Nitrones for understanding and ameliorating the oxidative stress associated with aging

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Received: 12 February 2009 / Accepted: 7 May 2009 / Published online: 29 May 2009
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Abstract Oxidative damage from reactive oxygen species (ROS) and the carbon-centred radicals arising from them is important to the process of aging, and age-related diseases are generally caused, exacerbated or mediated by oxidative stress. Nitrones can act as spin traps to detect, identify, quantify and locate the radicals responsible using electron paramagnetic resonance (EPR or ESR) spectroscopy, and a new carnitine-derived nitrone, CarnDOD-7C, designed to accumulate in mitochondria is reported. Nitrones also have potential as therapeutic antioxidants, e.g. for slowing cellular aging, and as tools for chemical

biology. Two low-molecular weight nitrones, DIPEGN-2 and DIPEGN-3, are reported, which combine high water-solubility with high lipophilicity and obey Lipinski's rule of five.

Keywords Aging · Oxidative stress · Reactive oxygen species · Radicals · Electron paramagnetic resonance spectroscopy · Nitrones · Antioxidants

Introduction

The oxidative damage caused to cells by reactive oxygen species (ROS), such as hydroxyl radicals, peroxy radicals and superoxide radical anions, and the carbon-centred radicals arising from them, appear to be important to aging (Halliwell and Gutteridge 1999) though it is not yet clear whether oxidative stress determines life span (Muller et al. 2007). What is clear is that with aging comes diseases caused, exacerbated or mediated by oxidative damage, including neurodegeneration, stroke, cancers, heart disease, arthritis, and autoimmune diseases (Halliwell and Gutteridge 1999; Muller et al. 2007). We will discuss here how nitrones can be used to detect, identify, quantify and locate the species responsible for oxidative stress; how they can ameliorate oxidative stress and how they can be used as biological tools to elucidate biological mechanisms of aging (chemical biology). In general we will concentrate on examples from our own work

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and will include some new compounds that may be of interest to the field of aging research.

Materials and methods

General information on synthesis

All reactions under an inert atmosphere were carried out using oven-dried glassware. Solutions were added via syringe. Reagents were obtained from commercial suppliers and used without further purification unless otherwise stated. Dichloromethane (DCM) was dried where necessary using a solvent drying system, Puresolv, in which solvent is pushed from its storage container under low nitrogen pressure through two stainless steel columns containing activated alumina and copper. Dimethylformamide (DMF) was stirred overnight with calcium hydride and distilled under reduced pressure. ^1H and ^{13}C NMR spectra were obtained on a Bruker DPX/400 spectrometer operating at 400 and 100 MHz, respectively. All coupling constants are measured in Hertz.

Synthesis of CarnDOD-7C

(R)-carnitine octyl ester chloride

Acetyl chloride (2.0 ml, 27.8 mmol) was added to 1-octanol (36.0 ml, 289 mmol) stirred at 0°C under argon. After 15 min, the mixture was allowed to warm to room temperature and *(R)*-carnitine inner salt (1.51 g, 9.3 mmol, 1 equiv) was added. The mixture was heated to reflux for 3 h. Excess 1-octanol was removed at 130°C under reduced pressure and the oil triturated with diethyl ether to give a gum. The diethyl ether was decanted off and the gum washed again with diethyl ether. The gum was dissolved in DCM, the solvent removed under reduced pressure to give the *(R)*-carnitine octyl ester chloride as a gum (2.89 g, 99%). ^1H NMR (400 MHz, CDCl_3) δ : 0.86 (3H, t, J 6.3 Hz), 1.26–1.27 (12H, m), 1.58–1.61 (2H, m), 2.66 (1H, dd, J 7.3 and 16.9 Hz), 2.76 (1H, dd, J 5.4 and 16.9 Hz), 3.45 (9H, s), 3.46–3.60 (1H, m), 4.03–4.01 (1H, m). ^{13}C NMR (100 MHz, CDCl_3) δ : 14.1, 22.7, 25.8, 28.5, 29.2, 31.8, 39.9, 55.1, 62.7, 62.9, 65.2, 70.5, 171.2. M/z (FAB): 274 (M^+ , 100%). HRMS calcd for $\text{C}_{15}\text{H}_{32}\text{O}_3\text{N}$ requires 274.2382, found 274.2383. $[\alpha]_{\text{D}}^{24} = -28.7$ ($C=1.0$, CHCl_3).

