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Potential implication of the chemical properties and bioactivity of nitrone spin traps for therapeutics

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Abstract

Nitrone therapeutics has been employed in the treatment of oxidative stress-related diseases such as neurodegeneration, cardiovascular disease and cancer. The nitrone-based compound NXY-059, which is the first drug to reach clinical trials for the treatment of acute ischemic stroke, has provided promise for the development of more robust pharmacological agents. However, the specific mechanism of nitrone bioactivity remains unclear. In this review, we present a variety of nitrone chemistry and biological activity that could be implicated for the nitrone's pharmacological activity. The chemistries of spin trapping and spin adduct reveal insights on the possible roles of nitrones for altering cellular redox status through radical scavenging or nitric oxide donation, and their biological effects are presented. An interdisciplinary approach towards the development of novel synthetic antioxidants with improved pharmacological properties encompassing theoretical, synthetic, biochemical and *in vitro/in vivo* studies is covered.

Among the most important signaling molecules in biological systems are the reactive oxygen species/reactive nitrogen species (ROS/RNS). ROS/RNS are small molecules, comprising a handful of atoms that come as radical or nonradical species, as well as in neutral or ionic forms. As natural by-products of O_2 metabolism, ROS/RNS play an important role in modulating cell function, cell signaling and immune response [1-3]. ROS are mostly O_2 derived [4] while RNS are NO-derived species in which the oxygen and nitrogen atoms participate in the redox reaction, respectively [5]. While the names ROS and RNS imply their reactive nature, their reactivity varies with different substrates. For example, $O_2^{\bullet-}$ is practically unreactive with most amino acids, lipids or nucleotides but exhibits high reactivity with Fe-S clusters and other radicals such as tyrosyl and NO. The ability of ROS to cause post-translational protein modification results in changes in protein conformation and can have physiological consequences. For example, NO's ability to bind to the heme of soluble guanylate cyclase is the main mechanism of NO's vasodilatory effect in the vasculature. In cerebral arteries, activation of NADPH-oxidase causes production of $O_2^{\bullet-}$, and $O_2^{\bullet-}$-derived ROS results in increased vasodilation and may play a physiological role as a signaling molecule in cerebral circulation [6]. Redox regulation by ROS through

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reversible or irreversible modification of thiol groups via oxidation, which also includes disulfide formation, nitrosylation and glutathiolation, has tremendous consequences in anti- or pro-apoptotic signal transduction processes [7,8]. Under oxidative-stress conditions, ROS unregulated production or concentrations can cause oxidative damage to key biomolecular systems such as lipids, nucleotides, carbohydrates and proteins, which can have significant effects in maintaining the integrity of cellular structure, controlling gene induction and can cause enzyme deactivation. Among the mechanisms that can trigger oxidative stress are downregulation and/or deactivation of antioxidant enzymes, mitochondrial dysfunction, activation of NADPH oxidase, aberrant activation of NF-κB and MAPK, and calcium signaling – all of which can result from ROS-mediated posttranslational protein modification. Oxidative stress most often results from the disturbance of the intracellular and extracellular redox environments, triggering a variety of events that can lead to apoptosis or necrosis. Oxidative stress has been implicated in the pathogenesis of cardiovascular diseases [9], cancer [10], neurodegeneration [11] and ischemic–reperfusion (I/R) injury [12,13], to name a few.

Antioxidant-based therapy based on *in vitro* observations has made some strides to minimize, prevent or ameliorate oxidative stress via a variety of different mechanisms, such as direct radical scavenging, bolstering of phase II enzyme expressions and/or activity, reversal of eNOS uncoupling or suppression of signal transduction and gene induction leading to apoptosis. Antioxidants can be categorized into subclasses of small molecules and macromolecules that are endogenously synthesized in the body, or exogenously introduced from diets. One of these classes is the antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), heme-oxygenase, NAD(P)H dehydrogenase quinone 1, glutathione peroxidase and glutathione reductase. Due to the size of these molecules, high-molecular-weight therapeutics has been the strategy of choice for their efficient delivery to cells that form the blood–brain barrier. Through the use of nanoparticulate systems, such as lyposomes or polymeric nanoparticles, antioxidant enzymes have been delivered to the cells imparting protective efficacies against oxidants. Poly(D,L,-lactide co-glycolide) polymer loaded with SOD was shown to protect cultured human neurons from H_2O_2-induced oxidative stress [14]. In *in vivo* models, immunotargeting using platelet–endothelial cell adhesion molecule antibodies conjugated to CAT showed immediate protective antioxidant effects in the pulmonary endothelium for the treatment of acute lung injury [15]. A gene therapy approach, however, can provide continuous delivery of the antioxidant enzymes at the site of the disease through just a single administration [16-18]. *In vivo* gene transfer of the cDNA encoding extracellular SOD exhibited substantial protection to the heart against myocardial stunning and decreased infarct size following myocardial infarction [19,20]. Inhibition of ROS-producing enzymes using small-molecule and peptide inhibitors has also shown promise in the treatment of vascular and renal dysfunctions [21]. For example, apocyanin, an NADPH oxidases inhibitor, has been employed in Type 2 diabetes rat models, showing reduction in markers of oxidative stress along with improvements in renal glomellular injury [22].

