Characterization of the Radical Trapping Activity of a Novel Series of Cyclic Nitrone Spin Traps*

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α-Phenyl-tert-butyl nitrone (PBN) is a nitrone spin trap, which has shown efficacy in animal models of oxidative stress, including stroke, aging, sepsis, and myocardial ischemia/reperfusion injury. We have prepared a series of novel cyclic variants of PBN and evaluated them for radical trapping activity in vitro. Specifically, their ability to inhibit iron-induced lipid peroxidation in liposomes was assessed, as well as superoxide anion (O2−) and hydroxyl radical (OH) trapping activity as determined biochemically and using electron spin resonance (ESR) spectroscopy. All cyclic nitrones tested were much more potent as inhibitors of lipid peroxidation than was PBN. The unsubstituted cyclic variant MDL 101,002 was approximately 8-fold more potent than PBN. An analysis of the analogs of MDL 101,002 revealed a direct correlation of activity with lipophilicity. However, lipophilicity does not solely account for the difference between MDL 101,002 and PBN, inasmuch as the calculated octanol/water partition coefficient for MDL 101,002 is 1.01 as compared to 1.23 for PBN. This indicated the cyclic nitrones are inherently more effective radical traps than PBN in a membrane system. The most active compound was a dichloro analog in the seven-membered ring series (MDL 104,342), which had an IC50 of 26 μM, which was 550-fold better than that of PBN.

The cyclic nitrones were shown to trap OH with MDL 101,002 being 20–25 times more active than PBN as assessed using 2-deoxyribose and p-nitrosodimethylamine as substrates, respectively. Trapping of OH by MDL 101,002 was also examined by using ESR spectroscopy. When Fenton's reagent was used, the OH adduct of MDL 101,002 yielded a six-line spectrum with hyperfine coupling constants distinct from that of PBN. Importantly, the half-life of the adduct was nearly 5 min, while that of PBN is less than 1 min at physiologic pH. MDL 101,002 also trapped the O2•− radical to yield a six-line spectrum with coupling constants very distinct from that of the OH adduct.

In mice, the cyclic nitrones ameliorated the damaging effects of oxidative stress induced by ferrous iron injection into brain tissue. Similar protection was not afforded by the lipid peroxidation inhibitor U74006F, thus implicating radical trapping as a unique feature in the prevention of cell injury. Together, the in vivo activity, the stability of the nitroxide adducts, and the abilities to distinguish between trapping of OH and O2•− suggest the cyclic nitrones to be ideal reagents for the study of oxidative cell injury.

In the central nervous system, both stroke and neurotrauma have been proposed to initiate a sequela of oxidative events, which ultimately lead to neuronal cell death (1). Studies have reported that the nitrone spin trap α-phenyl-tert-butyl nitrone (PBN)1 can significantly ameliorate neuronal cell loss and neurologic deficits induced by stroke in a gerbil model of global ischemia (2–4). Furthermore, PBN was shown by ESR spectroscopy to trap lipid-derived radicals in cortical tissue of these animals. Recent work has shown remarkable protective effects afforded by PBN in focal ischemia employing both transient (5) and permanent (6) ischemia.

There are a number of additional situations of oxidative stress in which PBN has demonstrated beneficial effects. In animal models of septic shock, where activation of inflammatory cells leads to an oxidative burst, PBN has been reported to reduce endotoxin-associated mortality (7). Bolli et al. (8) have utilized PBN to demonstrate radical formation following reperfusion in a dog model of myocardial dysfunction referred to as “stunning.” Of interest was the finding that administration of the spin trap significantly improved post-ischemic contractile recovery. In Langendorf heart preparations subjected to doxorubicin-mediated formation of superoxide and hydrogen peroxide, treatment with PBN prevented the cardiotoxicity associated with the redox cycling of doxorubicin (9).

Historically, nitrone spin traps such as PBN have been utilized to trap short-lived reactive radicals like OH as the resultant nitroxide is a more stable radical and can be detected by ESR spectroscopy. Nitrones react more rapidly with carbon-centered radicals than the oxygen-centered radicals that are thought to be the primary radicals generated in vivo. Reactions of these initial radicals with cellular biomolecules such as lipids lead to the formation of secondary carbon and oxygen-centered radicals. PBN has been used recently in a number of in vivo or ex vivo studies as an analytical tool to demonstrate radical formation as evidenced by trapping of the secondary radicals. Mason and colleagues have demonstrated radical adducts of PBN in bile of vitamin E and selenium-deficient rats challenged with either copper (10) or carbon tetrachloride (11). Human patients undergoing elective cardioplegia were shown to have radicals present in coronary sinus blood using PBN (12). More recently, investigators have demonstrated that nitrones like PBN can inhibit the oxidation of lipids including low density lipoproteins (13) and proteins such as glutamine synthetase (14).

The ability of nitrones such as PBN to trap radicals to form more stable adducts, coupled with its apparent beneficial phar-

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1 The abbreviations used are: PBN, α-phenyl-tert-butyl nitrone; BHT, butylated hydroxytoluene; cLogP, computer-determined octanol-water partition coefficient; DETAPAC, diethylene-triaminepentaacetic acid; HOMO, highest occupied molecular orbital; LUMO, lowest unoccupied molecular orbital; p-NDA, para-nitrosodimethylaniline; TBA, thiodarbiduric acid.

