Bioorganic & Medicinal Chemistry Letters 20 (2010) 7405–7409

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Cholesterol-based α-phenyl-*N*-*tert*-butyl nitrone derivatives as antioxidants against light-induced retinal degeneration

Fanny Choteau [a], Grégory Durand [a,*], Isabelle Ranchon-Cole [b], Christine Cercy [b], Bernard Pucci [a,*]

[a] *Laboratoire de Chimie Bioorganique et des Systèmes Moléculaires Vectoriels, Université d'Avignon et des Pays de Vaucluse, Faculté des Sciences, 33 Rue Louis Pasteur, F-84000 Avignon, France*

[b] *Laboratoire de Biophysique des Handicaps Sensoriels (UE 2667), Université d'Auvergne, 28 Place Henri Dunant, F-63000 Clermont-Ferrand, France*

ARTICLE INFO

Article history:
Received 2 September 2010
Revised 6 October 2010
Accepted 7 October 2010
Available online 20 October 2010

Keywords:
α-Phenyl-*N*-*tert*-butyl nitrone
Amphiphiles
Antioxidants
Reactive oxygen species
Retinal degeneration

ABSTRACT

Two cholesterol-based α-phenyl-*N*-*tert*-butyl nitrone derivatives were synthesized as antioxidants against light-induced retinal degeneration. Whereas nitrone **10** significantly protected retina against bright fluorescent light exposure when injected into the vitreous at 1 mM, no protection was observed with nitrone **6**. The parent compound α-phenyl-*N*-*tert*-butyl nitrone also exhibited protective activity at 9 mM but not at 1 mM. This suggests that nitrone **10** may be a candidate for the treatment of retinal diseases.

© 2010 Elsevier Ltd. All rights reserved.

The retina is a light sensitive tissue lining the inner surface of the eye and is constituted of several cellular types. Among them are the photoreceptor cells whose function is to transform the light signal into an electrophysiological signal, which is then transmitted to the brain. Retinal degeneration is characterised by a progressive loss of photoreceptor cells leading to visual loss. In inherited and aged-related retinal degeneration, the photoreceptor cells undergo apoptosis as the final common death pathway converging the primary defects.[1] Because apoptotic cell death underlies light-induced retinal damage,[2] the excessive light-induced photoreceptor cells death represents a suitable model system to study retinal degeneration.[3] For instance, synthetic antioxidants and free radical scavengers such as dimethylthiourea,[4,5] cyclic nitroxides,[6] edaravone[7] and α-phenyl-*N*-*tert*-butyl nitrone (PBN)[8] have already shown potency against light-induced retinal degeneration.

Indeed, since the seminal work of Novelli et al.,[9] nitrone spin-traps have been widely used as protective agents in several biological models with particular attention to PBN (Fig. 1).[10] Originally designed as a probe for the scavenging of oxygen and carbon-centred free radicals, PBN has also been shown to have several pharmacologic effects, such as preventing the induction of inducible nitric oxide synthase (iNOS), inhibiting the expression of multiple cytokine genes, activating transcription factors, or inhibiting the expression of multiple apoptosis-associated genes.[11,12] On the basis of these data, the selective targeting of nitrones as therapeutics has been an active field of research for the past decade. For example, with the aim to target cellular membranes, lipophilic nitrones bearing one or two long alkyl chains have been developed[13–15] as well as a lipophilic β-cyclodextrin cyclic nitrone conjugate[16] or a cholesteryl ester of DEPMPO[17] (Fig. 1).