Small endogenously synthesized molecules such as α-lipoic acid (α-LA) have been shown to bolster antioxidant defenses through induction of phase II enzymes (e.g., heme-oxygenase) via stimulated translocation of Nrf2 [23]. Supplementation of α-LA has been

shown to ameliorate oxidative stress-associated insulin resistance in whole body and skeletal muscle in the obese rats [24]. Gluthathione (GSH) is also one of the most ubiquitous small molecules present in biological systems. In humans, aerosolized thiols such as glutathione and *N*-acetylcysteine have been employed as therapeutic strategy against pulmonary cystic fibrosis to target inflammatory responses caused by neutrophil-dependent reactions catalyzed by myeloperoxidase [25].

Supplementation by diet-derived natural anti-oxidant vitamins has failed to reduce cardiovascular disease and has even been detrimental with single antioxidant therapy using vitamin E in clinical trials in adults [26]. Moreover, the use of vitamins E and C and β-carotene alone or in combination blunts the protective HDL2 cholesterol response to HDL cholesterol-targeted therapy and, therefore, can be harmful [27,28]. However, early detection and treatment with vitamins C and E in hyperlipidemic children has been promising showing restored endothelial function that may retard the development of atherosclerosis [29]. Several plant-derived antioxidants, including isoflavones, indole-3-carbinol and 3,3′-diindolylmethane, curcumin, epigallocatechin-3-gallate, resveratrol, lycopene and vitamins, exhibit inhibitory effect against oxidative stress and NF-κB activation in the development of cancers [30].

The use of synthetic antioxidants is attractive as it becomes possible to manipulate their physicochemical properties as well as their target specificity to enzyme, organelle or cell type for optimal pharmacological activity [31]. Molecules containing multiple α-LA moieties forming lyposomes can increase α-LA bioavailability [32]. Low-molecular-weight SOD mimetics show pharmacological potential with strong anti-inflammatory and cytoprotective properties [33], for example, the SOD/CAT mimetic, EUK-134, completely abolished the H_2O_2-mediated ROS release as well as the incidence of arrhythmias in rat hearts [34]. Synthetic phenolic-based compounds such as isoeugenol [35] showed improved antioxidant capacity compared with trolox and butylatedhydroxytoluene, while hydroxylated resveratrol analogues [36] show selective inhibition of COX-2 *in vitro*. Ebselen, a synthetic lipid-soluble selenium-based compound shows glutathione peroxidase 1-mimetic properties with anti-inflammatory and antioxidant activities by inhibiting 5-lipoxygenase, NOS, NADPH oxidase, PKC and ATPase activities [21]. Iron chelators have also shown promise as therapeutic agents, since evidence show that iron accumulates at the sites of tissue deterioration and, therefore, relieving iron overload at these sites is important [37-39]. Iron, through Fenton-type reactions, can generate radicals, therefore their sequestration can be an effective strategy to prevent oxidative damage to cellular systems. In neurodegenerative diseases, it has been shown that iron chelators not only suppress radical production, but also promote stabilization of HIF-1α and increase transcription of HIF-1-related survival genes – important for its neuroprotective effects [40].

Mitochondrial dysfunction has been implicated in the early development of diseases. Therefore, drugs that target the innermitochondria membrane agents through the use of electron acceptors, electron donors and hydride acceptors can be an effective therapeutic strategy [41]. Using nitroxides conjugated to a modified gramicidin S moiety, triphenylphosphonium lipophilic cationic compounds, sulfonylureas, anthracyclines, SS tetrapeptides with 2′,6′-dimethyltyrosine moiety, or to mitochondria-specific proteins/lipids,

were successfully used in preventing superoxide radical production in cellular systems and in protecting cells against apoptosis [41]. Nitroxides have found therapeutic applications by altering tissue redox status and have the ability to alter many metabolic processes [42,43]. Although nitroxides impart SOD-mimetic properties, in biological systems they can be rapidly reduced to hydroxylamines by enzymes such as CYP450 and nitroreductases. Nitroxide specificity to radicals is dependent on their structure, cellular compartmentalization property and rate of bioreduction. Since SOD is ubiquitous in cells, the nitroxide mode of action is only relevant under conditions where the $O_2^{\bullet -}$ flux overwhelms that of SOD. For example, in conditions of ionizing radiation, nitroxides can serve as protective agents against radiation-induced oxidative damage via their direct reaction with radicals.