CarnDOD-7C

A 0.12 M solution of *(R)*-carnitine octyl ester chloride (1 equiv), DCC (1.5 equiv), *N*-[4-dodecyloxy-2-(6'-carboxyhex-1'-yloxy)benzylidene]-*N*-*tert*-butylamine *N*-oxide [1.04 equiv, prepared by the same route as reported for DOD-8C (Hay et al. 2005), except using ethyl 7-bromoheptanoate instead of methyl 8-bromooctanoate], and 4-(dimethylamino)pyridine (5 mol%) in dry DCM was stirred at room temperature for 22 h. The solution was filtered and the filtrate was evaporated under reduced pressure. The residue was redissolved in DCM and again filtered and concentrated. Finally, column chromatography (SiO_2 , $\text{CHCl}_3/\text{MeOH}$, 9/1) gave the ester as a viscous oil (62%, 278 μmol). ^1H NMR (400 MHz, MeOD) δ : 0.91–0.93 (6H, m), 1.32–1.50 (32H, m), 1.60 (9H, s), 1.64–1.72 (4H, m), 1.80–1.90 (4H, m), 2.44 (2H, t, J 7.4 Hz), 2.80 (2H, d, J 6.0 Hz), 3.22 (9H, s), 3.72 (1H, broad d, J 14.2 Hz), 3.86–3.91 (1H, m), 4.05–4.13 (6H, m), 5.65–5.70 (1H, m), 6.59–6.62 (2H, m), 8.16 (1H, s), 9.13 (1H, d, J 8.7 Hz). ^{13}C NMR (100 MHz, MeOD) δ : 14.5, 23.7, 23.8, 25.7, 27.1, 27.2, 28.5, 29.7, 29.8, 30.0, 30.3, 30.4, 30.5, 30.7, 30.8, 33.0, 33.1, 35.0, 38.1, 54.6, 66.2, 66.4, 69.3, 69.5, 71.4, 100.1, 106.4, 113.2, 129.5, 131.8, 161.0, 164.6, 170.9, 174.0. M/z (FAB): 761 (M^+ , 100%), 691 (50), 256 (70), 87 (78). HRMS calcd for $\text{C}_{45}\text{H}_{81}\text{O}_7\text{N}_2$ requires 761.6044, found 761.6045. $[\alpha]_{\text{D}}^{24} = -11.3$ ($C=1.0$, MeOH).

Synthesis of DIPEGN-2 and DIPEGN-3

Glycol monochlorohydrin or chloroethanol (2.2 equiv) was added to a stirred 0.6–1.2 M solution of 2,4-dihydroxybenzaldehyde (1 equiv), K_2CO_3 (3 equiv) and KI (2.2 equiv) in dry DMF under argon. The mixture was maintained at 100–130°C for 4–7 days. The mixture was allowed to cool, toluene was added and the solvent removed under reduced pressure. The addition of toluene and removal of solvent was carried out ten times. The residue was dissolved in DCM, filtered, dried over magnesium sulfate and the solvent removed under reduced pressure to give a diPEGylated aldehyde in sufficient purity for the next step. A 0.19 M solution of the acetate salt of *N*-*tert*-butylhydroxylamine (1.5 equiv) in EtOH was added to a flask containing the aldehyde (1 equiv) and NaHCO_3 (1.5 equiv). The mixture was stirred and heated under reflux under argon for 3–7 days. The

residue obtained from the above procedure was dissolved in methanol and cooled to 0°C. DCM:ether (50:50) was added to precipitate inorganic impurities, which were then removed by filtration. The filtrate was evaporated under reduced pressure and the residue further purified as indicated below.

