino function can be abstracted and donated to free radical molecules. The remaining unpaired free electron can then be stabilized within resonance structures of lazabemide, as opposed to being lost to neighboring phospholipid molecules and further contributing to the peroxidation reaction (Fig. 7).

Free radical-induced damage to neuronal membrane lipids has been implicated in the pathogenesis of neurodegenerative diseases, including AD [4]. AD brain homogenates have demonstrated an increased potential for lipid peroxidation when stimulated *in vitro* with Fe^{2+}-ascorbate or Fe^{2+}-H_2O_2, suggesting an increase in the amount of polyunsaturated fatty acids and/or decreased antioxidant levels [5–7]. Evidence in support of an association between oxidative stress and the development of AD also comes from clinical studies that indicated a benefit for the antioxidant vitamin E among patients with moderately severe impairment [21]. The beneficial activity of vitamin E was similar to that reported for the MAO-B inhibitor selegiline [21]. Vitamin E (α-tocopherol) is a lipid-soluble antioxidant that attenuates the peroxidation chain reaction by trapping free radicals in the membrane. Although the changes were modest, vitamin E did slow the clinical deterioration among patients who had already been diagnosed with this disease. These clinical findings have stimulated larger

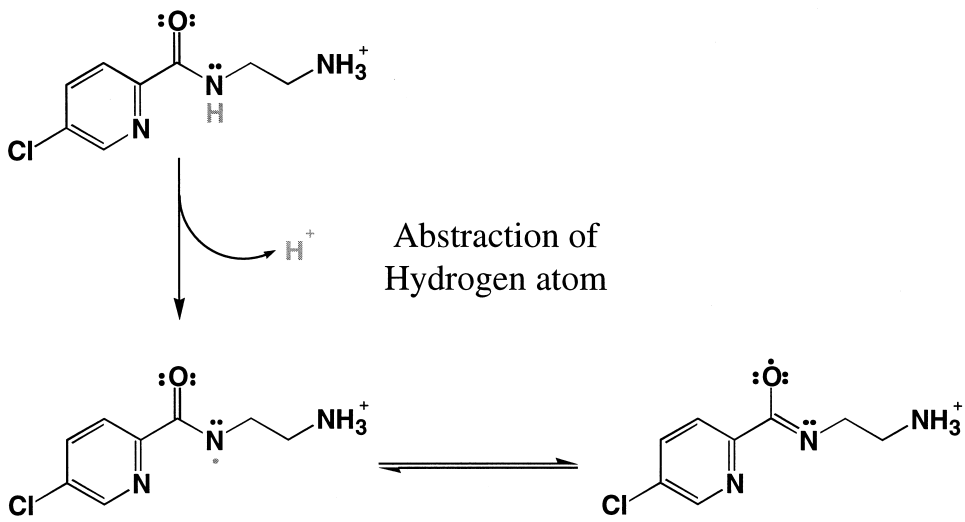

FIG. 7. Chemical antioxidant mechanism for lazabemide. The covalent chemical structure of lazabemide includes a hydrogen atom that can be donated to unstable free radicals. The remaining unpaired free electron can be stabilized in the calculated resonance structures for lazabemide.

trials to test the benefit of antioxidants in the treatment of AD and are supported by *in vitro* studies showing that vitamin E reduces neurotoxicity following Aβ treatment [22, 23]. It has been proposed that antioxidant effects also underlie the beneficial effects of estradiol in the treatment of AD among postmenopausal women [24, 25] and further support a role for oxidative stress in AD pathophysiology.

An important source of free radical-induced neuronal damage may be an elevation in the levels of Aβ, the key protein constituent of neuritic plaques, a hallmark pathologic lesion in AD brain tissue [26]. An increase in the production and abnormal accumulation of Aβ in the brain has been implicated in the etiology of AD [26–28]. Several studies have shown that Aβ forms free radicals following solubilization that result in lipid peroxidation [29]. It has been proposed that radical formation by Aβ may be due to the oxidation of methionine to the corresponding sulfoxide and may proceed through a radical intermediate [30]. The effects of Aβ on lipid peroxidation appear to be biphasic and sequence-specific [31].

In conclusion, the findings from the present study demonstrated that the MAO-B inhibitor lazabemide had potent and concentration-dependent lipid antioxidant activity, independent of MAO-B interactions. The antioxidant activity of lazabemide was significantly ($P < 0.001$) more potent than that of either selegiline or vitamin E alone, under identical conditions. The antioxidant activity of the amphipathic lazabemide molecule is attributed to strong physicochemical interactions with the membrane lipid bilayer, as determined by small angle x-ray diffraction approaches. By partitioning into the membrane hydrocarbon core, lazabemide can effectively inhibit the propagation of free radicals by electron-donating and resonance-stabilization mechanisms. Thus, lazabemide is able to inhibit oxidative damage to neurons by two separate, but complementary mechanisms: (i) inhibiting the formation of free radicals associated with excessive MAO-B activity, and (ii) interfering with the propagation of reactive oxy-radicals through cellular membranes, as demonstrated in this study.

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