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Comparison of the Radical Trapping Ability of PBN, S-PBN and NXY-059

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The nitrones α-phenyl-N-*tert*-butyl nitrone (PBN), sodium 2-sulfophenyl-N-*tert*-butyl nitrone (S-PBN) and disodium 2,4-disulfophenyl-N-*tert*-butyl nitrone (NXY-059) are neuroprotective in a variety of rodent models. The objective of the current studies was to compare the ability of PBN, S-PBN, and NXY-059 to form radical adducts and to prevent salicylate oxidation in an aqueous system. For the electron spin resonance (ESR) studies, hydroxyl radicals were generated with ultraviolet (UV) light and hydrogen peroxide. Secondary radicals were then produced by the addition of methanol, ethanol, isopropanol, dimethylsulfoxide, tetrahydrofuran or 1,4-dioxane. In addition, competition spin trapping studies were performed using PBN-α-^{13}C and either S-PBN or NXY-059. In the salicylate studies, PBN, S-PBN and NXY-059 were compared to a variety of other antioxidants and reference compounds (cysteine, glutathione, ascorbate, uric acid, Tempo, Trolox, and Tirilizad) for their ability to prevent 2,3- and 2,5-dihydroxybenzoic acid formation induced by hydroxyl radical generating systems. All 3 nitrones trapped carbon- and oxygen-centered radicals to produce ESR-detectable radical adducts. Each nitrone also prevented salicylate oxidation, with PBN being the most effective. The ability of these 3 nitrones to prevent salicylate oxidation resembled that of most of the other compounds tested.

Keywords: Salicylate, Spin-trapping, Free radical, Phenyl-N-*tert*-butyl nitrone, 2-Sulfophenyl-N-*tert*-butyl nitrone, 2,4-Disulfophenyl-N-*tert*-butyl nitrone

INTRODUCTION

The nitrones α-phenyl-N-*tert*-butyl nitrone (PBN), sodium 2-sulfophenyl-N-*tert*-butyl nitrone (S-PBN) and disodium 2,4-disulfophenyl-N-*tert*-butyl nitrone (NXY-059) have been shown to be neuroprotective in a number of animal models. For example, both PBN [1–4] and NXY-059 [5] are efficacious in rat models of acute focal stroke, while S-PBN [6] is efficacious in a rat model of histotoxic hypoxia. In addition, S-PBN is also efficacious in a number of other animal models of neurotoxicity [7–15]. NXY-059, which was more efficacious than PBN in a transient middle cerebral artery occlusion focal stroke model in the rat [5], is currently under evaluation as a potential therapeutic for acute

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focal stroke in human clinical trials. These three nitrones differ structurally by the number of sulfo groups attached to the phenyl ring (Figure 1).

PBN is a well-known electron spin resonance (ESR) spin trapping reagent [16–19]. It is generally assumed that nitrones that are active in *in vivo* models exert their effect by scavenging deleterious free radicals. Although PBN has been characterized as an antioxidant [20–23], the antioxidant potential of S-PBN and NXY-059 has not been reported. It is important to note that when PBN and NXY-059 were tested in the same transient middle cerebral artery occlusion focal stroke rat model, NXY-059 was much more efficacious [5].

The objectives of the studies reported here were to compare the ability of PBN, S-PBN, and NXY-059 to act as a radical trap based on ESR studies, and to compare their ability to prevent the oxidation of salicylate in a pure chemical environment.

METHODS AND MATERIALS

Chemicals

PBN, S-PBN, NXY-059 and PBN-α-^{13}C were synthesized at Centaur Pharmaceuticals, Inc., from the corresponding aldehyde and N-*tert*-butylhydroxylamine. Other chemicals were purchased in the USA as follows: Trolox, uric acid, 2,3-dihydroxybenzoic acid (2,3-DHBA), 2,5-dihydroxybenzoic acid (2,5-DHBA), sodium phosphate dibasic heptahydrate, Tempo, DMSO, DMSO-d_6, methanol, ethanol, tetrahydrofuran (THF), 1,4-dioxane, isopropanol, and 2,2′-azobis(2-methylpropionamidine) dihydrochloride from Aldrich (Milwaukee, WI). ^{13}C-methanol, 1-^{13}C-ethanol, and benzaldehyde-[carbonyl-^{13}C] from Cambridge Isotope Laboratories, Inc. (Andover, MA). Salicylic acid sodium salt, hydrochloric acid, ferrous sulfate heptahydrate, sodium citrate trisodium salt dihydrate, acetic acid glacial, ascorbic acid, L-cysteine, and glutathione from Sigma (St. Louis, MO). Methanol and acetonitrile from J. T Baker (Phillipsburg, NJ). Hydrogen peroxide (aqueous solution, 30%) from Fisher Scientific (Pittsburgh, PA). Tirilizad was kindly provided by AstraZeneca.