N-[2,4-Bis-[2-(2-hydroxyethoxy)ethoxy]benzylidene]-*N*-tert-butylamine *N*-oxide DIPEGN-2

The residue was purified by column chromatography on silica using gradient elution [DCM to DCM:methanol (9:1)] to give the nitron as an oil (56%, 1.21 mmol). ¹H NMR (400 MHz, CDCl₃) δ: 1.49 (9H, s, CH₃), 3.28 (2H, broad singlet, OH), 3.58–3.55 (4H, m), 3.67–3.64 (4H, m), 3.80–3.74 (4H, m), 4.08–4.05 (4H, m), 6.47–6.42 (2H, m), 7.91 (1H, s), 9.25 (1H, d, *J* 8.8 Hz). ¹³C NMR (100 MHz, CDCl₃) δ: 28.2, 61.3, 67.3, 68.0, 69.2, 69.3, 70.1, 72.5, 72.6, 99.6, 105.0, 113.6, 124.9, 130.2, 158.0, 161.1. *m/z* (FAB+): 386 (100%, *M* + *H*⁺).

N-[2,4-Bis-[2-[2-(2-hydroxyethoxy)ethoxy]ethoxy]benzylidene]-*N*-tert-butylamine *N*-oxide DIPEGN-3

The residue was dissolved in water and extracted first with diethyl ether (2 ×), and then with DCM (6 ×). The last four DCM extracts were evaporated under reduced pressure to give the nitron as an oil (69% yield, 6.85 mmol). ¹H NMR (400 MHz, CDCl₃) δ: 1.49 (9H, s), 3.18 (2H, broad singlet, OH), 3.51–3.48 (4H, m), 3.64–3.56 (12H, m), 3.83–3.75 (4H, m), 4.11–4.06 (4H, m), 6.50–6.40 (2H, m), 7.87 (1H, s, CH), 9.28 (1H, d, *J* 8.6 Hz). ¹³C NMR (100 MHz, CDCl₃) δ: 28.0, 61.3, 67.2, 67.9, 69.3, 69.4, 69.9, 70.0, 70.1, 70.5, 70.7, 72.4, 72.4, 99.6, 105.0, 113.6, 124.6, 130.0, 157.9, 161.0. *m/z* (FAB+): 474 (10%, *M* + *H*⁺), 330 (100).

Electron paramagnetic resonance spectroscopy

Iron(II) sulfate (50 μl of a 1.66 mM aqueous solution) and hydrogen peroxide (10 μl of a 3.3 mM aqueous solution) were added to a solution of CarnDOD-7C in methanol (100 μl of a 10 mM solution). The solution [6.25 mM CarnDOD-7C, 0.21 mM hydrogen peroxide, 0.52 mM iron(II) sulfate in water:methanol (6:10)] was then immediately transferred to a quartz flat cell and placed in the electron paramagnetic resonance (EPR)

Fig. 1 Reaction of a hydrogen atom transfer agent with radicals

spectrometer for analysis. Spectra were acquired on a Bruker e-scan bench-top EPR machine (<http://www.bruker-biospin.com>) with a permanent magnet and a magnetic sweep circuit (centre of field = 0.345 T, sweep width 25 mT) operating at a frequency of 9.8 GHz (X-band). Hyperfine couplings were derived from simulations using WINEPR SimFonia (Bruker).