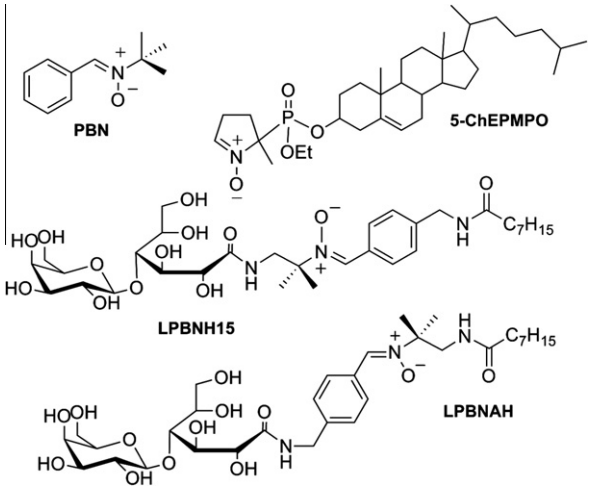


Figure 1. Chemical structures of PBN, 5-ChEPMPO, LPBNH15 and LPBNAH.

* Corresponding authors. Tel.: +33 (0)4 9014 4445 (G.D.); tel.: +33 (0)4 9014 4442 (B.P.).

E-mail addresses: gregory.durand@univ-avignon.fr (G. Durand), bernard.pucci@univ-avignon.fr (B. Pucci).

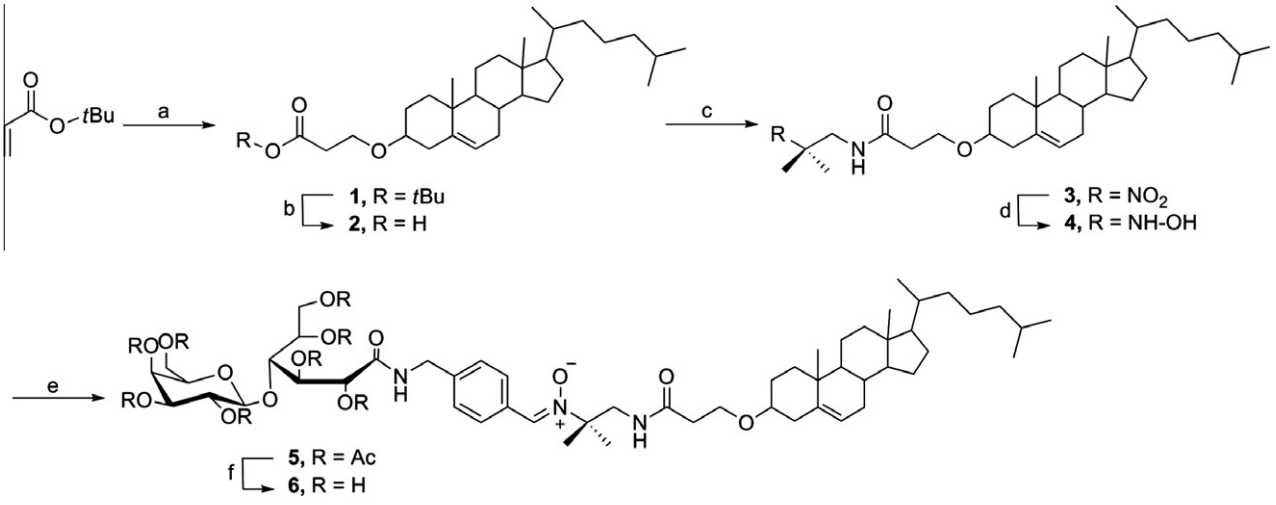
0960-894X/$ - see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2010.10.037

In our group, we have demonstrated that amphiphilic nitrones, comprised of a hydrophilic polar head and a lipophilic group, exhibited high potency in preventing oxidative stress-mediated damages in in vitro,[18,19] ex vivo[20] and in vivo models.[21,22] The amphiphilic nature is very likely responsible for improved membrane crossing ability and therefore enhanced bioavailability and bioactivity. Very recently, two amphiphilic amide nitrones called LPBNH15 and LPBNAH (Fig. 1) were found to rescue cell cultures and aquatic organisms exposed to lethal doses of various oxidotoxins.[23] They were also found to decrease electron and proton leakage in mitochondria at nanomolar concentrations suggesting that amphiphilic amide nitrones are not only free radical scavengers but may act as bioenergetic agents.[23] However, because of their relatively short C_7H_{15} alkyl chain, these amphiphilic amide nitrones, exhibiting very good water solubility, may have a poor membrane crossing ability and so would exhibit limited efficiency as neuroprotective agents.