Electron Spin Resonance (ESR) Studies

Radicals were generated in the presence of PBN, S-PBN, NXY-059 or PBN-α-^{13}C as follows: Ultraviolet (UV) light was used with hydrogen peroxide to generate hydroxyl free radicals in the presence of the nitrone. Different secondary radicals were produced in UV-irradiated hydrogen peroxide solutions by adding appropriate secondary target compounds (see Table I) to the nitrone / hydrogen peroxide mixtures before irradiation. The ESR hyperfine splitting constants, a_H, a_N and a_{13C} were calculated for each radical adduct from duplicate spectra and reported in gauss (G). For the compounds shown in Table I, solutions were bubbled with argon gas to remove air before UV irradiation. In order to generate a bulky oxygen-centered radical, the UV-initiated decomposition of the azo compound, 2,2′-azobis(2-methylpropionamidine) dihydrochloride, was performed in the presence of the air. The azo compound decomposition generated tertiary carbon-centered radicals, which further reacted with dissolved molecular oxygen to produce the oxygen-centered radicals.

Solutions were prepared using 100 mM, pH 7.4 phosphate buffer as the solvent. The final concentration of the nitrone was 50 mM. The final concentration of hydrogen peroxide was 1% (V/V) and of the secondary target was 20% (V/V). UV light was generated with a 100-W mercury lamp (Oriel model #6281) assembled with an igniter (Oriel model #66001) which was power-supplied with a universal Oriel unit (Oriel #68805). The electric current for the mercury lamp was 5 A. Solutions were mixed in an ESR flat cell and were irradiated with UV light

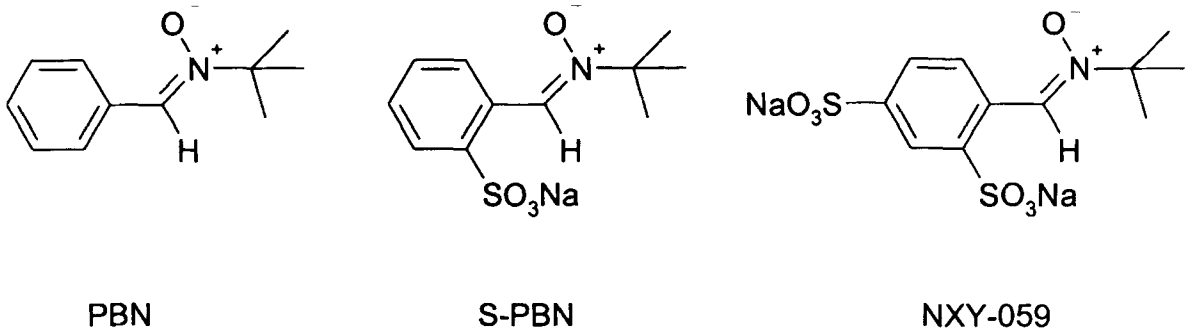


FIGURE 1 Structures of PBN, S-PBN and NXY-059

directly in the ESR cavity for 10–20 sec, depending on the radical system. Controls run with only the nitrone did not yield detectable radical adducts under the ESR conditions used.

ESR spectra were recorded using a Bruker spectrometer operating at 9.77 GHz with a modulation frequency of 50 kHz and a TM_{110} cavity. Instrumental settings were 0.2 G modulation amplitude, 2×10^5 receiver gain, 1 sec time constant, 100 G field width, and a 480 sec scan time.