Results and discussion

One way of detecting, quantifying and locating ROS is to use small molecules that sense ROS and report their presence through switching on or switching off fluorescence, a process that lends itself to the technique of confocal microscopy. A range of such fluorescent probes that distinguish between different ROS have been developed (Soh 2006; Wardman 2007), and new ones continue to appear, a particularly impressive example being the hydrogen peroxide-sensitive mitochondria-targeted probe (Dickinson and Chang 2008). However, many fluorescent probes are susceptible to artefacts, and the process of irradiation can sometimes lead to the generation of ROS (Wardman 2007). An alternative is to use electron paramagnetic resonance (EPR or ESR) spectroscopy (Rosen et al. 1999). With the exception of hydrogen peroxide, the ROS responsible for oxidative damage are free radicals, i.e. they are molecules that have an unpaired electron. EPR spectroscopy detects only species containing unpaired electrons and can be used *in vitro* and to some extent *in vivo*.

Unfortunately, radical ROS and the carbon-centred radicals arising from them are generally too short-lived, due to their high reactivity, to be present at sufficient concentration for detection by EPR spectroscopy. A solution to this is to use molecules that

Fig. 2 Reaction of nitron spin traps with radicals

Fig. 3 Dual detector probe detects and differentiates between hydroxyl radicals, methyl radicals and iron(III) ions

react with the ROS to generate a new less-reactive, longer-lived radical that can be detected by EPR spectroscopy. Two classes of such molecules that are commonly used are hydroxylamines, which act as hydrogen-atom transfer agents, and nitron spin traps; molecules from both classes react to form stable nitroxide radicals (Soule et al. 2007). The distinction is important though—hydroxylamines give the same nitroxide radical regardless of the radical ROS or carbon-centred radical ($Y\cdot$) being detected, e.g. TEMPO-H gives TEMPO upon reaction with either hydroxyl radicals or methyl radicals ($Y\cdot = \text{HO}\cdot$ or $\text{CH}_3\cdot$, Fig. 1). This is useful for quantification as the EPR signal is uncomplicated, grows progressively with exposure and is easily measured, but it does not allow the reactive radical $Y\cdot$ to be identified. The nitron spin traps on the other hand react with different reactive radicals ($Y\cdot$) to give different nitroxide adducts (Fig. 2), which can have distinctive EPR spectra allowing identification of each reactive radical $Y\cdot$ involved. A good example of this from our own work is the dual detector probe (Fig. 3, Caldwell et al. 2007), which gives distinctive EPR signals for the three different nitroxides 1–3 resulting from reaction with hydroxyl radicals, methyl radicals and iron(III) ions (redox-active metal ions implicated in oxidative stress), respectively. Unfortunately, nitron spin traps are less effective for quantification than hydroxylamines as some of the adducts are not very stable.

In addition to quantification and identification, spin traps can be designed to locate radical ROS or carbon-centred radicals in different sub-structures and organ-

elles. A good example from our own work is the detection of radicals in membranes using the nitron DOD-8C (Hay et al. 2005), which orientates in lipid membranes with the hydrophilic carboxylate group towards the aqueous phase and the nitron moiety sunk into the membrane (Fig. 4). An anisotropic EPR spectrum for a spin adduct of this nitron confirms the trapping of radicals within membranes.

Mitochondria are believed to be the main endogenous source of the ROS that lead to cellular aging and consequent neurodegeneration (Lin and Beal 2006), particularly when there is a large membrane potential across the inner mitochondrial membrane (James et al. 2005). Indeed, murine life span can be extended by overexpression of catalase targeted to mitochondria, where the increased removal of hydrogen peroxide will reduce ROS production and consequent damage (Schriner et al. 2005). There has therefore been interest in targeting nitron spin traps to mitochondria.

