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Synthesis and Antioxidant Efficiency of a New Amphiphilic Spin-Trap Derived from PBN and Lipoic Acid

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Abstract—The synthesis of a new amphiphilic antioxidant called PBNLP and derived from both α-phenyl-*N*-*tert*-butyl nitrone (PBN) and lipoic acid was described. Grafting a lactobionamide moiety onto the aromatic group of the PBN provided the water solubility of this compound. In vitro preliminary biological evaluations of its antioxidant capacity were performed using the KRL biological test based on free radical-induced hemolysis. The PBNLP induces a protection of erythrocytes against exogenous free radicals higher than that measured with lipoic acid or PBN alone or with lipoic acid or PBN derivatives in admixtures.

The generation of Reactive Oxygen Species (ROS) is associated with numerous and various pathological states such Alzheimer's disease,[1] chronic neurodegenerative disorders,[2] ischemia-reperfusion or stroke.[3] Floyd et al.[4,5] were the first to show that nitrone-based free radical spin traps, especially α-phenyl-*N*-*tert*-butyl nitrone (PBN), exhibit neuroprotective activity. In a previous paper,[6] we have described the synthesis of a novel series of amphiphilic glycosylated nitrones derived from PBN. Preliminary investigations on their anti-apoptotic effect in cultured neuronal cells showed that they drastically inhibit caspase-3 activity following H_2O_2-induced apoptosis. Furthermore, we have underlined that the hydrophilic lipophilic balance and the amphiphilic properties of these molecules plays a key role on their protective activity. In the present paper, we reported the synthesis of an amphiphilic hybrid of PBN and lipoic acid. The latter and its reduced form, dihydrolipoic acid, which are natural metabolic substances, appeared like ideal antioxidants for the treatment of brain and neurodegenerative disorders in which free radical processes are involved.[7,8] Synergistic interactions between lipoate and other natural antioxidants like vitamin C and E that resulted from lipoate recycling, were previously described.[9–14] In order to improve these synergetic effects, lipoate was recently connected to Trolox®, a water-soluble analogue of vitamin E, which is a strong inhibitor of lipid peroxidation.[14] In a similar way, Harnett et al.[15] have synthesized a hybrid of lipoate and of a nitric oxide synthase inhibitor, capable of protecting neuronal cells against glutamate toxicity.

Thereafter, we decided to prepare a new amphiphilic hybrid of PBN and lipoate, called PBNLP. The nitronyl function is inserted into the heart of the surfactant between the hydrophobic tail and the polar head (Scheme 1). Whereas the hydrophobic part is made up of lipoate group, a lactobionamide moiety linked to the para position of PBN aromatic group forms the polar head and gives the water solubility to the whole molecule. To evaluate the biological effect of this new amphiphilic molecule PBNLP and to specify the possible beneficial effect of the chemical association of water-soluble derivatives of PBN, lactobioamide PBN (LAMPBN) and of lipoic acid, lipoylglytris (LGT) (Scheme 1, see[16,17] for synthesis processes), we have assessed the antioxidant efficiency of PBNLP as compared to specific antioxidant capacities of PBN, lipoic acid, LAMPBN and LGT and to the antioxidant effectiveness of LAMPBN and LGT or lipoate in admixtures.