Application of nitrones as potential pharmacological agents was proposed by Novelli *et al.*, demonstrating that administration of α-phenyl-*tert*-butyl nitrone (PBN) confers protection on rats from lethal whole-body trauma or circulatory shock [44,45]. Due to their efficacy in trapping free radicals generated inside the cells and tissues, nitrones have been used as pharmacological agents against several ROS-related disorders, such as brain injury [46,47], renal injury [48], visual loss [49], neuronal damage [50] and other age-related diseases [51]. The PBN derivative 2,4-disulfophenyl-*N*-*tert*-butylnitrone, referred to as **NXY-059**, was the first compound to reach Phase III clinical trials in the USA for the treatment of acute ischemic stroke [52]. Very recently, the cardio-protective functions of the cyclic nitrone, 5,5-dimethyl-1-pyrroline *N*-oxide (**DMPO**), against I/R injury have been reported [53]. The anticancer role of PBN and its derivatives against various types of cancer is also well documented [54,55]. The PBN-derived nitrones are reported to inhibit tumor growth and progression in hepatocellular carcinoma [56,57], brain cancer [58,59] and colon cancer [60] models. However, experimental evidence suggests other mechanisms for its protection are involved other than its free radical-trapping ability. Aside from the free radical-scavenging property of nitrones, PBN and DMPO exhibit NO-releasing properties [61,62], which may be partly responsible for their pharmacological properties.

What are nitrones?

Nitrones are *N*-oxides of imines with a general formula of $R_1R_2C = NR_3^{+} O^{-}$ (where R1, R_2, R_3 = any alkyl group; R_1 or R_2 = H). Nitrones have been widely employed as synthetic intermediates [63] and as a spin-trapping reagent for the detection of radicals in solution [64]. Nitrone chemistry parallels that of carbonyl compounds in which the nitronyl-carbon (α-carbon) (Figure 1) is susceptible to nucleophilic attack. Another unique property of nitrones is their ability to undergo a cycloaddition reaction, a property that has been widely exploited in the synthesis of more complex organic molecules [65].

Nitrones can undergo a radical addition reaction to form an aminoxyl-based paramagnetic adduct that is persistent enough to be detected by electron paramagnetic resonance (EPR) spectroscopy (Figure 2), and has become a popular analytical tool for the detection of radicals in chemical and biological systems [66], and in the synthesis of polymers [67].

To date, there are two major types of spin traps, the cyclic (DMPO and 5-diethoxy-phosphoryl-5-methyl-1-pyrroline *N*-oxide [DEPMPO]) and linear (PBN and NXY-059) forms (Figure 3). EPR spin trapping using DMPO has undoubtedly contributed significantly towards the understanding of some important free radical-mediated biochemical processes [68-72]. Nitrones have also found application in the fields of photodynamic therapy [73,74], fuel cell research [75], nanotechnology [76] and catalysis [77].

Importance of superoxide radical scavenging

Superoxide radical ($O_2^{\bullet-}$) is a paramagnetic and anionic molecule formed from the one-electron reduction of triplet oxygen and is a major precursor of the most highly reactive species known to exist in biological systems (Figure 4). Superoxide can undergo further reduction to form ROS, causing oxidation of proteins, carbohydrates, lipids and DNA. Therefore, formation of $O_2^{\bullet-}$ signals the first sign of oxidative burst and, therefore, its detection and/or sequestration in biological systems is important.

Superoxide can be enzymatically generated in biological systems from NO synthase uncoupling [78], stimulation of NADPH oxidase [79], CYP450 [80], FAD-containing monooxygenase [81], xanthine oxidase [82], or electron leakage from the mitochondrial electron transport chain (ETC) complexes [83]. Chemically, $O_2^{\bullet-}$ can also be formed directly from alkali metal salts (NaO_2 and KO_2), or from redox cycling xenobiotics [84], photosensitization of quinones [85,86] and water-soluble fullerene C_{60} [87]. In chemical and biological systems, $O_2^{\bullet-}$ itself is generally poorly reactive, showing high reactivity to only a few species such as tyrosyl radicals, metal ions and metalloproteins (e.g., aconitase), making $O_2^{\bullet-}$ perhaps a more detrimental oxidant due to its reaction selectivity [88].