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macrolide activity in animal models, suggests that nitrones may represent a valuable therapy for treatment of oxidative injury. However, PBN suffers from a requirement for relatively high doses to achieve protective activity. Furthermore, many radical adducts of PBN, particularly the \( \cdot \text{OH} \) adduct, are unstable and undergo decomposition reactions, which limit its use in ESR spectroscopy. Efforts to synthesize phenyl substituted analogs of PBN with improved adduct stability have met with limited success (15, 16). We have recently prepared a series of novel cyclic variants of PBN and examined their utility as therapeutic agents in animal models of ischemia/reperfusion injury (17) and endotoxic shock (18, 19). In each case, the cyclic nitrones were much more active than PBN. Recently, we have also reported on the ability of these compounds to function as effective inhibitors of \( \text{Cu}^{2+} \)-dependent oxidation of low density lipoproteins (20). Of great interest was the demonstration of both lipid and protein radical trapping. Herein, we describe the synthesis of the cyclic nitrones and characterization of their ability to function as radical traps in a number of in vitro assays and by ESR spectroscopy.

MATERIALS AND METHODS

The isoquinoline-based nitrones were synthesized from the appropriate formamide by a multistep procedure (21). First, the formamide was cyclized to a 3,3-disubstituted 3,4-dihydroisoquinoline by a modified Bischler-Napieralski reaction essentially following the literature procedure (22). Where regioisomers resulted from cyclization, the mixture of dihydroisoquinolines was readily separated by silica gel chromatography. For the six-membered ring nitrones, the dihydroisoquinoline was first reduced with sodium borohydride in methanol and the resulting imines were made in a similar fashion, but the yield in the modified Bischler-Napieralski cyclization was much lower. The resulting imines could be oxidized directly to the nitrones using the same conditions, but this required multiple additions of 30% hydrogen peroxide and several days to approach completion. The benzazepine-based nitrones were made in a similar fashion, but the yield in the modified Bischler-Napieralski cyclization was much lower. The resulting imines had to be immediately reduced to the secondary amines before oxidation to the 6,7-nitrones. All final products gave satisfactory elemental and spectral data. Complete details of the synthesis have been submitted for publication elsewhere.²

Chemicals

2-Deoxy-o-ribose, FeCl₃, FeCl₂, EDTA, 30% \( \text{H}_₂\text{O}_₂ \), ascorbic acid, TBA, trichloroacetic acid solution, BHT, NADPH, p-NDA, GSH, DETAPAC, FMN, NADP, xanthine, xanthine oxidase (from butternick), and 1,1,3,3-tetraethoxypropane were purchased from Sigma. Soybean phosphatidylincholine was a product of Avanti Polar Lipids (Birmingham, AL) while PBN, \( ^{13}\text{C} \)ethanol, and \( ^{13}\text{C} \)formate were purchased from Aldrich. All other chemicals were of the highest grade available.

Molecular Modeling

Three structures (syn and anti forms of PBN, and MDL 101,002) were constructed using SYBYL 6.0 (24). These structures were then optimized using the TRIPOS 52 Force field (25) and then further optimized using the semi-empirical quantum program MOPAC (26). The HOMO and LUMO were plotted for the PBN in both the Hartree-Fock method. (27) using a split valence basis set (6–31 G*) with the unrestricted Hartree-Fock method.

Several of the structures were subjected to an estimated logP (octanol/water partition coefficient) via a fragment-based approach (28). The methods are implemented in the program PCMODELS (29), which comprises an estimation of logP based on structural considerations (represented as \( \text{cLogP} \)) and an estimation of the molar refractivity, which is not utilized here.

² R. C. Bernotas, D. A. Hay, A. A. Carr, T. R. Nieduzak, G. Adams, D. F. Ohlweiler, and C. E. Thomas submitted for publication.

Radical Trapping and Antioxidant Activity

Radical trapping in vitro by the cyclic nitrones was evaluated by: (a) examining the ability of the nitrones to inhibit oxidation of soybean phosphatidylincholine liposomes and (b) assessing \( \cdot \text{OH} \) trapping using bleaching of p-NDA.

In Tables I-III, the values represent the IC₅₀ as determined from a single experiment employing at least five concentrations. In most instances, the numbers reflect the mean of duplicate experiments and MDL 101,002 was generally run as a "positive" control to ensure reproducibility of the methodology. While evaluating the effect of structural variation and ring substitution on activity, for any values that appeared to be unusual relative to other analogs, the assay was repeated and the average of two experiments with similar values are reported.

Inhibition of Lipid Peroxidation—For determination of the ability to inhibit lipid peroxidation, liposomes were prepared from soybean phosphatidylincholine by ethanol injection. An aliquot of phosphatidylincholine was dried in a small glass vial under \( \text{N}_₂ \), and resolubilized in ethanol at a volume of 10 \( \mu \text{L} \) of liposomes. Typically, liposomes were prepared in a volume of 8 \( \mu \text{L} \)tube and then all preparations were combined to provide a homogeneous mixture for the assay. The ethanol containing the lipid was taken up in a Hamilton syringe and injected into the appropriate volume of 50 \( \text{mM} \) \( \text{NaCl} \), 10 \( \text{mM} \) Tris, pH 7.0, at 37°C with mixing to achieve a final lipid concentration of 0.963 mM.