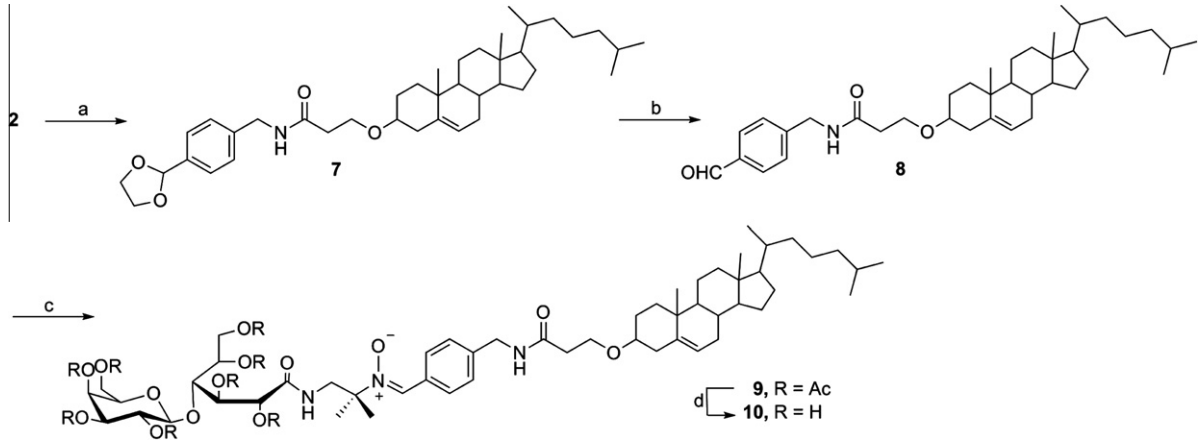
Therefore, with the expectation that the presence of a bulky lipophilic group would impart better membrane affinity and higher lipophilicity, we have designed two new amide nitrones **6** and **10**, in which the alkyl chain is replaced by a cholesterol moiety. Whereas in nitrone **6** the cholesterol is grafted onto the *N-tert* butyl group, in nitrone **10** the cholesterol is grafted onto the aromatic ring. In order to evaluate the neuroprotective effect of these new compounds, we used the light-induced retinal degeneration model.[5] Albino rats were treated by intravitreal injection of nitrone agents 18 h before being exposed for 24 h to a bright fluorescent light, and the retinal status was evaluated by histology.

The convergent synthetic pathway of the cholesteryl nitrone **6**, bearing cholesterol on the *N-tert*-butyl group, is based on three key steps:[24] (i) synthesis of a polar lactobionamide benzaldehyde from 4-cyanobenzaldehyde according to the previously reported procedure;[25] (ii) synthesis of a lipophilic cholesterol-based *N-tert*-butylhydroxylamine from 2-methyl-2-nitropropanol (Scheme 1); (iii) formation of the nitronyl function by condensation of the hydroxylamine to the benzaldehyde under inert atmosphere (Scheme 1). Cholesterol was first grafted onto *tert*-butyl acrylate in the presence of NaH to give, after purification by flash chromatography, compound **1** in 72% yield. Removal of the *tert*-butyl protective group was carried out under acidic condition in a 1:1 (v/v) TFA/CH_2Cl_2 mixture to give compound **2** in quantitative yield. The coupling reaction of 2-methyl-2-nitro-propylamine[18] to compound **2** was next achieved using DCC/HOBt as coupling reagents to lead to compound **3** in 96% yield. Then, the nitro group of **3** was selectively reduced using Zn/NH_4Cl in a 3:1 (v/v) THF/H_2O mixture, according to our general procedure,[23] to give hydroxylamine **4** in 40% yield. The nitrone functional group was obtained by reaction of the hydrophobic cholesterol-based *N-tert*-butyl hydroxylamine **4** with *N*-(4-formylbenzyl)octa-*O*-acetyl-lactobionamide[25] in a 3:2 (v/v) THF/AcOH mixture at 60 °C. In order to avoid the oxidation of the hydroxylamine, the reaction was carried out in the dark and under argon atmosphere and small amounts of hydroxylamine were regularly added to the mixture. It is important to note that despite the bulky cholesterol and lactobionamide groups, the kinetic of the coupling reaction was dramatically increased by addition of acetic acid to the milieu as previously observed.[23] Extensive purification by flash chromatography on silica gel (eluent: EtOAc) and gravity size exclusion chromatography on Sephadex LH-20 resin (eluent: CH_2Cl_2/MeOH 1:1 (v/v)), led to the pure nitrone **5** in 58% yield. Finally, Zemplén de-O-acetylation of the lactobionamide group followed by purification by size exclusion chromatography led to the lipophilic nitrone **6** in 84% yield.