Traps versus probes

One can make a generalization that all spin traps are redox probes, but not all redox probes are spin traps. EPR spectroscopy exploits the magnetic moment of an unpaired electron where the absorption of microwave radiation by the unpaired electrons as a function of magnetic field strength is measured. EPR spectroscopic detection of $O_2^{\bullet-}$ can either involve spin-quenching (or spin-loss) or spin-formation techniques where loss or formation of a signal is measured, respectively (Figure 5). An example of the spin-quenching method is through the use of the triarylmethyl (trityl)-based radical, which is considered an 'inert' free radical because of its inherent stability [89,90]. The redox reaction of trityl with $O_2^{\bullet-}$ yields molecular oxygen and the EPR silent trityl anion. The synthetic trityl radicals TAM OX063 and perchlorotriphenylmethyl (PCM-TC) have been shown to give high reactivity to $O_2^{\bullet-}$ with second-order rate constants of 3.1×10^3 M^{-1} s^{-1} and 8.3×10^8 M^{-1} s^{-1}, respectively [89,90]. Trityl radicals show inertness toward a majority of the common oxidoreductant species; however, they exhibit reactivity with other radical species, such as $HO_2^{\bullet}$, $RO_2^{\bullet}$ and $HO^{\bullet}$ [90].

A spin-generating system uses a diamagnetic probe that, upon its reaction with a free radical, forms an EPR-detectable paramagnetic species. Nitrones and hydroxylamines fall under this category. Original research on enzyme-catalyzed oxidation of hydroxylamines to

nitroxides dates back to 1977 [91,92]. Hydroxylamines are *N*-hydroxy-pyrrole or piperidine derivatives that are able to undergo oxidation by $O_2^{\bullet-}$ via a simple electron transfer mechanism to yield a para-magnetic aminoxyl species and H_2O_2. The commonly used hydroxylamines TEMPONE-H, CP-H and PP-H are able to extracellularly or intracellularly scavenge $O_2^{\bullet-}$ radicals [93,94]. The rate of hydroxylamine oxidation is dependent on structure. In the case of PP-H, its rate of reaction to $O_2^{\bullet-}$ was found to be $840 \pm 60\ M^{-1}\ s^{-1}$. Although the hydroxylamine PP-H was shown to have tenfold higher reactivity with $O_2^{\bullet-}$ than the nitrone DEPMPO, the paramagnetic species generated does not allow discrimination between the different radicals generated since this reaction is not specific to $O_2^{\bullet-}$ and other radicals can cause one-electron oxidation of the hydroxylamine. Moreover, the H_2O_2 generated from the redox reaction can lead to the production of other ROS and is, therefore, susceptible to false interpretation [95]. Addition of SOD can serve as control, but in many biological systems this is not possible.

Nitrones, however, can undergo addition of a free radical at the α-carbon (C-2) to form an aminoxyl product in which the unpaired electron is almost equally distributed between the N and O atoms (Figure 6).

By virtue of resonance stabilization between the aminoxyl-O and -N (with a stabilization energy of ΔG_{298K} ~30 kcal/mol) [96], the spin adduct is more persistent than the transient free radical being trapped, thus allowing for their spectrum to be observed by EPR. The spectra arising from various radical adducts allow for their discrimination due to the characteristic spectral profiles they exhibit and is by far a better technique in identifying specific free radicals. For example, DMPO-OOH exhibits a unique 12-line EPR spectrum compared with the four-line EPR spectrum for DMPO-OH, the latter nitroxide may be derived from the reduction of DMPO-OOH by gluthathione (GSH) [97]. Spin trapping has also been employed to detect enzymatically generated $O_2^{\bullet-}$, such as from xanthine oxidase [98], the mitochondrial ETC [83,99], thioredoxin reductase [100] and NADPH oxidase [101]. Nitrones have also been successfully used to detect $O_2^{\bullet-}$ generation in human epithelial cells [102], human neutrophils [103], reperfused cardiac tissue [104] and, as a secondary radical adduct from small animals using an *ex vivo* technique [105]. *Ex vivo* spin trapping was best exemplified through the I/R studies of Boll and colleauges [106], and Zweier *et al.* [107] in which the spin trap was administered to the animals before the onset of ischemia. The reperfusates were then collected and radical adduct generation was detected by EPR spectroscopy.