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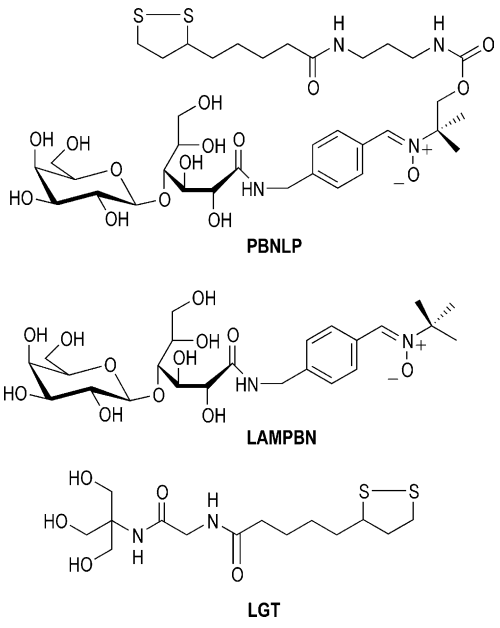
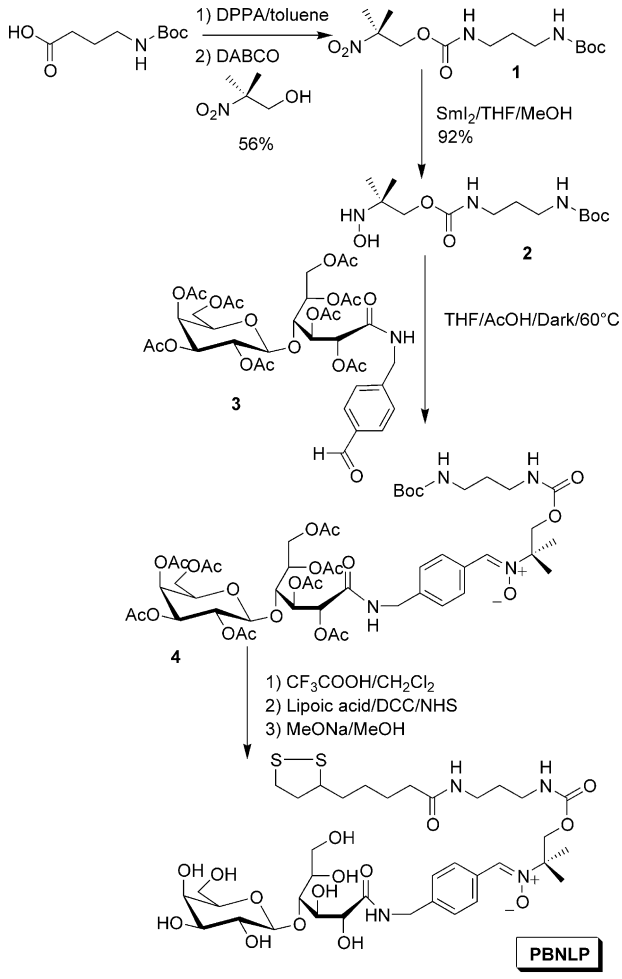


Scheme 1. Structure of PBN and lipoate derivatives.

In the chosen PBNLP synthetic route (Scheme 2) the grafting of the lipoate group has to be carried out after the nitronyl function preparation. Indeed, the nitronyl function is currently obtained by binding the benzaldehyde group to a hydroxylamine group, which can be easily prepared by reduction of a nitro function with the Kagan reagent, SmI_2. Nevertheless, this type of reduction is prevented by the presence of the lipoate group, whose disulfide group could oxidize the Kagan reagent and hence provide a dihydrolipoate function. Taking into account such a reaction, we chose to synthesize first the nitronyl function and then to graft the lipoate moiety. Therefore, we used the 2-methyl-2-nitropropanol as starting material. According to the fact that the final opening of acetylated lactobionamide using the Zemplen method could induce an ester group hydrolysis, we decided to link the lipoate moiety to the *t*-butyl nitrone by a carbamate bond. However, Harnett et al.[15] have already observed the formation of an oxadiazoline-5-one through a cycloaddition reaction between an isocyanate group and the nitronyl function. Coupling trials with decyl isocyanate and *t*-butyl nitrone have shown that such a cycloaddition reaction was mainly achieved. To avoid these problems, we have inserted a γ-aminobutyric acid (GABA) as spacer arm between the nitrone function and the lipoate group. First, the Boc-GABA was bound to the hydroxyl group of the 2-nitro-2-methyl propanol through an urethane bond. The reaction was performed in toluene at 60 °C in the presence of diphenyl phosphoryl azide (DPPA) and diaminobicyclooctane (DABCO). The obtained compound **1** was reduced in a very good yield (92%) with Kagan reagent. The hydroxylamine **2** was then grafted onto the aldehyde **3** in a THF/acetic acid mixture (9/1). This very slow reaction was achieved in 8 days. The nitrone **4** was isolated in 68% yield after purification on silica gel column.

After hydrolysis of the *t*-butyloxy group of compound **4** with a trifluoroacetic acid/CH_2Cl_2 mixture at room temperature, succinimidyl ester of lipoic acid was condensed on the resulting amino group in 70% yield. The synthesized compound was then purified by silica gel column chromatography and size exclusion chromatography on Sephadex LH20 column. Hydrolysis of acetyl groups with catalytic amounts of MeONa in dry MeOH according to the Zemplen method led to glycosylated nitrones in quantitative yield. PBNLP compound was carefully purified by C18 reverse phase HPLC (mobile phase; methanol–water) and was obtained pure as a white powder. It was next fully characterized by ^1H, ^{13}C NMR, DEPT and HMQC NMR sequences. Mass spectrometry (positive FAB) allowed the observation of the characteristic fragmentations of PBNLP hydrophobic *N* terminal part.[18]



Scheme 2. Synthesis of PBNLP.