The lipophilic alkyltriphenylphosphonium (TPP) cation has been pioneered as a targeting group for antioxidants by the groups of Murphy and Smith working in collaboration (Murphy and Smith 2007). TPP cations permeate biological membranes easily and accumulate up to a 1,000-fold in the mitochondrial matrix due to the large mitochondrial membrane potential across the inner mitochondrial membrane set up by the electron transport chain. A few nitron spin traps (MitoPBN, MitoBMPO, MitoDEPMPO in Fig. 5), have been designed to accumulate in mitochondria using the TPP group to scavenge and/or detect radicals generated there (Hardy et al. 2007a, b;

Fig. 4 Interaction of DOD-8C with membranes

Fig. 5 Nitrone spin traps incorporating the mitochondria-targeting TPP cation

Xu and Kalyanaraman 2007; Murphy et al. 2003). Although the TPP-group is effective and relatively non-toxic, it is not the only lipophilic cation that could act as a targeting group (Skulachev 2007; Antonenko et al. 2008; Robertson and Hartley 2009). Indeed, we have recently reported nitrone spin traps **4** and **5** bearing a pyridinium ion as a lower-molecular-weight alternative (Fig. 6; Robertson and Hartley 2009).

Different methods of targeting compounds to mitochondria are also conceivable. Recently, a 4-amino-TEMPO (the oxidized form of a hydroxylamine hydrogen atom transfer agent) conjugated to a pentapeptide fragment of the mitochondrial membrane-targeting antibiotic Gramicidin S has been reported by Wipf and co-workers (Wipf et al. 2005). Another potential method that attracted our attention is to attach the amino acid, (–)-(R)-carnitine (Skulachev 2007), which is used in the transport of fatty acids into the matrix of the mitochondrion for catabolism (Lohninger et al. 2005). We here report that we have prepared a nitrone-carnitine conjugate, CarnDOD-7C (Fig. 7), related to DOD-8C (Fig. 4, Hay et al. 2005).

Fig. 6 Nitrone spin traps bearing lipophilic pyridinium cations

Fig. 7 Reaction of CarnDOD-7C with hydroxymethyl radicals

Use of an octyl ester (Cipollone et al. 2000) leaves open two possible mechanisms for accumulation in the mitochondria: firstly, CarnDOD-7C is a lipophilic cation and could potentially accumulate in the same way as TPP cations; secondly, it could take advantage of active transport by carnitine acyltransferase. Either way, it would be expected to embed in the inner mitochondrial membrane in the same way that DOD-8C interacts with membranes. In vitro, hydroxyl radicals can be produced by the Fenton reaction between iron(II) salts and hydrogen peroxide and these will react with methanol to give hydroxymethyl radicals (Rosen et al. 1999), in the same way as hydroxyl radicals generate other carbon-centred radicals by reaction with lipids and other biomolecules in nature. When CarnDOD-7C is reacted with hydroxymethyl radicals generated in this way, it gives rise to a strong EPR signal, which has hyperfine splittings consistent with nitroxide **6** (i.e. the main signal present is a triple doublet due to coupling to the nitrogen nucleus and the α -proton, Fig. 8). Thus, CarnDOD-7C may be useful for detection of carbon-centered radicals within the inner mitochondrial membrane. On the other hand the adducts formed by reaction between hydroxyl radicals and acyclic nitrone spin traps fragment rapidly, and it was no surprise that a signal for the hydroxyl adduct of CarnDOD-7C could not be detected.

There is evidence that carnitine supplementation may reverse the age-associated decline of brain function (Lohninger et al. 2005; Shea 2007). The combination in CarnDOD-7C of carnitine and a nitrone moiety may

Fig. 8 EPR spectrum of nitroxide **6** $g=2.0047$, $A_N=15.62$ G (t), $A_H^\beta=4.31$ G (d)

provide double benefit because acyclic nitrones themselves have shown promise for the treatment of age-related diseases (Floyd et al. 2008). One possible mechanism for their action is the scavenging of the free radicals that lead to oxidative damage. As discussed above, nitrones react with these highly reactive oxygen-centered and carbon-centered radicals ($Y^\bullet$) to give less reactive nitroxides (Fig. 2), in effect they convert the highly damaging radicals responsible for oxidative stress into innocuous products. Furthermore, stability of the nitroxides towards fragmentation is not important to antioxidant activity if the overall pathway (including fragmentation) takes a reactive radical to benign products. This combined with growing evidence that nitrones reduce oxidative stress in other ways also, mean that new acyclic nitrones continue to be developed as potential therapeutics (Floyd et al. 2008).