The synthesis of the reverse analogue **10**, bearing the cholesterol on the aromatic ring, used a connected synthetic pathway based on three key steps:[24] (i) synthesis of a polar lactobionamide *N-tert*-butylhydroxylamine from 2-methyl-2-nitropropanol according to the procedure recently described;[23] (ii) synthesis of a lipophilic cholesterol-based *N*-4-formyl benzylamide from 4-cyanobenzaldehyde (Scheme 2); (iii) condensation of the hydroxylamine to the benzaldehyde to lead to the nitrone derivative (Scheme 2). First, the cholesterol derivative **2** was grafted onto 4-[1,3]dioxolan-2-ylbenzylamine[25] in the presence of DCC/HOBt to give compound **7** in 72% yield. Then, dioxolane group removal was carried out in a 7:3 (v/v) AcOH/H_2O mixture to give compound **8** in quantitative yield. Nitrone **9** was prepared following the same procedure as described above. The lactobionamide-based *N-tert*-butyl hydroxylamine previously published[23] was grafted onto the cholesterolyl benzaldehyde **8** in a 3:2 (v/v) THF/AcOH mixture. The reaction was carried out in the dark under argon atmosphere and small amounts of hydroxylamine were regularly added to the mixture. After purification by flash chromatography on silica gel (eluent: EtOAc) and gravity size exclusion chromatography on Sephadex LH-20 resin (eluent: CH_2Cl_2/MeOH 1:1 (v/v)), acetylated nitrone **9** was isolated in 82% yield. Finally, Zemplén de-O-acetylation gave the lipophilic nitrone **10** in 96% yield. The lipophilic nitrones **6** and **10** and their acetylated derivatives **5** and **9** were



Scheme 1. Synthesis of **6**. Reagents and conditions: (a) cholesterol, NaH, THF, rt, 16 h, 72%; (b) TFA/CH_2Cl_2 2:8 (v/v), rt, 2 h, 81%; (c) 2-methyl-2-nitro-propylamine, DCC, HOBt, DIEA, CH_2Cl_2, 1 h, rt, 96%; (d) Zn dust, NH_4Cl, THF/H_2O 3:1 (v/v), 0 °C, 1 h, 40%; (e) *N*-(4-formylbenzyl)octa-*O*-acetyllactobionamide, THF/AcOH 3:2 (v/v), dark, 60 °C, 24 h, 58%; (f) MeONa, MeOH, rt, 3 h, 84%.



Scheme 2. Synthesis of **10**. Reagents and conditions: (a) 4-[1,3]dioxolan-2-ylbenzylamine, DCC, HOBt, DIEA, CH_2Cl_2, rt, 16 h, 72%; (b) AcOH/H_2O 7:3 (v/v), rt, 12 h, 92%; (c) *N*-(octa-*O*-acetyl-lactobionyl)-2-methyl-2-hydroxylaminopropanamide, THF/AcOH 3:2 (v/v), dark, 60 °C, 8 h, 82%; (d) MeONa, MeOH, rt, 3 h, 96%.

characterised by ^1H and ^{13}C NMR as well as mass spectrometry. The purity of **6** and **10** was confirmed by C18 reverse phase HPLC (UV detection at 298 nm) and was higher than 95%.