TABLE I Secondary Radicals Generated in the Presence of Nitrones in ESR Studies

Secondary Targets[a]	*Radical Source*	*Secondary Radicals Generated*
CH_3OH	$CH_3OH + H_2O_2 + UV$	$\bullet CH_2OH$
CD_3OH	$CD_3OH + H_2O_2 + UV$	$\bullet CD_2OH$
$^{13}CH_3OH$	$^{13}CH_3OH + H_2O_2 + UV$	$\bullet ^{13}CH_2OH$
C_2H_5OH	$C_2H_5OH + H_2O_2 + UV$	$\bullet CH(CH_3)OH$
$CH_3{}^{13}CH_2OH$	$CH_3{}^{13}CH_2OH + H_2O_2 + UV$	$\bullet ^{13}CH(CH_3)OH$
$(CH_3)_2CHOH$	$(CH_3)_2CHOH + H_2O_2 + UV$	$\bullet C(CH_3)_2OH$
DMSO	$DMSO + H_2O_2 + UV$	$\bullet CH_3$
DMSO-d_6	DMSO-d_6 + H_2O_2+ UV	$\bullet CD_3$
THF	$THF + H_2O_2 + UV$	
1,4 dioxane	1,4-Dioxane + H_2O_2 + UV	

a. The solution was bubbled with argon gas to remove air before UV-irradiation.

Although we wished to compare the abilities of PBN, S-PBN and NXY-059 to trap radicals, we did not expect their respective hyperfine splitting constants to differ greatly. Thus, in order to look at competition between the nitrones, PBN-α-^{13}C, which is a ^{13}C-PBN analogue with a single ^{13}C at the α-carbon of the nitrone, was used as an internal spin trapping reference compound. The ^{13}C, which has a spin of ½, adds an additional hyperfine interaction that we hoped would prevent radical adduct lines from being superimposed. In this manner, we hoped to find clean wing lines that could be monitored for PBN-α-^{13}C and either S-PBN or NXY-059 for the competition studies. For the competition studies, an equimolar mixture of PBN-α-^{13}C and either S-PBN or NXY-059 were exposed to one of the aforementioned radical generating systems prior to irradiation. The relative concentrations for all radical adducts was determined based on the line intensity of clean wing lines for each adduct following the peak-height method, and, where necessary, employing the relationship

$$[A]/[B] = n_A h_A (\Delta H_{pp,A})^2 / n_B h_B (\Delta H_{pp,B})^2$$

where the n's are the number of lines for each spectrum, h's are peak-to-peak heights and the ΔH_{pp}'s peak-to-peak line widths [24].

HPLC / EC Analysis for Salicylate Assays

Compounds were tested for their ability to prevent the formation of 2,3- and 2,5-DHBA following incubation with salicylate in the presence of a hydroxyl radical generating system. Two different methods of generating hydroxyl radicals were used: autoxidation of ferrous iron and a ferrous iron / hydrogen peroxide system. In both systems, a 30 min room temperature incubation was chosen based on results from previous studies which demonstrated that 2,3- and 2,5-DHBA had not reached a plateau by 30 min and were on a linear part of the curve. The antioxidants tested in these studies were PBN, S-PBN, NXY-059, cysteine, glutathione, ascorbate, uric acid, Tempo, Trolox, and Tirilizad. Salicylic acid solution was prepared using 100 mM phosphate buffer (pH 7.6). All other reagents were prepared using milli-Q water.

$FeSO_4$ System: In a 1.5 ml centrifuge tube, the following reagents were added in sequence to yield the final concentrations specified: 100 μM salicylic acid and either 0, 10, 50, 100 or 200 μM antioxidant. The solution was vortexed for 2 sec, then $FeSO_4$ (200 μM final concentration) was added to initiate the reaction. The solution was vortexed for 4 sec, kept at room temperature for 30 min, and then immediately injected onto the HPLC column for analysis of formation of 2,3- and 2,5-DHBA formed by salicylate oxidation. For the control reactions, water was substituted for either the antioxidant or $FeSO_4$. All assays were run using 3–6 repetitions per reaction.

$FeSO_4$ / H_2O_2 System: In a 1.5 ml centrifuge tube, the following reagents were added in sequence to yield the final concentrations specified: 100 μM salicylic acid, either 0, 10, 50, 100 or 200 μM antioxidant, and 200 μM $FeSO_4$. The solution was vortexed for 2 sec, then H_2O_2 (200 μM final concentration) was added to initiate the reaction. The solution was vortexed for 4 sec, kept at room temperature for 30 min, and then immediately injected onto the HPLC column for analysis of formation of 2,3- and 2,5-DHBA formed by salicylate oxidation. For the control reactions, water was substituted for either the antioxidant, $FeSO_4$ or H_2O_2. All assays were run using 3–6 repetitions per reaction.