The liposomes were added to 25-ml beakers in a Dubnoff metabolic shaker at 37°C. To the liposomes were added the test compound (in buffer or ethanol), histidine-FeCl₃ (250:50 \( \mu \text{M} \) final), FeCl₂ (50 \( \mu \text{M} \) final, prepared in \( \text{N}_₂ \)-purged water) and sufficient buffer to achieve a final lipid concentration of 0.5 mM. Oxidations were initiated by the addition of \( \text{H}_₂\text{O}_₂ \) and carried out under an air atmosphere with shaking. One mL aliquots were removed at 0.2, 4, 6, 10, 12, and 15 min and added to 2 mL of 0.67% thiobarbituric acid (TBA): 10% trichloroacetic acid (2:1) in 0.25 N HCl, containing 0.05 mL of 2% \( \text{BHT} \) to terminate oxidation (30) followed by heating at 100°C for 20 min. After cooling, the tubes were centrifuged at 3,000 rpm for 10 min and the absorbance of the resultant supernatant read at 532 nm - 580 nm. Quantitation of TBARS was determined by comparison to a standard curve of malondialdehyde equivalents generated by acid catalyzed hydrolysis of 1,1,3,3-tetraethoxypropane. The IC₅₀ was determined with the 15 min time point using GraphPad InPlot 4. The data reported represent the value determined from a curve generated with at least 5 concentrations of nitrone. MDL 101,002 was routinely run to verify the reproducibility of the data over time.

Assessment of Cyclic Nitrones as \( \cdot \text{OH} \) Traps Using p-NDA—Cyclic nitrones were evaluated for \( \cdot \text{OH} \) trapping activity by a variety of tests. The primary assay was dependent upon the ability of the compounds to inhibit the \( \cdot \text{OH} \)-dependent bleaching of p-NDA (31). The p-NDA was prepared at 1 \( \mu \text{L} \) in 50 \( \mu \text{L} \) \( \text{NaCl} \), pH 7.0. The hydrous radical was generated using 10% \( \text{H}_₂\text{O}_₂ \) (in \( \text{N}_₂ \)-purged water) and sufficient buffer to achieve a final concentration of 2.5 mM while \( \text{H}_₂\text{O}_₂ \) was prepared from a 30% stock solution (8.8 M) at 1.25 mM in the buffer. Test compounds were solubilized in buffer or ethanol at a concentration of 1 \( \mu \text{M} \) or 5 \( \mu \text{M} \), depending upon solubility.

Assay mixtures in glass cuvettes contained 0.02 mL of \( \text{H}_₂\text{O}_₂ \), 0.02 mL of test compound, 0.10 mL of p-NDA, and 50 \( \mu \text{L} \) NaCl, pH 7.0, to a final volume of 0.98 mL. The oxidation was initiated by the addition of 0.02 mL of \( \text{Fe}^{2+} \), and the bleaching of p-NDA was monitored as the loss in absorbance at 440 nm for 100 s. To generate concentration curves, serial dilutions of the test compounds were made such that a constant volume of 0.02 mL was added to the reaction mixture. Ethanol itself is an \( \cdot \text{OH} \) trap, thus, controls contained an equal volume of ethanol for any test compound requiring this vehicle. The IC₅₀ values were determined by GraphPad InPlot 4 and represent the amount of spin trap required to inhibit the bleaching of p-NDA by 50%. The values presented are from a minimum of two determinations for each concentration of nitrone.

ESR Spin Trapping—The cyclic nitrones were examined as spin traps using ESR spectroscopy and compared to PBN. For evaluation of \( \cdot \text{OH} \) trapping ability and the stability of the resulting radical adducts, the spin trap's reagent was used to generate \( \cdot \text{OH} \). The nitrones were prepared in phosphate buffered saline at a concentration of 100 \( \mu \text{M} \). FeCl₃ was prepared in \( \text{N}_₂ \)-purged, double-distilled water at a concentration of 1 \( \mu \text{M} \), while 30% \( \text{H}_₂\text{O}_₂ \) (8.8 M) was diluted in phosphate-buffered saline to a concentration of 8.8 mM. To a glass test tube was added 0.408 mL of spin trap, 0.307 mL of \( \text{H}_₂\text{O}_₂ \) (0.3 \( \mu \text{M} \)), and 0.075 mL of \( \text{Fe}^{2+} \) (0.15 \( \mu \text{M} \)). The mixture was immediately transferred to a quartz flat cell and placed in the cavity of the spectrometer. The spectra were recorded on a Bruker 300E spectrometer with the following parameters: magnetic
field strength 3480 G, microwave power 20 milliwatts, modulation amplitude 1.15 G, scan range 100 G, scan time 82 s, and receiver gain 2 \times 10^3.

Trapping of \( \text{OH} \) by the cyclic nitrones was confirmed using \(^{13}\text{C}\) ethanol and \(^{13}\text{C}\) formate. The reaction of \( \text{OH} \) with these substrates yields the \( \alpha \)-hydroxyethyl and \( \text{CO}_2 \) radicals, respectively, which are subsequently trapped by the nitrones. The \(^{13}\text{C}\) nucleus further splits the signal. Ethanol or formate, as well as their \(^{13}\text{C}\) isotopes, was included at 5% final concentration and Fenton's reagent (Fe\(^{2+}/\text{H}_2\text{O}_2\)) was used as described above. The parameters were magnetic field strength 3480 G, microwave power 10 milliwatts, modulation amplitude 1.025 G, scan range 100 G, scan time 168 s, and receiver gain 1 \times 10^4. The ability of MDL 101,002 and PBN to trap superoxide anion was also assessed using ESR spectroscopy. The radical was generated using either FMN/NADPH or xanthine/xanthine oxidase. The iron chelator DETAPAC was included to minimize formation of \( \text{OH} \). The reaction mixtures contained 0.375 ml of spin trap at 50 mm in phosphate-buffered saline, 0.083 ml of 3 mm FMN, 0.017 ml of 30 mm NADPH, and 0.025 ml of 1 mm DETAPAC. For enzymatic generation of \( \text{O}_2^- \), xanthine oxidase was included at 0.05 units and xanthine was 0.33 mM. Immediately after the addition of NADPH or xanthine oxidase, the mixtures were transferred to a quartz flat cell and the spectra recorded. The spectrometer parameters were as described above for the \( \text{OH} \) studies.