Despite the presence of a lactobionamide group, the two nitrones were found to be insoluble in water at concentration ~0.5 g/L demonstrating that the cholesterol group provides a high lipophilic character to the molecule. The relative lipophilicities of these two analogues were further measured by a chromatographic method.[24] Whereas, PBN exhibits a log k'_W value of ~1.7,[23] the two cholesteryl nitrones **6** and **10** were found to be much more lipophilic with values of 8.83 and 8.98, respectively. When comparing to the log k'_W value of amphiphilic amide nitrones LPBNAH (2.76) and LPBNH15 (2.86),[23] this demonstrates the effect of the very hydrophobic cholesterol group on the overall lipophilicity of the nitrone derivatives. One can also note that the grafting of the cholesterol onto the aromatic ring or the *N*-*tert*-butyl group does not affect the lipophilicity of the nitrone. This confirms our recent data on amphiphilic amide nitrones showing that reverse analogues exhibit only slightly higher log k'_W values.[23]

We next focused our attention on the effects of this novel series of cholesterol-based nitrones against retinal damage induced by light exposure. Although, the primary function of photoreceptors is to absorb light, they are very sensitive to light[26] and it has been demonstrated that excessive light exposure induces reactive oxygen species overproduction,[3] which can lead to retinal dysfunction and cell death. To evaluate the protective effect of nitrones on photoreceptor cells, we measured the thickness of the outer nuclear layer (ONL), which contains the photoreceptor nuclei, after exposure to damaging light.[24] An unexposed-untreated-group was done in parallel for control as well as a DMSO 2%-treated group. Indeed, both cholesterol-based PBN derivatives **6** and **10** were insoluble in PBS 1× at 1 mM and so DMSO was used instead, whereas PBN was normally dissolved in PBS 1×. However, it has to be noted that the effects of intravitreal injection of PBS 1× and DMSO 2% against the damaging effect of light were previously assayed, and as a result no protection of the ONL thickness was observed.[2] Therefore, in the present study DMSO 2% group is used as equivalent to an untreated-group. The ONL thickness measurements give an estimate of the number of photoreceptor cells in the retina and therefore are a good estimate of the potency of the nitrone, the thicker the layer, the stronger the protection by the nitrone. In the control group, the ONL was 35–40 μm thick all along the retina. As already observed by us[2,8] and others,[27,28] a 24-h exposure to bright fluorescent light induced a significant reduction of the ONL thickness of the retina, with a stronger effect in the superior side. As shown in Figure 2C, in the DMSO-exposed group the area under the ONL thickness curve of the inferior side is significantly reduced by 23% ($p = 0.047$) compared to the control, while that of the superior side is reduced by 51% ($p = 0.004$). Injection of PBN at 9 mM into the vitreous offered a significant protection ($p < 0.009$) to the retina from the damaging effect of light compared to the DMSO-exposed group, with the ONL thickness in both inferior and superior sides similar to that of the control (Fig. 2A). Obviously, no significant reduction of the area under the inferior or the superior ONL thickness in the PBN group was also observed (Fig. 2C). This is in full agreement with our previous observations where PBN was found to exhibit neuroprotective effects against light-induced retinal degeneration when injected intraperitoneally.[8] However, when PBN was injected intravitreally at 1 mM, no protection was observed showing that this concentration is too low to provide protection. With regard to the cholesterol-based PBN derivatives administered into the vitreous at 1 mM, the outer nuclear layer was thicker in the **10**-treated group than in the DMSO-exposed one (Fig. 2B). The area under the superior side of the ONL thickness curve of the **10**-treated group was also significantly higher ($p < 0.003$) than that of the DMSO-exposed group. Therefore, nitrone **10** at 1 mM can efficiently protect retina from light-induced damage, this protection being similar to that of PBN at 9 mM. On the contrary, the outer nuclear layer thicknesses in the **6**-treated group and in the DMSO-exposed group were similar, and the area under the curve was not significantly different between these two groups. This clearly demonstrates that nitrone **10** but not nitrone **6** protects the photoreceptor cells from light-induced degeneration. Since both compounds possess a very close lipophilic character, as demonstrated by their log k'_W values, one can expect them to have similar membrane crossing ability. Therefore, the difference of protection is very likely due to other parameters. As reported with LPBNAH and LPBNH15, a simple reversal of the position of the polar head and the hydrophobic chain on the nitrone group can alter the scavenging properties but also the antioxidant potency of the nitrones.[23] Although LPBNAH and LPBNH15 are structural isomers as they are differentiated only from the position of the polar head and the hydrophobic chain, LPBNH15 exhibited higher potency than LPBNAH.[23] The data herein supports the conclusion that amphiphilic nitrones possessing the polar head group on the *tert*-butyl group and the lipophilic group on the aromatic ring are more potent than their reverse analogues in preventing oxidative stress-mediated damages. Moreover, as the injection of nitrones was done 18 h before inducing the light stress and considering the short half-live of PBN in the retina (~2 h),[8] it is likely that the concentration of molecules still present into the