The HPLC apparatus consisted of a Hewlett Packard 1050 Liquid Chromatography System with pump, autosampler (Hewlett Packard, Wilmington, DE, USA) and an electrochemical detector (Oklahoma Medical Research Foundation, OK, USA) with a carbon glass electrode and Ag/AgCl reference electrode from Bioanalytical Systems (West Lafayette, IN, USA). The separation was performed on a Keystone BDS Hypersil C18 column, 300 mm × 3 mm ID, 5 μm particle size (Keystone, Bellefonte, PA, USA) with a 2 μM precolumn filter (Rheodyne, Cotati, CA, USA).

The data were collected using a Hewlett Packard 35900E dual channel interface, ChemStation software, Version 6.01, and a Pentium computer (Hewlett Packard, Wilmington, DE, USA).

HPLC analyses were performed using an isocratic mobile phase consisting of (V/V) 25% methanol / 75% 0.03 M sodium citrate, 0.03 M acetic acid, pH 3.6 buffer at 0.4 ml/min and 30°C. The mobile phase was prepared using milli-Q water (Millipor, Milford, MA, USA) and filtered before use with a 0.45 µm filter (Nalgene, Rochester, NY, USA). The electrochemical detector potential was +0.9 V and the injection volume was 10 µl.

Data were analyzed using Prism2 software, version 2.01 (GraphPad Software, Inc., San Diego, CA, USA). IC_{50} curves were fit using a sigmoidal dose-response (variable slope) nonlinear fit equation (Prism 2 software, version 2.01). Goodness of fit of the IC_{50} curve was expressed in terms of the correlation coefficient (R^2).

RESULTS

ESR Spin Trapping Studies

PBN, S-PBN and NXY-059 all trapped radicals and produced radical adducts that were sufficiently stable to allow ESR detection and characterization (see Table II for the ESR hyperfine splitting constants).

TABLE II ESR Hyperfine Splitting Constants (G) for PBN, S-PBN and NXY-059 Radical Adducts

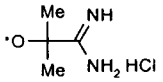
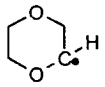
Radical Trapped	*PBN*			*S-PBN*			*NXY-059*		
	a_N	a_H	a_{13C}	a_N	a_H	a_{13C}	a_N	a_H	a_{13C}
	15.64	4.08	–	15.48	4.47	–	15.30	4.03	–
•OH	15.64	2.76	–	15.87	5.34	–	15.75	4.98	–
•CH_2OH	16.07	3.87	–	15.91	5.64	–	15.77	5.32	–
•CD_2OH	16.08	3.89	–	15.91	5.68	–	15.79	5 34	–
•$^{13}CH_2OH$	16.08	3.89	3.89	15.90	5.64	2.82	15.77	5.29	2.95
•$CH(CH_3)OH$	16.16	3.36	–	15.93	4.16	–	15.81	3.78	–
•$^{13}CH(CH_3)OH$	16.17	3.48	3.48	15.96	4.08	3.30	15.84	3.76	3.54
•$C(CH_3)_2OH$	16.15	3.57	–	16.17	3.95	–	16.04	3.46	–
•CH_3	16.51	3.56	–	16.36	6.44	–	16.24	6.12	–
•CD_3	16.48	3.62	–	16.38	6 50	–	16.24	6.17	–
	16.07	3.28	–	16.01	3.34	–	15.91	2.95	–
	15.96	3.01	–	15.80	3.83	–	15.64	4.30	–

TABLE III ESR Hyperline Splitting Constants (G) for PBN-α-^{13}C Radical Adducts

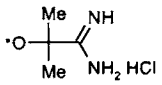
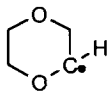
Radical Trapped	*Radical Adduct*		
	a_N	a_H	a_{13C}
	15.61	4.11	5.61
•OH	15.62	2.71	4.36
•CH_2OH	16.06	3.87	6.17
•CD_2OH	16.08	3.89	6.17
•$CH(CH_3)OH$	16.16	3.37	6.01
•$C(CH_3)_2OH$	16.12	3.61	6.04
•CH_3	16.47	3.57	5.97
•CD_3	16.48	3.60	6.01
	16.05	3.34	5.94
	15.98	3.12	5.97