The overall antioxidant effectiveness of PBN and lipoate derivatives was evaluated in a dynamic way using the KRL biological test based on free radical-induced hemolysis (Laboratoires Spiral, France).[19,20] Diluted blood samples without and in the presence of different doses of compounds were submitted to organic free radicals produced in the aqueous phase at 37 °C under air atmosphere from the thermal decomposition of a 50 mM solution of 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH). Hemolysis was recorded by turbidimetry using a 96-well microplate reader. Results were expressed as the time that is required to reach 50% of

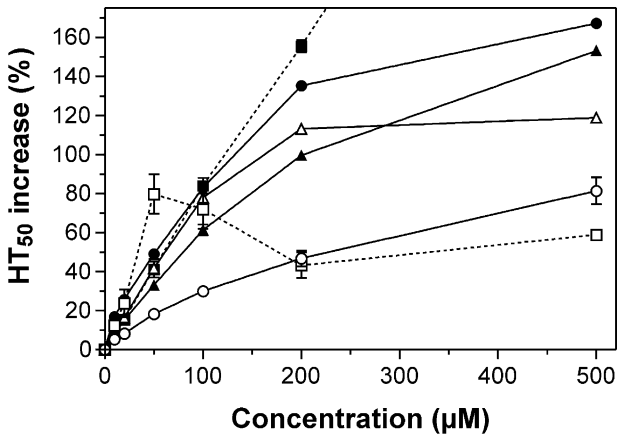


Figure 1. Dose–response studies of the antiradical efficiency of the new amphiphilic compound PBNLP (●) as compared to antiradical efficiencies of lipoic acid (□), LGT (△), PBN (○), LAMPBN (▲) and Trolox® (■). Results are expressed as percentages of compound-induced increase in the control half-hemolysis time (HT_{50}). They are means ± standard deviations (SD) of three separated experiments.

maximal hemolysis (half-hemolysis time, HT_{50} in min), which refers to the whole blood resistance to free radical attack. The overall antiradical efficiency was defined as the percentage of the compound-mediated HT_{50} increase as compared to control. Preliminary cytotoxic assays without AAPH addition showed that in the tested concentration range from 0 to 500 μM, lipoic acid, LGT, PBN, LAMPBN and PBNLP exhibited no cytolytic activities towards blood samples.

At concentrations below 100 μM, lipoic acid showed antioxidant efficiency higher than PBNLP that could result from the hydrophobic character of lipoic acid favouring its insertion and/or its diffusion across cell membranes and hence temporarily improving the cell resistance to free radical attacks (Fig. 1).

However, in the whole concentration range the new amphiphilic compound PBNLP exhibited a higher global protective activity towards free radical aggression of blood samples as compared to unmodified PBN and lipoate alone. Nevertheless, PBNLP was only slightly more active than its individual constituents, the water-soluble derivatives of PBN and lipoate that unexpectedly suggested a negative interaction between LAMPBN and LGT. Moreover, at low concentrations below 50 μM this new compound presented an antiradical capacity higher than Trolox® although at more than 200 μM, it presented a lower activity than Trolox®, which totally prevented hemolysis probably by scavenging radicals in the aqueous phase as soon they are produced (Fig. 1).

In good agreement with our previous results on the protective effect of PBN derived amphiphilic compounds on H_2O_2-mediated cell apoptosis,[6] the hydrophilic lipophilic balance and thereby the amphiphilic character of PBNLP, which could act in the extra- as in the intra-cellular medium, may play a determinant role on the higher improvement of cellular resistance against radicals as compared to unmodified PBN and lipoic acid alone. To verify this assumption and the possible negative interaction between LAMPBN and LGT that would have limited PBNLP beneficial effects, we have compared PBNLP antiradical efficiency with LAMPBN/LGT mixtures one. The experimental activities of PBNLP and mixtures at 1.33/0.67, 1/1 and 0.67/1.33 molar ratios were put together with their theoretical activities, which were calculated by summing experimental HT_{50} increases obtained with molar equivalents of pure LAMPBN and LGT (Table 1). As regards the mixtures, no additional antioxidant effect was observed whatever the concentration used. By contrast, our results showed a strong antagonistic effect between the two PBNLP constituents that was also observed between LAMPBN and lipoate (results not shown). Thus, LAMPBN would be unable to recycle lipoate in contrast to that was previously described with natural antioxidants.[9–14] According to the enhanced antioxidant efficiencies of mixtures that were noted when increasing LGT (or lipoate) molar proportion at the expense of LAMPBN molar proportion, this antagonism might result from the counteracted effect of LAMPBN on lipoic acid.