Ferrous Iron-induced Oxidative Stress in Mice

The efficacy of the nitrones as radical traps and antioxidants in vivo was assessed using a model of ferrous iron-induced oxidative stress in mice. Ferrous iron was prepared at 50 mm in \( N_2 \)-purged double-distilled water. Five \( \mu l \) was administered into the left intracerebroventricular region using a 250-\( \mu l \) Hamilton syringe with a Chaney adapter to allow delivery of the small volume. The syringe is fitted with a 25-gauge needle (0.75 inch) with a metal sleeve that sets penetration depth at 3–5 mm. Where indicated, mice were administered the nitrone intraperitoneally, 20 min prior to iron injection. The iron induced convulsions within several min and led to death over a 20–70-min period. The effect of the nitrones on convulsions and mortality was monitored.

RESULTS

Molecular Modeling—The structures of the cyclic nitrones are illustrated in Fig. 1. A theoretical determination of the effect of cyclization on radical trapping was conducted using molecular modeling. Previous (32) frontier molecular orbital calculations done on PBN with O-centered and C-centered radicals showed that electron transfer from the HOMO of the spin trap to the LUMO plays an important role in O-centered radicals, in contrast to C-centered radicals where electron transfer occurs from the HOMO of the radical to the LUMO of the spin trap. In addition, the geometry of the transition state of the radical with the spin trap PBN was rationalized. The anti form of PBN was the conformation that was used in this discussion with the syn form not expected to follow the pathway. Initially, MDL 101,002 was not expected to follow the mechanism discussed (32) since it structurally resembled the syn form of PBN. However, after further calculation, it was found that MDL 101,002 resembled the anti form of PBN in terms of the frontier molecular orbitals (Fig. 2). For PBN, of the two forms, the anti form had the lower heat of formation (by 13.3 kcal) in keeping with the crystal structure (33). MDL 101,002 had a higher energy for the HOMO than PBN, thereby making it more reactive as per the reactivity scheme discussed (32). The energies of the HOMO and LUMO for the various structures are shown in Table I. In addition to the carbon \( \alpha \) to the nitrone, which is the reactive center toward the radical, had a higher HOMO coefficient in MDL 101,002 than PBN (in the anti form).

Effect of Nitrones on Iron-induced Liposomal Peroxidation—The effect of the cyclic nitrones on iron-induced oxidation of liposomes was determined by the TBARS test. MDL 101,002 demonstrated a dose-dependent protection with a 15-min IC\(_{50}\) of 1.67 mm, which is an 8-fold improvement over PBN (Fig. 3). The remaining cyclic nitrones were examined in a similar fashion, and the IC\(_{50}\) values are reported in Table II. The addition of a 6- or 7-methoxy had little effect on the ability to inhibit lipid peroxidation, while substitution in the 8-position (MDL 102,839) led to a slight enhancement in activity. The same effect of methoxy substitution was evident in the spirocyclohexyl (MDL 102,832) series.

The effect of halogen substitution on inhibition of lipid peroxidation was also investigated. A fluoro substituent in the 6- or 7-position increased potency approximately 2-fold. Monochloro substitution at any position on the phenyl ring increased activity 7–10-fold, relative to MDL 101,002. The 7-Cl substituted spirocyclohexyl compound (MDL 101,694), the 6,8-dichloro derivative of MDL 101,002 (MDL 100,630), and the
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TABLE I

| Compound          | Energy of HOMO | Energy of LUMO |
|-------------------|----------------|----------------|
| MDL 101,002       | -0.2766        | 0.0867         |
| PBN (anti form)   | -0.2946        | 0.0958         |
| PBN (syn form)    | -0.3223        | 0.1208         |

Fig. 3. Effect of MDL 101,002 on iron-induced peroxidation of soybean phosphatidylcholine liposomes. Liposomes were treated with a mixture of Fe(II) (50 μM) and histidine-Fe(III) (250 μM) in the presence or absence of varying concentrations of MDL 101,002. At the times indicated, 1-ml aliquots were removed and the degree of lipid oxidation determined by TBARS as described under "Materials and Methods." The data represent a single experiment; the IC50 of MDL 101,002 has been confirmed in at least five independent experiments.