Table III lists the hyperfine splitting constants obtained when the radicals were trapped with PBN-α-^{13}C. The a_N and a_H values of PBN-α-^{13}C (Table III) are very consistent with those for PBN (Table II), showing that PBN-α-^{13}C was a suitable internal reference standard for the competition studies. An example spectrum from these studies is shown in Figure 2 for the adducts for the •CD_2OD radical. The results of these competition studies are shown in Table IV. Because in each study we normalized the adduct concentration to that for PBN-α-^{13}C, comparisons in Table IV should only be made within a row. It is clear that, with the exception of bulky oxygen-centered radicals, NXY-059 yielded higher steady-state radical adduct concentrations than either PBN-α-^{13}C or S-PBN. For all but the bulky oxygen-centered radical, NXY-059 > S-PBN > PBN-α-^{13}C with respect to radical adduct signal intensity.

Salicylate Studies

In the salicylate studies, the 3 nitrones were compared to other antioxidants for their potential to prevent the formation of 2,3-DHBA and 2,5-DHBA induced by a hydroxyl radical generating system. The 2,5-DHBA results are shown in Table V, and analogous results were obtained by analyzing the extent of formation of 2,3-DHBA (data not shown). Whether autoxidation of ferrous iron or ferrous iron / hydrogen peroxide was used to generate hydroxyl radicals, PBN was the most effective of the 3 nitrones tested, and NXY-059 the least effective. The ability of these nitrones to scavenge hydroxyl radicals resembled that of the other antioxidants tested with one main exception. Although Tirilizad has been reported to have antioxidant activity [25], in our studies it consistently potentiated the formation of the salicylate oxidation products. In the ferrous iron / hydrogen peroxide system, ascorbate also potentiated the formation of 2,3- and 2,5-DHBA, presumably by redox cycling the iron back to the ferrous state.

DISCUSSION

It is clear from the ESR studies that the three nitrones PBN, S-PBN and NXY-059 all trap carbon- and oxygen-centered radicals to produce radical adducts. In addition, the salicylate studies show that these compounds prevent other molecules from being oxidized in an environment in which radicals are generated. Thus, all three nitrones show some antioxidant abilities, presumably as a result of radical scavenging.

All but one of the hyperfine splitting constants obtained for the adducts of PBN in the aqueous solvent system used in this study fall within the corresponding range of values reported in the NIEHS Spin-Trap Database ([26], see Table VI). The exception was a_H for •CH_3, which was 0.02 less than the respective minimum reported value.