Furthermore, we found that despite this antagonistic action, the PBNLP-mediated enhancement of cellular resistance to radical aggression is significantly higher

Table 1. Experimental and theoretical antiradical capacity of the new amphiphilic molecule PBNLP as compared to LAMPBN and LGT mixtures

Compd	Conc.	50 μM		100 μM		200 μM		500 μM	
	Mol. ratio	% HT_{50}	% HT_{50}	% HT_{50}	% HT_{50}	% HT_{50}	% HT_{50}	% HT_{50}	% HT_{50}
		Exp.	Theo.	Exp.	Theo.	Exp.	Theo.	Exp.	Theo.
PBNLP	1	49.0	74.8	82.9	139.1	135.2	212.7	167.2	272.0
LAMPBN/LGT	1.33/0.67	41.5	70.1	47.4	127.8	49.8	201.1	86.3	281.1
LAMPBN/LGT	1/1	44.6	74.8	51.6	139.1	53.7	212.7	86.5	272.0
LAMPBN/LGT	0.67/1.33	56.6	76.8	60.8	132.1	62.0	188.4	88.6	247.5

Note. Experimental (Exp.) and theoretical (Theo.) results are expressed as average percentages (three separated experiments) of compound-induced increase in the control half-hemolysis time (% HT_{50}). Experimental antiradical efficiencies are raw percentages obtained or percentages extrapolated from dose–response curves. Theoretical antiradical capacity of one PBNLP mole is the sum of experimental capacities of one LAMPBN mole and of one LGT mole. Theoretical antiradical capacity of mixtures that provides 1.33, 1 or 0.67 LAMPBN moles and 0.67, 1 or 1.33 LGT moles is the sum of experimental capacities of 1.33, 1 or 0.67 LAMPBN moles and of 0.67, 1 or 1.33 LGT moles, respectively. According to quantification limits of the KRL hemolysis test, percentage differences between experimental and theoretical HT_{50} increases less than 5% are considered as non-significantly different at $P < 0.05$.

than the antioxidant protection induced by mixtures of LAMPBN and LGT at concentrations above 50 μM (Table 1) and by mixtures of LAMPBN and lipoate in the whole concentration range from 0 to 500 μM (Results not shown). Nevertheless, we observed that the overall experimental antioxidant efficiency of one mole of PBNLP kept lower than the theoretical antioxidant capacities of LAMPBN and LGT mixtures independently of the molar ratio (Table 1). Thus, the combination of water-soluble derivatives of PBN and lipoic acid in an amphiphilic molecule PBNLP did not induced synergistic interactions between these two compounds, but it markedly diminished their antagonistic actions highlighted by the free radical-mediated hemolysis test.

It is noteworthy that the measured antiradical efficiency using this hemolysis test reflected the overall antioxidant behavior of the tested compounds including exogenous radical scavenger activity but also metal ion chelator properties, protective actions on the intrinsic antioxidant defense system and regenerative effects on antioxidant molecules involved in the regulation of the intra- and extra-cellular redox status. Thus, numerous chemical mechanisms at cell cytoplasmic, cell membranes and/or extra-cellular levels may be involved in the antagonism that occurred between LAMPBN and LGT or lipoate. Therefore, further studies are needed to thoroughly investigate specific chemical mechanisms underlying antagonistic antioxidant effects between the water-soluble derivatives of PBN and lipoate that are profoundly reduced by their chemical association in PBNLP.

All together, these findings pointed out that the chemical association of water-soluble derivatives of PBN and lipoic acid has a strong beneficial effect on blood resistance to free radical attack as compared to these derivatives in admixtures and to PBN and lipoic acid alone. Moreover, it suggested that the PBNLP beneficial effect is partly governed by the amphiphilic character of the molecule that probably increased its bioavailability and by the reduction of negative chemical interactions between the two PBNLP constituents LAMPBN and LGT. These preliminary encouraging results on the in vitro protection of blood against oxidative stress emphasized the benefit of the synthesis of new amphiphilic antioxidant molecules such as PBNLP to improve their biological effects. In vivo studies are now needed to further confirm the beneficial effect of PBNLP and to investigate the therapeutic benefit of modified antioxidant molecules as compared to unmodified molecules. The use of the free radical-mediated hemolysis test could also be helpful for this purpose.

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