TABLE II

| Compound          | IC50       | cLogP      |
|-------------------|------------|------------|
| PBN               | 14.30      | 1.23       |
| Six-membered ring series |
| MDL 101,002 (R = H) | 1.67       | 1.01       |
| MDL 102,336 (5-Cl)  | 0.15       | 1.73       |
| MDL 100,426 (6-Cl)  | 0.11       | 1.73       |
| MDL 100,777 (7-Cl)  | 0.24       | 1.73       |
| MDL 101,872 (8-Cl)  | 0.21       | 1.73       |
| MDL 102,663        | 0.28       | 1.41       |
| Seven-membered ring series |
| MDL 101,002 (R = H) | 0.026      | 3.00       |
| MDL 105,185 (8-Cl)  | 0.06       | 2.29       |
| MDL 104,342 (7,9-Cl2) | 0.026    | 3.00       |
| Spirocyclohexyl series |
| MDL 102,832 (R = H) | 0.12       | 1.97       |
| MDL 101,354 (6-Cl)  | 0.12       | 2.04       |
| MDL 101,882 (8-OCH3) | 0.08      | 2.04       |
| MDL 101,694 (7-Cl)  | 0.03       | 2.68       |
| Others              |
| MDL 102,663        | 0.28       | 1.41       |
| MDL 100,818        | 82.4       | Not determined |

Fig. 4. Correlation of cLogP values versus IC50 values for inhibition of lipid peroxidation for selected cyclic nitrones. The IC50 values were determined in the liposome experiments, and the LogP values are computer-estimated. The data are those presented in Table I. The data represent one to three separate determinations. While the overall degree of peroxidation varies slightly between experiments, when calculated as a percentage of control (no nitrone), the variation is less than 15% between experiments. For all compounds, a minimum of five concentrations were run, and MDL 101,002 was done in parallel as a positive control. The estimated LogP values are computer-generated.

When the effect of halogen substitution on 'OH trapping was studied, no clear structure activity relationship was evident. In general, fluoro or chloro substituents tended to decrease 'OH trapping activity in all of the three series of structures. Interestingly, the dichloro compound MDL 100,630 had comparable activity relative to the corresponding monochloro compounds and, in the seven-membered ring series, the 7,9-dichloro analog (MDL 104,342) was nearly 10 times more active compared to the corresponding unsubstituted compound MDL 102,389 (IC50 values of 1.76 mM versus 18.1 mM). Determination of rate constants (k) for reaction with 'OH using the 2-deoxyribose assay (34, 35) revealed that all cyclic nitrones were more reactive than PBN with k increased 3.5-fold (MDL 101,882) to 15-fold (MDL 102,073) (data not shown).

ESR Spectra of Spin Adducts of PBN and MDL 101,002—Electron spin resonance spectroscopy was used to study radical trapping by MDL 101,002 and to examine the stability of the...
TABLE III

IC50 values for inhibition of \( \cdot \)OH-induced bleaching of p-nitroso-dimethylaniline

| Compound                | IC50  |
|-------------------------|-------|
| PBN                     | 72    |
| Six-membered ring series|       |
| MDL 101,002 (R = H)     | 2.8   |
| MDL 102,336 (5-Cl)      | 13.1  |
| MDL 100,426 (6-Cl)      | 13.5  |
| MDL 100,777 (7-Cl)      | 14.6  |
| MDL 101,872 (8-Cl)      | 20.2  |
| MDL 100,630 (6,8-Cl2)   | 6.4   |
| MDL 104,698 (6-F)       | 37.4  |
| MDL 101,111 (7-F)       | 5.0   |
| MDL 100,094 (6-OCH3)    | 0.8   |
| MDL 102,073 (7-OCH3)    | 1.3   |
| MDL 102,839 (8-OCH3)    | 14.3  |
| MDL 101,842 (6,7-(OCH3)2| 10.2  |
| Seven-membered ring series|     |
| MDL 102,389             | 18.1  |
| MDL 105,185 (8-Cl)      | 23.7  |
| MDL 104,342 (7,9-Cl2)   | 1.8   |
| Spirocyclohexyl series  |       |
| MDL 102,832 (R = H)    | 15.5  |
| MDL 101,694 (7-Cl)      | 20.2  |
| MDL 101,354 (6-Cl)      | 7.3   |
| MDL 101,882 (6-Cl)      | 9.2   |
| Others                  |       |
| MDL 102,663             | 11.5  |
| MDL 100,818             | >20 (limited by solubility) |

The relative \( \cdot \)OH trapping efficiency of the nitrone adducts were determined by their ability to prevent the \( \cdot \)OH-mediated bleaching of \( p \)-nitroso-dimethylaniline. The amount of nitroso required to decrease the rate of loss of absorbance at 440 nm by 50% over the first 100 s of the reaction was used to calculate the IC50. The results are the means of duplicate determinations, except where unexpected results were obtained (i.e. MDL 104,698 versus MDL 101,111) the experiment was conducted at least three times.

resultant nitroxide adducts. As observed in Fig. 5, the \( \cdot \)OH adduct of PBN displays a doublet of triplets, which decays rapidly at pH 7.4. The MDL 101,002-\( \cdot \)OH adduct also gives a six-line spectrum with hyperfine splitting constants of \( a^H = 15.2 \) G and \( a^N = 8.2 \) G. It is apparent that cyclization increases the \( \mu \) hydrogen coupling markedly in comparison to PBN for which \( a^H = 2.9 \) G and \( a^N = 15.6 \) G. The spectra shown are on the same scale; thus, the greater signal height for MDL 101,002 reflects either a more efficient trapping of \( \cdot \)OH, a decreased rate of decay of the adduct, or both. The relative stability of the \( \cdot \)OH adduct of MDL 101,002 is illustrated in Fig. 6. Spectra were recorded at 4-min intervals, and the adduct was still readily detectable at 20 min. When signal height was plotted versus time, a biphasic decay curve was evident with a half-life of approximately 4.9 min under these experimental conditions.

