Fine-Tuning the Amphiphilicity: A Crucial Parameter in the Design of Potent α-Phenyl-*N*-*tert*-butylnitrone Analogues

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Abstract: A new series of hydrophilic, lipophilic, and amphiphilic α-phenyl-*N*-*tert*-butylnitrone (PBN) derivatives were synthesized to explore the relationship between their hydrophilic−lipophilic properties and antioxidant potency. Very potent protective effects of amphiphilic lactobionamide and tris(hydroxymethyl)aminomethane PBN derivatives were observed in mitochondrial preparations, in cell cultures, and in rotifers exposed to unspecific and mitochondria targeted oxidotoxins.

A wide body of evidence suggests that reactive oxygen species (ROS) are implicated in a number of disease states[1] and the pathophysiology of aging.[2] Since the first work of Novelli et al.,[3] nitrone spin traps, typified by α-phenyl-*N*-*tert*-butylnitrone (PBN), have been widely used as antioxidants in several biological models including protection against death after endotoxic shock,[4] protection from doxorubicin cardiotoxicity,[5] and protection from focal and global ischemia reperfusion injury[6,7] and in increasing the life span.[8] PBN is a small molecule with hydrophilic and lipophilic properties allowing for a rapid permeation of all tissues including the heart, the liver, and the brain,[9,10] has a moderate half-life, and is devoid of acute toxicity.[11] However, the requirement of high doses of PBN (100−300 mg/kg body weight) to display significant protective activity has stimulated the development of intrinsically more potent analogues. Such efforts have been mainly focused on the synthesis of phenyl substituted analogues such as imidazolylnitrones,[12] cyclic nitrones,[13] azulenylnitrones,[14] and sulfonate nitrones.[15] Because mitochondria are the main source and target of ROS,[16] an alternative approach consisting of the selective targeting of antioxidants to mitochondria has also been developed by Murphy et al.[17]

Over the past 10 years, our work was devoted to the design and synthesis of amphiphilic nitrones. With the expectation that amphiphilic compounds possessing a hydrophilic polar head and a lipophilic alkyl chain would exhibit improved bioavailability and membrane crossing ability, we synthesized a fluorinated glycolipidic nitrone derived from PBN.[18] More recently we reported the synthesis of a new series of amphiphilic PBN derivatives in which the nitrone function was fitted into the core of the molecule.[19] The preliminary biological evaluations have shown that all these amphiphilic compounds were far more potent than the parent antioxidant compound PBN. Moreover, we observed that the more lipophilic compounds exhibited the highest antiapoptotic activity in cultured skin fibroblasts with the neurogenic ataxia retinitis pigmentosa (NARP) mutation.[19c] Very recently, we have validated the amphiphilicity mediated mitochondria targeting concept using a broad spectrum of antioxidant agents.[20] We demonstrated that the protective activities of different antioxidant agents can be greatly enhanced by grafting them on fluorinated amphiphilic amino acid carriers, confirming and extending previous preliminary findings. Amphiphilicity of nitrones is a key feature in determining bioactivity and protection against the oxidative toxicity in vitro and in vivo, as one of these compounds called LPBNAH[21] exhibited exceptionally high antioxidant activity serving as a lead structure (Figure 1).

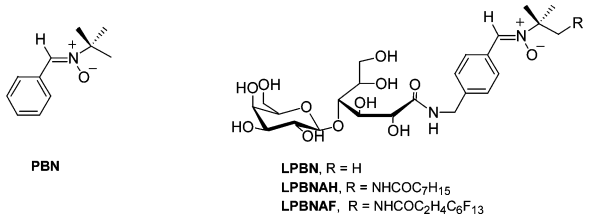

Figure 1. Lactobionamide PBN derivatives previously developed.