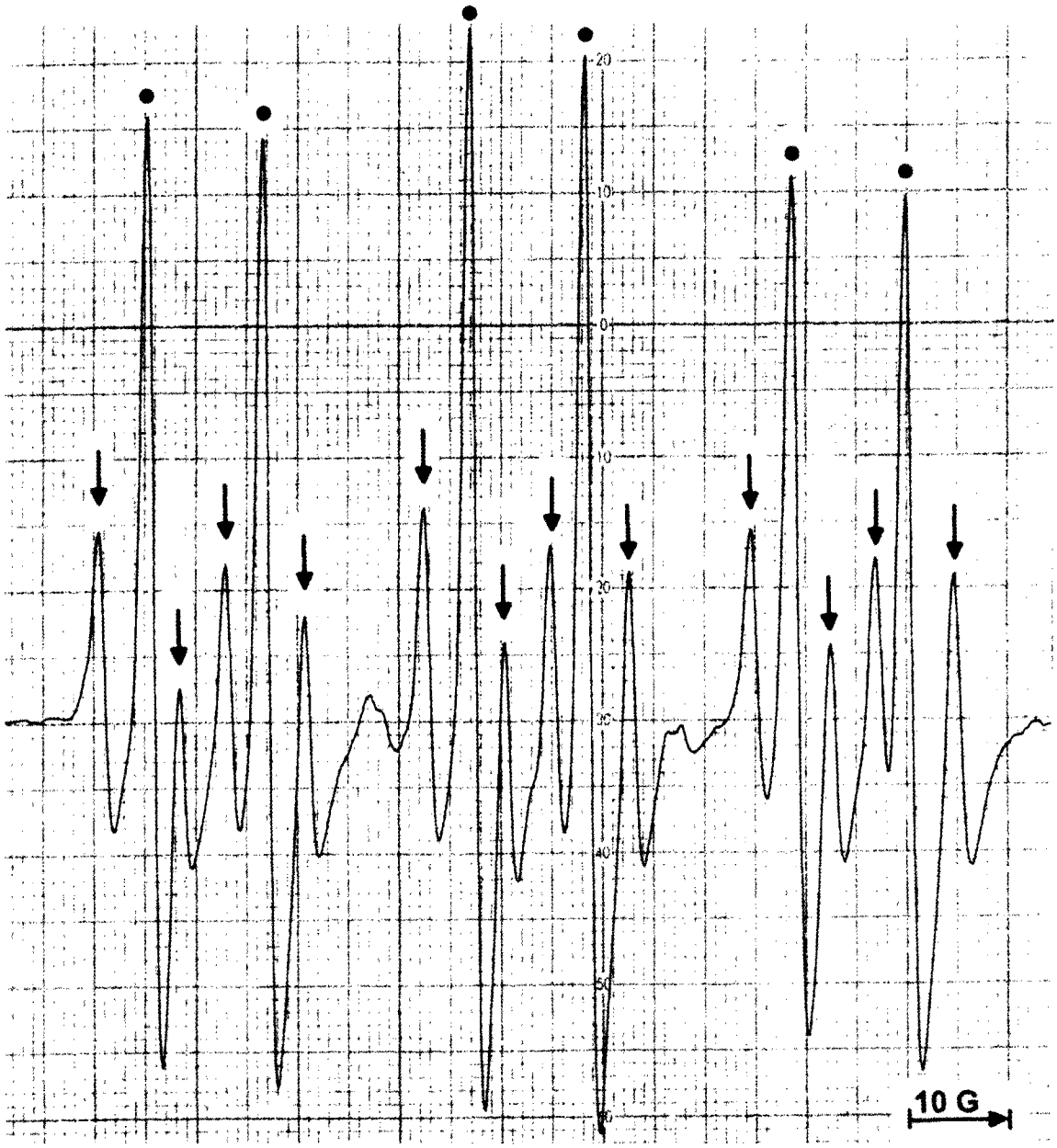


FIGURE 2 ESR spectrum obtained following irradiation of a mixture containing PBN, PBN-α-^{13}C, CD_3OD, and hydrogen peroxide. Solutions were prepared using 100 mM, pH 7.4 phosphate buffer as the solvent. The final concentration of PBN and PBN-α-^{13}C were both 50 mM. The final concentration of hydrogen peroxide was 1% (V/V) and of CD_3OD was 20% (V/V). UV light was generated with a 5 A, 100-W mercury. Solutions were mixed in an ESR flat cell and were irradiated with UV light directly in the ESR cavity for circa 15 sec. The ESR spectrum was recorded using a Bruker spectrometer operating at 9.77 GHz with a modulation frequency of 50 kHz and a TM_{110} cavity. Instrumental settings were 0.2 G modulation amplitude, 2×10^5 receiver gain, 1 sec time constant, 100 G field width, and a 480 sec scan time. The arrows indicate the lines for the $\bullet CD_2OD$ adduct of PBN-α-^{13}C, whereas the closed circles indicate the lines for the $\bullet CD_2OD$ adduct of PBN

TABLE IV ESR Spin Trapping Competition of S-PBN or NXY-059 with PBN-α-^{13}C

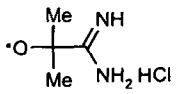
Radical Trapped	*Relative Radical Adduct Concentration*		
	PBN-α-^{13}C	*S-PBN*	*NXY-059*
	1	0.8	0.9
•OH	1	1.4	2.1
•CD_2OH	1	1.7	3.1
•CH_2OH	1	1.7	2.8
•CD_3	1	1.6	2.2
•CH_3	1	1.6	2.4