The trapping of \( \cdot \)OH by MDL 101,002 was confirmed by using \(^{13}\)C formate and \(^{13}\)C ethanol. Trapping of the \( \cdot \)CO2 radical generated by the action of \( \cdot \)OH on 5% formate yields a six-line spectrum (\( a^N = 16.0 \) G and \( a^H = 10.2 \) G) (Fig. 7). When \(^{13}\)C formate is used, the signal is further split into 12 lines (\( a^N = 16.2 \) G, \( a^H = 10.3 \) G, and \( a^C = 12.6 \) G) due to the interaction of the unpaired electron with the \(^{13}\)C nucleus, thereby confirming that the \( \cdot \)OH was generated and that the six line spectrum with MDL 101,002 is indeed the \( \alpha \)-hydroxy adduct. Likewise, the inclusion of 5% ethanol in the reaction mixture yields a rather complicated spectrum, which represents trapping of the \( \alpha \)-hydroxyethyl radical with splitting constants of \( a^N = 16.6 \) G and \( a^H = 11.0 \) G. Again, the signal is further split when \(^{13}\)C ethanol is used. The splitting constants are similar for \( a^N \) and \( a^H \) being 15.7 and 15.0 G, respectively, with \( a^C = 10.6 \) G. Additional splitting of the signal may be derived from an amnonitroxyl radical resulting from decomposition of the nitroxide adduct.

Trapping of \( \cdot \)O2 by MDL 101,002 was also tested using: 1) the reduction and autoxidation of FMN or 2) xanthine and xanthine oxidase to generate the radical. A six-line spectrum was observed for MDL 101,002 with splitting constants of 5% ethanol and \( a^N = 4.3 \) G (Fig. 8). Induction of 200 units of SOD nearly totally abolished the signal, thus insuring the adduct arose from trapping of \( \cdot \)O2. Irrespective of the radical generating system, the adduct intensity with MDL 101,002 was approximately twice that of PBN. A compilation of splitting constants for PBN, POBN, and MDL 101,002 with various radical species...
is shown in Table IV.

Effect of Nitrones on Iron-induced Mortality—Injection of ferrous iron into brain parenchyma of mice induced convulsions and resulted in death 20-70 min post-injection. Pretreatment of mice with MDL 101,002 and, to a lesser extent, PBN decreased convulsions and prolonged the time to death (Fig. 9). Conversely, the 21-aminosteroid Tirilazad (U74006F), a well known lipid peroxidation inhibitor, was essentially without effect on the time to death, as was the anticonvulsant valproic acid (data not shown). The time to onset of convulsions was monitored and was 16.0 ± 2.7 min for control (n = 17), 17.8 ± 4.2 min (n = 6) for Tirilazad, and 97.3 ± 15.6 min (n = 16) for the nitrones.

**DISCUSSION**

Structure-Activity Relationships for Radical Trapping by Cyclic Nitrones—The results described herein indicate that cyclic variants of PBN function as more effective radical traps and antioxidants than does PBN. This was first tested by evaluating the ability of the compounds to function as inhibitors of liposomal oxidation. For this purpose, an initiating system comprising histidine-Fe³⁺ liposomal oxidation. For this purpose, an initiating system ing the ability of the compounds to function as inhibitors of antioxidants than does PBN. This was first tested by evaluat-

variants of PBN function as more effective radical traps and
clicNitrones—

radical is a less efficient means to inhibit lipid peroxidation than H atom or electron transfer as characteristic of phenolic antioxidants but, nonetheless, the cyclic nitrones can still completely prevent peroxidation in iron-challenged liposomes.

It was determined that all of the cyclic nitrones were considerably more active inhibitors of lipid peroxidation than was PBN in this system. The unsubstituted cyclic variant MDL 101,002 was approximately 8-fold more potent than PBN. An analysis of the variants of MDL 101,002 revealed a correlation of activity with lipophilicity (Fig. 4). This is not unexpected, as it is known that the ability of antioxidants to inhibit lipid oxidation is determined to an extent by the accessibility of the antioxidant to the lipid radicals. However, it is clear that lipophilicity does not solely account for the difference between MDL 101,002 and PBN, in as much as the cLogP for MDL 101,002 is 1.01 as compared to 1.23 for PBN. This indicates that the cyclic nitrones are inherently more effective traps in a membrane system. This is supported by the methoxy variants, which have even greater water solubility but are nonetheless more active inhibitors of lipid peroxidation than is PBN. This may reflect the more favorable HOMO and LUMO values for the cyclic nitrones as compared to PBN.

The utility of the nitrones to ameliorate in vivo oxidative damage was demonstrated in the iron-injected mouse model. Pre-administration with the nitrones minimized convulsions and delayed the time to death. The anticonvulsant valproic acid was without effect, suggesting that the pharmacologic action of the nitrones derived from radical trapping. Interestingly, the potent lipid peroxidation inhibitor U74006F also had no effect. These data imply that the efficacy of the nitrones stems, at least in part, from trapping of primary radicals. We have previously shown the nitrones to prevent protein oxidation (20). It would be of great interest to compare the nitrones and U74006F for effects on both lipid and protein oxidation in this model system; such data could provide significant new insight into the mechanism of iron-induced, radical-dependent damage in biological systems.