To extend and explore the importance of the amphiphilicity of nitrone derivatives in determining their antioxidant potency, we present here the synthesis of new hydrophilic, amphiphilic, and lipophilic analogues respectively called TGPBN, TGPBNAH, and EPBNAH. In comparison with these newly designed nitrone derivatives, we have also included in the antioxidant and protective activity assessments one hydrophilic nitrone LPBN[22] and two amphiphilic compounds LPBNAH and LPBNAF.[19a,c,21,23] Amphiphilic nitrones such as LPBNAH exert profound protective effects in vitro and in vivo exceeding the antioxidant activity of many other compounds in potency.

The tris(hydroxymethyl)aminomethane (Tris) derivatives TGPBN and TGPBNAH were synthesized as outlined in Scheme 1. The synthesis of the polar head was easily carried out in two steps. First, the glycine derivative **1** was obtained by reaction of *N*-benzyloxycarbonylglycine with Tris in the presence of 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoleine (EEDQ) in refluxing ethanol[24] followed by the acetylation of the hydroxyl groups with a mixture of acetic anhydride/pyridine. Then benzyloxycarbonyl group removal was achieved by catalytic hydrogenolysis in ethanol and the resulting amino compound **2** was immediately used without purification. At the same time, the nitrone derivatives were prepared by condensation of the *N*-*tert*-butylhydroxylamines to benzaldehyde groups under inert atmosphere in the dark, according to the already published procedure.[19] The condensation of the *N*-*tert*-butylhydroxylamine to 4-formylbenzoic acid in EtOH at 65 °C led to *N*-*tert*-butyl-α-(4-carboxyphenyl)nitrone, which was converted to *N*-hydrosuccinimide compound **3**.[20] The Tris polar head derived amino compound **2** was grafted in CH_2Cl_2 under basic conditions on the activated nitrone. Then the Zemplén de-O-acetylation procedure led, after purification by flash chromatography and recrystallization, to the hydrophilic PBN derivative **4**, called TGPBN.

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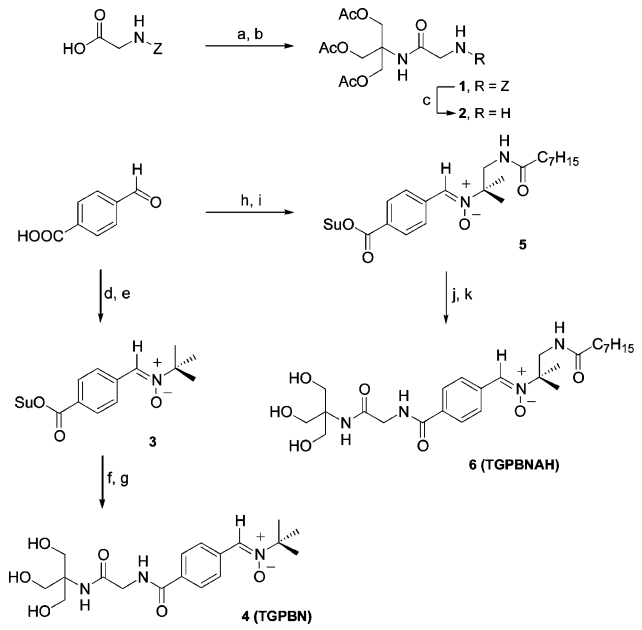
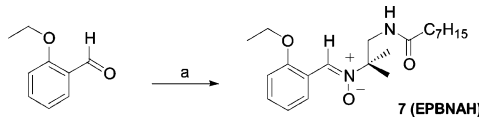
Scheme 1. Synthesis of Tris PBN Derivatives[a]



[a] Reagents: (a) EEDQ, EtOH, reflux, 58%; (b) 1:1 Ac_2O/pyridine (v/v), room temp, 95%; (c) 7 bar of H_2, Pd/C, ethanol, room temp, 100%; (d) *N*-*tert*-butylhydroxylamine, ethanol, 65 °C, 50%; (e) HOSu, DCC, dioxane, room temp, 67%; (f) **2**, DIEA, CH_2Cl_2, room temp, 38%; (g) MeONa, methanol, room temp, 76%; (h) octanoic acid (2-hydroxyamino-2-methylpropyl)amide, 1:1 EtOH/pyridine (v/v), 65 °C, 68%; (i) HOSu, DCC, CH_2Cl_2, room temp, 42%; (j) **2**, DIEA, CH_2Cl_2, room temp, 79%; (k) MeONa, methanol, room temp, 56%.