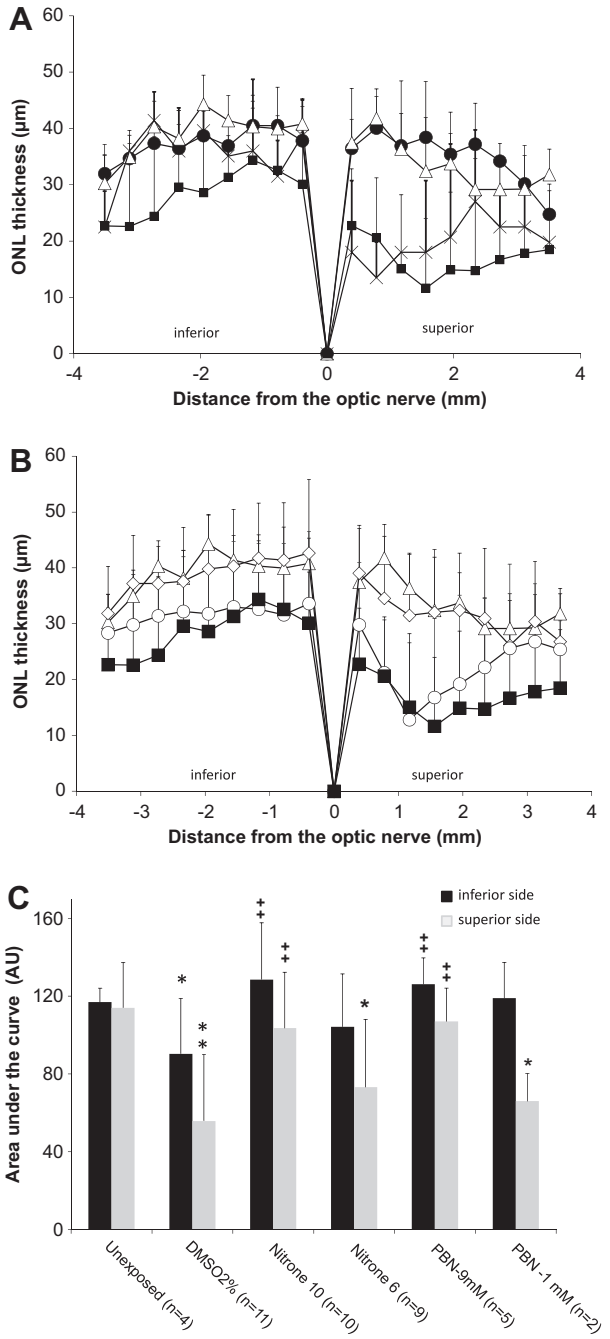


Figure 2. Effects of nitrones on retinal damage induced by exposure to light. The outer nuclear layer was measured from the optic nerve (0) to the superior and inferior ora serata. Control rats were untreated and unexposed to the damaging light. Two-microliter intravitreal injections of compounds were done 18 h before exposure to a 24-h bright fluorescent light. (A) Control rats (●), PBN at 9 mM (△), PBN at 1 mM (×), and DMSO 2% (■). (B) Nitrone **10** (◇) and nitrone **6** (○) at 1 mM, PBN at 9 mM (△), and DMSO 2% (■). (C) Area under the ONL thickness curves in the superior and the inferior retina calculated using the programme Origin 6.0 (Microcal). * compared to unexposed group; + compared to DMSO 2% group. One symbol: p <0.05; two symbols: p <0.01.

retina at the time of light exposure is extremely low. This suggests, in agreement with the literature,[10–12] that the protective effects provided by the treatment with nitrone agents is not dependent on their free radical scavenging capacities. Therefore, further experiments are needed to elucidate the molecular mechanism explaining the neuroprotective effect of PBN and nitrone **10** compared to nitrone **6**.

In conclusion, two novel amide nitrones bearing both a cholesterol and lactobionamide group were synthesized through a convergent synthetic route. We investigated the protective effects of these two nitrone derivatives against light-induced retinal damage as well as those of the parent compound PBN. Treatment with PBN at 9 mM resulted in a significant protection of the retina while no protection was observed when used at 1 mM. The nitrone **10** bearing the cholesterol group on the aromatic ring was found to significantly protect the retina against fluorescent light exposure at only 1 mM but no protection was observed with nitrone **6**, its reverse analogue, which bears the cholesterol group on the *tert*-butyl group. These findings confirm that oxidative stress plays a crucial role in light-induced retinal damage and that administration of nitrone-type antioxidants **10** and PBN prevents light-induced photoreceptor degeneration. This suggests that the cholesterol-based nitrone **10** may be a candidate for the treatment of retinal diseases.

Acknowledgements

F.C. is the recipient of a fellowship from the 'Région Provence Alpes Côte d'Azur' and Targeting System Pharma company.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.10.037.