The LUMO for MDL 101,002 is lower than for PBN, which should increase its reactivity with O-centered radicals (32). An evaluation of this was performed by study of reaction of the nitrones with ‘OH and O₂⁻ the two radicals of most relevance to biological systems. The nitrones were tested to ascertain whether they could compete for ‘OH with p-NDA, which reacts rapidly and stoichiometrically with ‘OH (31). This assay is quite stringent in that it is difficult to compete with p-NDA for ‘OH. Nonetheless, in a biological milieu, the diffusion distance of ‘OH is miniscule, and thus we chose this assay to aid in determining whether the nitrones could trap ‘OH in the pres-
ence of a highly competitive substrate. As expected, the nitrones react with $'OH$, but much less readily than does P-NDA, as evidenced by the requirement for millimolar concentrations to compete with 100 $\mu M$ P-NDA. The reactivity of PBN was poor, as shown by an estimated $IC_{50}$ value of 72 mM. Conversely, the cyclic nitrones were able to inhibit P-NDA bleaching in the low millimolar to high micromolar range. In the 101,002 series, the addition of methoxy substituents had little effect on $'OH$ trapping when assayed by the p-NDA or 2-deoxyribose method. The presence of chloro substituents on the phenyl ring tended to decrease reactivity with $'OH$ with the exception of MDL 100,630.

When the nitrone-containing ring was expanded to seven atoms by an additional methylene (MDL 102,389), a significant loss of $'OH$ trapping activity relative to MDL 101,002 was observed. There was a slight additional loss of activity with chloro substitution, although the 7,9-C$_2$H$_2$ analog MDL 104,342 exhibited good activity with an $IC_{50}$ of 1.76 mM. Coupled with an $IC_{50}$ value of 26 $\mu M$ against lipid peroxidation, this compound represents the most active nitrene radical trap that has been synthesized in this series and, to the best of our knowledge, exceeds the activity of any other nitrene. Linking of the gem-dimethyls with a three-methylene chain to produce the spirocyclohexyl compound MDL 102,832 decreased $'OH$ trapping activity as assessed by the p-NDA assay. As in the other two classes of cyclic nitrones, methoxy substitution improved activity slightly while chloro substitution decreased activity. A dichloro analog in this series was not synthesized.

Utility of Cyclic Nitrones as Spin Traps for $'OH$ and $O_2$$^\cdot$—The results using ESR spectroscopy confirm that MDL 101,002 is a superior trap for $'OH$ than is PBN as the $'OH$ adduct of MDL 101,002 had an intensity nearly 7 times that of PBN when determined 30–45 s after addition of Fe$^{2+}$. The data obtained in the 2-deoxyribose and p-NDA assays indicate that the rate of trapping of $'OH$ by MDL 101,002 is greater than that of PBN, which likely contributes to the greater intensity of its adduct signal. Conversely, the $\alpha$-hydroxy nitrooxide adduct of MDL 101,002 may be more stable than that of PBN, which is on the order of 1 min at physiologic pH (37). The data in Fig. 5 show that the $'OH$ adduct is readily detectable even at 20 min with no evidence for the formation of secondary ESR-detectable adducts derived from decomposition of MDL 101,002-$'OH$. With the Fenton’s system, $'OH$ generation may continue for several minutes; thus, we cannot definitively determine the lifetime of the adduct under these conditions. When catalase and DETA-PAC were added to the mixture to quench $'OH$ formation 30 s after addition of Fe$^{2+}$, the adduct was detectable for 4–8 min (results not shown). More extensive studies in this regard, as well as an examination of phenyl ring substitution on $'OH$ trapping as determined by ESR spectroscopy, will be reported elsewhere.

MDL 101,002 and PBN were also examined for their ability to trap $O_2$$^\cdot$ using ESR spectroscopy. The reaction of superoxide with MDL 101,002 produced an adduct with a more intense six-line spectrum with a $\beta$ hydrogen coupling constant which was approximately half that of the $'OH$ adduct. This is similar to the differences in $\beta$ hydrogen coupling for the $'OH$ and $O_2$$^\cdot$ adducts of 5,5-dimethylpyrroline-1-oxide, where it is speculated that trapping of $O_2$$^\cdot$ induces a conformational change such that the altered bond angles bring the $\beta$ hydrogen in closer proximity to the nitrogen nucleus. These data suggest that the cyclic nature of our nitrones leads to a significant difference in trapping and adduct conformation relative to PBN as predicted by molecular modeling studies. Thus, MDL 101,002 is much better suited to distinguishing between $'OH$ and $O_2$$^\cdot$ than is PBN, but offers the advantage over 5,5-dimethylpyrroline-1-oxide of readily trapping C-centered radicals as suggested by the studies in liposomes. The greater trapping efficiency of the cyclic compounds, coupled with the difference in $\beta$ hydrogen splitting between $'OH$ and $O_2$$^\cdot$ adducts, suggests MDL 101,002 to be a superior spin trap to PBN for in vitro studies when a continuous flux of radicals is being generated.

In summary, the data presented herein suggest that cyclic nitrones represent valuable tools for the study of oxidative injury. Correlations between molecular modeling and experimental data allowed us to confirm previous calculations with PBN (32), which implied that electron transfer from the HOMO of the spin trap to the LUMO is critical for trapping of O-centered radicals. In vivo, the cyclic nitrones are capable of decreasing the effects of iron-induced oxidative stress, apparently by virtue of radical trapping. Of great significance is the fact that the nitroxides generated via radical trapping are much more stable than those of PBN and the adducts with $'OH$ and $O_2$$^\cdot$ are clearly distinguishable. Furthermore, we have prepared a series of spin traps which encompass a wide range of lipophilicity. Together, these properties suggest the compounds could be extremely useful in the study of radical trapping in biological systems and could be used to probe radical formation at the cellular level. To this end, we are currently using the nitrones in a cell culture model of ischemia/reperfusion injury to explore the site and type of radical(s) generated. Such information will greatly enhance our understanding of ischemia-induced cell injury.