Scheme 2. Synthesis of EPBNAH[a]



[a] Reagents: (a) octanoic acid (2-hydroxyamino-2-methylpropyl)amide, EtOH, 55 °C, 80%.

The same procedure was followed for the synthesis of **6**. The alkylamide substituted *N*-*tert*-butylhydroxylamine was grafted to 4-formylbenzoic acid followed by activation of the resulting nitrone with *N*-hydrosuccinimide. Then, after grafting of the Tris moiety on the activated carboxylic acid group and removal of the *O*-acetyl protecting groups followed by the purification by C18 reverse-phase HPLC (eluent, methanol/water), the amphiphilic PBN derivative **6**, also called TGPBNAH, was obtained as a white foam.

The ethoxy derivative was synthesized in one step by condensation of the alkylamide substituted *N*-*tert*-butylhydroxylamine to the commercially available 2-ethoxybenzaldehyde in ethanol under inert conditions (Scheme 2). Purification by flash chromatography and recrystallization led to the lipophilic PBN derivative **7**, also called EPBNAH.

The relative lipophilicities (log k'_W) of all these compounds as shown in Table 1 were measured by the chromatographic technique we used in a previous work.[19c] The TGPBN and the LPBN compounds, both grafted by a polar group on the aromatic ring, were found to be less hydrophobic and highly water soluble. In contrast, the ethoxy derivative devoid of any polar group and bearing the octanamide group on the *N*-*tert*-butyl part exhibited the highest lipophilicity. Finally, the amphiphilic compounds, endowed with a polar head and an alkyl chain, showed intermediate values. But surprisingly, unlike the hydrophilic compounds LPBN and TGPBN, no significant difference in the lipophilicities of LPBNAH and TGPBNAH was measured. Such a result clearly indicates that the volume and/or the nature of the polar group has only a low impact on the log k'_W of compounds, demonstrating that the main structural parameter able to modify this property is the nature of the hydrophobic chain. Thus, because of the very high hydrophobicity of the perfluorinated chain, LPBNAF exhibited a log k'_W value as high as the highly lipophilic EPBNAH.

Table 1. Relative Lipophilicities and Inhibition of Hydrogen Peroxide, Peroxynitrite, and Doxorubicin Induced Inactivation of the Mitochondrial Iron Sulfur Cluster N2 in Complex **I** by Nitrone Antioxidants[a]

compd (10 μM)	log k'_W	hydrogen peroxide (10 μM)	peroxynitrite (10 μM)	doxorubicin (10 μM)
PBN	1.75 (1.64[b])	35.0 ± 0.7	27.6 ± 0.9	44.3 ± 1.3
TGPBN	0.87	61.8 ± 2.4	43.0 ± 1.4	63.5 ± 0.7
LPBN	0.59	25.1 ± 0.4	17.3 ± 0.4	13.5 ± 0.3
TGPBNAH	2.75	59.2 ± 1.8	40.5 ± 1.2	83.5 ± 3.2
LPBNAH	2.67 (2.76[b])	78.3 ± 2.7	72.5 ± 2.8	90.7 ± 3.5
LPBNAF	4.44 (4.65[b])	51.9 ± 2.1	39.3 ± 1.5	57.3 ± 1.8
EPBNAH	4.46	nd[c]	nd[c]	nd[c]

[a] Submitochondrial particles from rat brain were incubated for 30 min with the oxidotoxin hydrogen peroxide, peroxynitrite, or doxorubicin in the absence or presence of protective nitrone compounds. The activity of the iron sulfur cluster N2 in complex **I** was assayed as decribed previously.[25] The percentage inhibition by the protective nitrone compounds of the oxidative inactivation in ferric cyanide reduction exerted at this rate-limiting site of the mitochondrial respiratory chain is shown. The findings are presented as the mean ± SEM ($N = 10$). All results were statistically significantly different from results of the control (vehicle-treated mitochondrial preparations) exposed only to the respective oxidotoxins, with $p < 0.01$ (ANOVA followed by Bonferroni *t*-test). [b] Data from ref 19c. [c] Not determined.