The nitrone NXY-059 is an interesting case in point (Fig. 9), as this compound was shown to be a very

promising drug candidate for the treatment of stroke in both mice and primates (Floyd et al. 2008). Ischemic stroke involves the blood supply being cut off from part of the brain, some cells are killed outright but most receive a reduced supply of oxygen and survive, but their metabolism changes so that when the oxygen supply is restored by blood reperfusing the area, there is a burst of free radicals causing extensive cell death, so called ischemia-reperfusion injury (Halliwell and Gutteridge 1999). Consequently, the use of an antioxidant might well reduce the severity of a stroke. We have shown that NXY-059 can act as a spin trap (Hay et al. 2005). However, NXY-059 is a double salt and its organic component is small and a dianion at physiological pH. It would therefore not be expected to be able to cross any membranes, let alone the blood–brain barrier. This may go some way to explaining why NXY-059 proved ineffective in phase III clinical trials. We reasoned that it would be possible

Fig. 9 Potential therapeutic antioxidants/ tools for chemical biology

to combine high water-solubility with sufficient lipophilicity to cross membranes if the charged sulfonate groups in NXY-059 were replaced with non-ionic hydrophilic groups, and chose short polyethylene glycol chains for this purpose. We here report that we have prepared nitrones DIPEGN-2 and DIPEGN-3 (Fig. 9). DIPEGN-2 in particular is drug-like in accordance with Lipinski's rules (Lipinski et al. 1997), i.e. it has ClogP=0.95, MW=385, seven hydrogen-bond acceptors, and two hydrogen-bond donors. However, what is unique about the compound is its combination of high water-solubility with high solubility in non-polar solvents. High water-solubility would be important for intravenous or oral drug delivery, and high lipophilicity would be necessary for crossing the blood–brain barrier. DIPEGN-2's water solubility is $>5 \text{ mg ml}^{-1}$ at room temperature and its solubility in non-polar solvents is also high, e.g. $>5 \text{ mg ml}^{-1}$ in DCM. Note that the dielectric constants (a measure of solvent polarity) of water and DCM are 80.1 and 8.93, respectively (Lide 2002). DIPEGN-2 and DIPEGN-3 are potential candidates as therapeutic antioxidants for the treatment of stroke and neurodegeneration and for slowing aging at a cellular level.

We have previously demonstrated that amphiphilic Bu-4C and Bu-6C are effective antioxidants, protecting human diploid fibroblasts from oxidative damage induced by high levels of hydrogen peroxide (Sklavounou et al. 2006). This means that nitrones Bu-4C and Bu-6C are potential therapeutic antioxidants, but they also proved to be useful chemical biological tools helping to provide evidence in support of telomere independent stress responses that show associated *p16* and *p21* expression, and indicating that antioxidant-interventions can enhance cell growth without limiting p53-dependent damage responses. In the same way, DIPEGN-2 and DIPEGN-3 may be useful chemical biological tools. The key advantages of chemical biology over molecular biology techniques are that the interventions can use native cells and wild-type organisms, and the effects are immediate, dose dependent and potentially reversible, unlike standard genetic techniques such as the use of knock-outs.

In summary, we have highlighted the potential of nitrones for the detection, identification, quantification and location of ROS and the carbon-centered radicals derived from them through EPR spectroscopy, and described how nitrones also have potential as thera-

peutic antioxidants and as probes for chemical biology. We have also reported a new spin trap that is expected to accumulate in mitochondria through targeting with carnitine and two novel potential antioxidants that combine high water-solubility with lipophilicity.

Acknowledgements Scottish Enterprise for Proof of Concept funding. SPARC and the BBSRC for the purchase of the bench-top EPR spectrometer used.

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