References and notes

1. Remé, C. E.; Grimm, C.; Hafezi, F.; Wenzel, A.; Williams, T. P. *News Physiol. Sci.* **2000**, *15*, 120.
2. Perche, O.; Doly, M.; Ranchon-Cole, I. *Invest. Ophthalmol. Vis. Sci.* **2007**, *48*, 2753.
3. Wenzel, A.; Grimm, C.; Samardzija, M.; Remé, C. E. *Prog. Retin. Eye Res.* **2005**, *24*, 275.
4. Organisciak, D. T.; Darrow, R. M.; Jiang, Y. L.; Marak, G. E.; Blanks, J. C. *Invest. Ophthalmol. Vis. Sci.* **1992**, *33*, 1599.
5. Ranchon, I.; Gorrand, J. M.; Cluzel, J.; Droy-Lefaix, M. T.; Doly, M. *Invest. Ophthalmol. Vis. Sci.* **1999**, *40*, 1191.
6. Tanito, M.; Li, F.; Elliott, M. H.; Dittmar, M.; Anderson, R. E. *Invest. Ophthalmol. Vis. Sci.* **2007**, *48*, 1900.
7. Imai, S.; Inokuchi, Y.; Nakamura, S.; Tsuruma, K.; Shimazawa, M.; Hara, H. *Eur. J. Pharmacol.* **2010**, *642*, 77.
8. Ranchon, I.; Chen, S.; Alvarez, K.; Anderson, R. E. *Invest. Ophthalmol. Vis. Sci.* **2001**, *42*, 1375.
9. Novelli, G. P.; Angiolini, P.; Tani, R.; Consales, G.; Bordi, L. *Free Radic. Res.* **1986**, *1*, 321.
10. Floyd, R. A.; Kopke, R. D.; Choi, C.-H.; Foster, S. B.; Doblas, S.; Towner, R. A. *Free Radic. Biol. Med.* **2008**, *45*, 1361.
11. Hensley, K.; Robinson, K. A.; Gabbita, S. P.; Salsman, S.; Floyd, R. A. *Free Radic. Biol. Med.* **2000**, *28*, 1456.
12. Floyd, R. A.; Hensley, K.; Kelleher-Andersson, J. A.; Wood, P. L. *Mech. Ageing Dev.* **2002**, *123*, 1021.
13. Morandat, S.; Durand, G.; Polidori, A.; Desigaux, L.; Bortolato, M.; Roux, B.; Pucci, B. *Langmuir* **2003**, *19*, 9699.
14. Stolze, K.; Udilova, N.; Rosenau, T.; Hofinger, A.; Nohl, H. *Biochem. Pharmacol.* **2003**, *66*, 1717.
15. Hay, A.; Burkitt, M. J.; Jones, C. M.; Hartley, R. C. *Arch. Biochem. Biophys.* **2005**, *435*, 336.
16. Han, Y.; Liu, Y.; Rockenbauer, A.; Zweier, J. L.; Durand, G.; Villamena, F. A. *J. Org. Chem.* **2009**, *74*, 5369.