Table 2. Inhibition of Hydrogen Peroxide, Peroxynitrite, and Doxorubicin Induced Cell Death in Mixed Cortical Cultures[a]

compd (10 μM)	hydrogen peroxide (200 μM)	peroxynitrite (200 μM)	doxorubicin (200 μM)
PBN	25.0 ± 0.9	20.6 ± 0.7	24.2 ± 0.8
TGPBN	54.4 ± 1.2	31.0 ± 1.0	63.5 ± 1.2
LPBN	18.3 ± 0.7	17.3 ± 0.4	18.5 ± 0.9
TGPBNAH	33.8 ± 0.7	30.5 ± 1.3	74.3 ± 0.6
LPBNAH	81.0 ± 1.0	61.0 ± 1.3	82.0 ± 1.3
LPBNAF	63.4 ± 1.1	40.1 ± 1.0	34.9 ± 0.8

[a] Cultures were treated for 24 h with the oxidotoxin at 200 μM. Antioxidants were added at 10 μM to evaluate their putative cytoprotective effects. Shown is the percentage inhibition of Trypan blue absorbance indicating enhanced survival of the cells. The findings are presented as the mean ± SEM ($N = 10$). All results were statistically significantly different from results of the control (vehicle), with $p < 0.01$ (ANOVA followed by Bonferroni *t*-test).

The protective potency of hydrophilic, amphiphilic, and lipophilic compounds was examined and compared in mitochondrial preparations, cell cultures, and aquatic organisms. (For experimental details, see the Supporting Information.) The amphiphilic PBN derivatives LPBNAH and TGPBNAH exerted superior antioxidant activity compared to the parent compound and the more hydrophilic or lipophilic nitrones as demonstrated in the Tables 1−3. Potent protection by amphiphilic nitrones against free radical damage to the highly vulnerable iron sulfur cluster N2 of complex **I** in mitochondrial preparations is shown in Table 1.[16,26] The iron sulfur cluster N2 is localized in an amphiphatic ramp extruding from the inner membrane to the mitochondrial matrix being exposed and accessible to water, oxygen, free radicals, and other reactive intermediates including hydrogen peroxide, peroxynitrite, and the amphiphilic quinone doxorubicin. Among all compounds, only EPBNAH alone at 10 μM strongly inhibited the activity of the iron sulfur cluster,

Table 3. Hydrogen Peroxide and Doxorubicin Toxicity and Antioxidant Protection: Percentage of Viable Rotifers after Treatment with Antioxidant Agents[a]

compd (10 μM)	hydrogen peroxide (200 μM)	doxorubicin (200 μM)
control	11.9 ± 0.4	15.0 ± 0.6
PBN	25.6 ± 0.8	18.7 ± 0.5[b]
TGPBN	33.0 ± 0.8	45.4 ± 0.9
LPBN	18.6 ± 0.7	18.2 ± 0.9[b]
TGPBNAH	24.1 ± 0.7	54.3 ± 1.0
LPBNAH	83.0 ± 1.1	85.8 ± 1.7
LPBNAF	40.6 ± 1.2	30.6 ± 1.2

[a] Rotifers were treated for 24 h with the oxidotoxin at 200 μM. Antioxidants were added at 10 μM to evaluate their putative protective effects in vivo. Shown is the percentage of viable organisms after exposure of the aquatic animals to the oxidotoxins hydrogen peroxide and doxorubicin. The findings are presented as the mean ± SEM ($N = 10$). Unless otherwise indicated, all results were statistically significantly different from results of the control (vehicle), with $p < 0.01$ (ANOVA followed by Bonferroni *t*-test). [b] Nonsignificant versus doxorubicin with vehicle.

indicating that this lipophilic and non-water-soluble derivative of PBN itself can act as a mitochondrial toxin. All amphiphilic derivatives and the hydrophilic TGPBN were much more potent than PBN in preserving the activity at this rate-limiting site of the mitochondrial respiratory chain.[16] In contrast, the highly water soluble LPBN exhibited very low protective activity.