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REFERENCES
1. Kontos, H. A. (1989) Chem. Biol. Interact. 72, 229–255
2. Oliver, C. N., Starke-Reed, P. E., Stadtmann, E. R., Liu, G. J., Carney, J. M., and Floyd, R. A. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 5144–5147
3. Phillips, J. W., and Clough-Helfman, C. (1990) Med. Sci. Res. 18, 403–404
4. Yue, T.-L., Gu, J.-L., Lyssko, P. G., Cheng, H.-Y., Barone, F. C., and Feuerstein, G. (1992) Brain Res. 574, 193–197
5. Zhao, Q., Pahlmark, K., Smith, M.-L., and Siejo, X. K. (1994) Acta Physiol. Scand. 152, 349–350
6. Cao, X., and Phillips, J. W. (1994) Brain Res. 644, 267–272
7. Hamburger, S. A., and McCay, P. B. (1989) Circ. Shock 29, 329–334
8. Bodi, R., Patel, B. S., Eradi, M. O., Li, X.-Y., Triana, J. F., Lai, E. K., and McCay, P. B. (1990) Am. J. Physiol. 259, H1901–H1911
9. Cova, D., DeAngelis, L., Monti, E., and Piccini, F. (1992) Free Radical Res. Commun. 15, 353–360
10. Kadiska, M. B., Hanna, P. M., Jordan, S. J., and Mason, R. P. (1993) Drug Metab. Dispos. 21, 224–227
11. Knecht, K. T., and Mason, R. P. (1988) Drug Metab. Dispos. 16, 813–817
12. Tortolani, A. J., Powell, S. R., Miski, V., Weglicki, W. B., Pogo, G. J., and Kramer, J. H. (1993) Free Radical Biol. Med. 14, 421–426
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13. Thomas, C. E., Ku, G., and Kalyanaraman, B. (1994) J. Lipid Res. 35, 610–619
14. Carney, J. M., Starke-Reed, P. E., Oliver, C. N., Landum, R. W., Cheng, M. S., Wu, J. F., and Floyd, R. A. (1991) Proc. Natl. Acad. Sci. U.S.A. 88, 3633–3636
15. Janzen, E. G., Hinton, R. D., and Kotake, Y. (1992) Tetrahedron Lett. 33, 1257–1260
16. Hinton, R. D., and Janzen, E. G. (1992) J. Org. Chem. 57, 2646–2651
17. Thomas, C. E., Carney, J. M., Bernotas, R. C., Hay, D. A., and Carr, A. A. (1994) Ann. N.Y. Acad. Sci. 738, 243–249
18. French, J. F., Thomas, C. E., Downs, T. R., Ohlweiler, D. F., Carr, A. A., and Dage, R. C. (1994) J. Org. Chem. 57, 2646–2651
19. Downs, T. R., Dage, R. C., and French, J. F. (1995) Int. J. Immunopharmacol. 17, 571–580
20. Thomas, C. E., Ohlweiler, D. F., and Kalyanaraman, B. (1994) J. Biol. Chem. 269, 28055–28061
21. Carr, A. A., Thomas, C. E., Bernotas, R. C., and Ku, G. (March 8, 1994) U.S. Patent 5,292,746
22. Larsen, R. D., Reamer, R. A., Corley, E. G., Davis, P., Grabowski, E. J. J., Reider, P. J., and Shinkai, I. (1991) J. Org. Chem. 56, 6034–6038
23. Murahashi, S., Shiota, T., and Imada, Y. (1991) Org. Synth. 70, 265–271
24. Tripos, Inc. (1993) SYBYL, Version 6.0, TRIPOS, Inc., St. Louis, MO
25. Clark, M., Cramer, R. D., III, and Van Opdenbosch, N. (1989) J. Comp. Chem. 10, 982–1012
26. QCPE (1989) MOPAC, Version 5.0, QCPE, Indiana University, Bloomington, IN
27. Wavefunction, Inc. (1993) Spartan, Version 3.0, Wavefunction, Inc., Irvine, CA
28. Leo, A. J. (1993) Chem. Rev. 93, 1281–1306
29. Daylight Chemical Informations Systems, Inc. (1994) PCMOLDS, Version 4.34, Daylight Chemical Informations Systems, Inc., Irvine, CA
30. Thomas, C. E., McLean, L. R., Parker, R. A., and Ohlweiler, D. F. (1992) J. Org. Chem. 27, 543–550
31. Bors, W., Michel, C., and Saran, M. (1979) Eur. J. Biochem. 95, 621–627
32. Abe, Y., Seno, S., Sakakibara, K., and Hirota, M. (1991) J. Am. Chem. Soc., Perkin Trans. 2, 897–903
33. Janzen, E., Zhang, Y., and Haire, D. L. (1994) J. Am. Chem. Soc. 116, 3738–3743
34. Halliwell, B., Gutteridge, J. M. C., and Aruoma, O. I. (1987) Anal. Biochem. 165, 763–767
35. Ching, T., Halnen, G. R. M. M., and Bast, A. (1993) Chem. Biol. Interact. 86, 119–127
36. Minotti, G., and Aust, S. D. (1987) J. Biol. Chem. 262, 1098–1104
37. Kotake, Y., and Janzen, E. G. (1991) J. Am. Chem. Soc. 113, 9503–9506
38. Buettner, G. R. (1987) Free Radical Biol. Med. 3, 259–303