17. Hardy, M.; Ouari, O.; Charles, L.; Finet, J.-P.; Iacazio, G.; Monnier, V.; Rockenbauer, A.; Tordo, P. *J. Org. Chem.* **2005**, *70*, 10426.
18. Durand, G.; Polidori, A.; Salles, J.-P.; Pucci, B. *Bioorg. Med. Chem. Lett.* **2003**, *13*, 859.
19. Durand, G.; Prosak, R. A.; Han, Y.; Ortial, S.; Rockenbauer, A.; Pucci, B.; Villamena, F. A. *Chem. Res. Toxicol.* **2009**, *22*, 1570.
20. Tanguy, S.; Durand, G.; Reboul, C.; Polidori, A.; Pucci, B.; Dauzat, M.; Obert, P. *Cardiovasc. Drugs Ther.* **2006**, *20*, 147.
21. Poeggeler, B.; Durand, G.; Polidori, A.; Pappolla, M. A.; Vega-Naredo, I.; Coto-Montes, A.; Boeker, J.; Hardeland, R.; Pucci, B. *J. Neurochem.* **2005**, *95*, 962.
22. Asanuma, T.; Yasui, H.; Inanami, O.; Waki, K.; Takahashi, M.; Iizuka, D.; Uemura, T.; Durand, G.; Polidori, A.; Kon, Y.; Pucci, B.; Kuwabara, M. *Chem. Biodivers.* **2007**, *4*, 2253.
23. Durand, G.; Poeggeler, B.; Ortial, S.; Polidori, A.; Villamena, F. A.; Böker, J.; Hardeland, R.; Pappolla, M. A.; Pucci, B. *J. Med. Chem.* **2010**, *53*, 4849.

24. Details for the synthesis and characterization of compounds **1**–**10** as well as for in vivo experiments are given in Supplementary data.
25. Ouari, O.; Chalier, F.; Bonaly, F.; Pucci, B.; Tordo, P. *J. Chem. Soc., Perkin Trans. 2* **1998**, 2299.
26. Noell, W. K.; Walker, V. S.; Kang, B. S.; Berman, S. *Invest. Ophthalmol.* **1966**, *5*, 450.
27. Rapp, L. M.; Naash, M. I.; Wiegand, R. D.; Joel, C. D.; Nielsen, J. C.; Anderson, R. E. In *Retinal Degeneration: Experimental an Clinical Studies*; LaVail, M. M., Hollyfield, J. G.; Anderson, R. E., Eds.; Alan R. Liss: New York, 1985; pp 421–437.
28. Káldi, I.; Martin, R. E.; Huang, H.; Brush, R. S.; Morrison, K. A.; Anderson, R. E. *Mol. Vis.* **2003**, *9*, 337.