In mixed cortical cultures, pronounced cytoprotection by the amphiphilic nitrone LPBNAH against all oxidotoxins was observed while the parent compound PBN exhibited a moderate protection (Table 2). There is a noticeable difference between LPBNAH and TGPBNAH activities, the latter compound efficiently protecting only against the doxorubicin, a specific mitochondria oxidotoxin. Such a result could indicate a real affinity of these amphiphilic compounds for the mitochondria compartments. The hydrophilic compound TGPBN was surprisingly potent in protecting against hydrogen peroxide and doxorubicin toxicity. Despite its high lipophilicity, LPBNAF was well tolerated and protective against the toxicity of all agents tested, whereas EPBNAH was highly toxic to the neuronal cells when used at 10 μM (data not shown).

In the studies on rotifer survival in vivo, a similar picture emerged as shown in Table 3. A limited protection by PBN was observed for the rotifers exposed to hydrogen peroxide, while no significant protection was observed when exposed to doxorubicin. The lipophilic compound EPBNAH was by itself toxic to rotifers (data not shown), whereas the amphiphilic and lipophilic LPBNAF exerted substantial protection against the toxicity of hydrogen peroxide and doxorubicin to these organisms. The two amphiphilic nitrones LPBNAH and TGPBNAH exerted a very pronounced protection against the mitochondria-selective oxidotoxicity of doxorubicin. The lactobionamide derivative LPBNAH was also very effective against the lethal toxicity of the nonselective agent hydrogen peroxide, as also shown in Tables 1 and 2. The particular potency of the TGPBN, bearing a small Tris polar head and a small hydrophobic *tert*-butyl group, might be due to its amphipathic nature. Such amphipathic character may provide protection in cytosolic and membranous compartments of the cells, as Thomas et al. have shown for the MDL 101,002 compound.[13c]

In conclusion, the PBN has shown moderate potency in all the tests presented in Tables 1−3 while the hydrophilic LPBN exhibited poor effectiveness and the lipophilic EPBNAH was toxic and ineffective. On the other hand, all the amphiphilic and amphipathic compounds have shown significant higher activities than the parent compound PBN. These findings indicate a crucial role for amphiphilicity in determining mitochondrial antioxidant protection by nitrone compounds in biochemical preparations, cell cultures, and aquatic organisms as previously proposed.[19−21,23] It is very likely that these agents exert their superior protective effects by interacting with specific sites in mitochondria, thereby maintaining the function and integrity of these organelles. The iron sulfur cluster N2 in complex **I** may be a major target of these drugs because it is very vulnerable to oxidative damage and inactivation by free radicals.[16] Our findings are in complete agreement with those of Kotamraju et al.,[26] demonstrating a protective effect of PBN in preserving and restoring the activity of iron sulfur clusters in complex **I** when exposed to doxorubicin. Such specific effects of mitochondrial antioxidants may be very important in determining the protective potency of nitrone compounds,[20,21] which exert substantial antioxidant activity in mitochondria such as the amphiphilic nitrone antioxidants investigated in this study. The superior bioavailability of certain amphiphilic nitrones is associated with potent mitochondrial protection as well as enhanced activity at sites, which are particularly vulnerable to oxidative damage. We are now investigating in great detail the interactions of amphiphilic PBN derivatives with key components of the mitochondrial respiratory chain like the iron sulfur cluster N2 in complex **I**. Selective protection at such sites as demonstrated in this study may enable the development of more potent antioxidant agents to prevent and treat degenerative diseases associated with oxidative stress and aging.

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Supporting Information Available: Experimental details for the preparation and characterization of **1**−**7** and for mitochondrial, cellular, and rotifer assays. This material is available free of charge via the Internet at http://pubs.acs.org.

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