TABLE V Salicylate Assay Results

A. Fe (II) System 2,5-DHBA Results.		
Compound	*IC_{50} (μM)*	*R^2*
PBN	141	1.00
S-PBN	178	0.98
NXY-059	441	0.99
Cysteine	278	0.98
Glutathione	185	1.00
Ascorbate	323	0.97
Uric Acid	104	0.99
Tempo	115	0.99
Trolox	208	0.99
Tirilizad	Increased	–
B. Fe(II) / H_2O_2 System 2,5-DHBA Results		
Compound	*IC_{50} (μM)*	*R^2*
PBN	178	0.99
S-PBN	199	0.96
NXY-059	298	0.91
Cysteine	189	0.96
Glutathione	127	0.98
Ascorbate	Increased	–
Uric Acid	164	0.98
Tempo	147	0.97
Trolox	129	0.96
Tirilizad	Increased	–

TABLE VI PBN Radical Adduct ESR Hyperfine Splitting Constant Comparison

Radical	*Spin-Trap Database Range*		*This Study*	
	a_N (G)	a_H (G)	a_N (G)	a_H (G)
•OH	15.50 – 15.74	2.70 – 2.91	15.64	2.76
•CH_3	16.46 – 16.65	3.58 – 3.62	16.51	3.56
•CH_2OH	15.99 – 16.20	3.80 – 4.00	16.07	3.87
•$CH(CH_3)OH$	16.00 – 16.35	3.30 – 3.50	16.16	3.36

The hyperfine splitting constant for a_H was profoundly affected by the presence of the sulfo group at the ortho position on the phenyl ring. In every instance, a_H for the S-PBN adducts was larger than for the corresponding PBN adducts. Even though the sulfo group is a strong electron-withdrawing group that would normally cause a decrease in a_H, the dihedral angle between the C-H bond and the semi-occupied *p*-orbital of the nitrogen atom is changed due to the 2-sulfo substituent in S-PBN. It is the dominating effect of this change in angle that is responsible for the increased a_H of the S-PBN adducts. Interestingly, the electron-withdrawing effect of the sulfo group becomes evident when the adducts of S-PBN and NXY-059 are compared. NXY-059, which has a sulfo substituent in the 2 and the 4 position of the phenyl ring, has smaller a_N and a_H values than S-PBN (see Table II). Thus, the 4-sulfo substituent in NXY-059 withdrew the odd-electron spin density from the nitroxyl moiety via the phenyl ring, while leaving the nitroxide conformation essentially unaffected.

On the basis of the ESR competition results alone, it might be suggested that NXY-059 is the best ESR spin trapping agent of the 3 nitrones tested. Certainly, it provided the most intense radical adduct signal and had a broader range of hyperfine splitting values for a_H than did PBN. However, this does not imply that NXY-059 is the most effective radical scavenger of the 3 nitrones. The salicylate results clearly show NXY-059 to be the least effective of these nitrones at preventing the oxidation of salicylate. Taken together, these results suggest that the stronger ESR adduct intensity for NXY-059 may be due to a slower decay of the radical adduct, rather than to a faster rate of radical trapping.

In general, although PBN is an antioxidant, it is not considered to be an extremely good antioxidant. For instance, Janzen *et al.* [20] reported that the IC_{50} for PBN in a lipid peroxidation inhibition assay was about 20 mM, whereas that for Trolox and BHT was 1.0 and 0.05 mM, respectively. In view of their negatively charged sulfo groups, we would not expect S-PBN and NXY-059 to partition into the lipid phase much, if at all. This would make them even less effective than PBN at trapping radicals in a lipid phase. In the hydrophilic environment of our salicylate studies, the nitrones performed in a similar way to most of the other antioxidants. However, this may simply reflect the strong oxidizing capacity of the hydroxyl radicals.

All three nitrones provide protection *in vivo* in rodent models of human disease. In a model of acute focal stroke, NXY-059 and PBN were given by intravenous delivery for the same length of time. In spite of the fact that NXY-059 was more efficacious than PBN at equimolar doses [5], the results described here do not suggest that NXY-059 is a much more effective antioxidant than PBN. It is possible, however, that NXY-059 is a particularly effective scavenger of some other specific types of radical species relevant to the damage occurring during ischemia and reperfusion. Another possibility is that NXY-059, due to its specific surface charge characteristics, concentrates at different critical sites of oxidative stress than PBN.

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