Like other probes mentioned above such as trityl and hydroxylamine, nitrone spin traps are also susceptible to the formation of artifactual adducts, which can be generated via nonradical addition mechanisms to nitrones. There are three accepted nonradical pathways for the formation of spin adducts: direct addition of nucelophiles to nitrones to form the hydroxylamine and subsequent oxidation to nitroxide mainly by O_2; metal-catalyzed (typically by Fe^{2+} or Cu^{+}) nucleophilic addition reaction to nitrones and subsequent electron-transfer with the metal; inverted spin trapping in which the nitrone is initially oxidized to the radical cation and addition of nucleophile forms the nitroxide. The nucleophilic addition/oxidation is usually referred to as the Forrester–Hepburn mechanism and is a common source of spin adduct artifacts in aqueous solution [108,109]. However,

control experiments through the use of chelating agents, such as SOD or CAT, can well differentiate such nonradical addition mechanism from the actual spin trapping reaction. In *in vivo* systems, the formation of nitrone-sulfite spin adduct was observed although the exact mechanism of the adduct formation is still a matter of controversy but nevertheless important in sulfur dioxide-exposure detoxification [110].

Reactivity of nitrones to superoxide

One direct proof for the reaction of nitrones with $O_2^{\bullet-}$ is through EPR spin trapping, which continues to be the most unequivocal detection technique for $O_2^{\bullet-}$ due to the distinctive EPR spectrum that the $O_2^{\bullet-}$ adduct imparts (Figure 7) compared with other radical adducts [111,112]. The limitations encountered using spin traps for the detection of $O_2^{\bullet-}$ is the slow reactivity of $O_2^{\bullet-}$ addition to nitrones, lack of target specificity making it difficult to identify the site of radical production in the cell and short adduct half-life, which limits their detectability. The first two limitations are important for the therapeutic applications of nitrones and efforts to overcome these problems will be discussed in detail in the following sections.

Why do nitrones trap superoxide radicals poorly?

From a purely thermodynamic standpoint, the order of increasing favorability as expressed by the free energies of reaction ($\Delta G_{rxn,aq,298K}$ [kcal/mol]) for the addition of different radicals to nitrones in general is: NO (14.1) < $O_2^{\bullet-}$ (10.0) < $HO_2^{\bullet}$ (-7.0) < $HS^{\bullet}$ (-13.6) < $^{\bullet}CH_3$ (-32.9) < $HO^{\bullet}$ (-38.9). The σ-type radicals (e.g., $HS^{\bullet}$, $^{\bullet}CH_3$ and $HO^{\bullet}$) have exhibited higher reactivity compared with the π-type radicals, NO, $O_2^{\bullet-}$ and $HO_2^{\bullet}$ [113-116]. This mirrors the computational findings by Boyd and Boyd [117] where the order of increasing formation energies for the nitroso HN=O spin adducts (in kcal/mol) is: NO (-7.8) < $HO_2^{\bullet}$ (-8.4) < $HS^{\bullet}$ (-21.5) < $HO^{\bullet}$ (-32.6) < $^{\bullet}CH_3$ (-53.9); and for CH_3N=O in kcal/mol is: NO (-8.1) < $HO_2^{\bullet}$ (-7.5) < $HS^{\bullet}$ (-18.2) < $HO^{\bullet}$ (-35.8) < $^{\bullet}CH_3$ (-50.1) at the MP2/6–31G(d,p)//HF/6–31G(d) level of theory.

In general, this trend in reactivity follows the order of the degree of spin density residing on the attacking atom of the radical; that is, 100% spin density on the O, S and C atoms of $HS^{\bullet}$, $^{\bullet}CH_3$ and $HO^{\bullet}$, compared with 70% (N), 50% (O) and 73% (terminal O) spin density distribution in NO, $O_2^{\bullet-}$ and HO_2•, respectively. The same partial electrostatic effect on radical reactivity was also observed for the radicals; that is, $HO^{\bullet}$ (O, -0.50 e), $^{\bullet}CH_3$ (C, -0.58 e), $HS^{\bullet}$ (S, -0.12 e), $HO_2^{\bullet}$ ($O_{terminal}$, -0.15 e), $O_2^{\bullet-}$ (O, -0.50 e) and NO (N, +0.20 e). Table 1 shows the charge and spin (where applicable) densities of $HO^{\bullet}$, $O_2^{\bullet-}$, $HO_2^{\bullet}$, HO_2^{-} and HO^{-}, as well as the free energies of their addition to DMPO. In general, addition of anions is endoergic compared with radical addition to nitrones, with the exception of $O_2^{\bullet-}$. Although both $O_2^{\bullet-}$ and $HO^{\bullet}$ have the same charge density (i.e., 0.50 e) on the O-atom, the higher spin density distribution on the hydroxyl-O than superoxide-O makes $HO^{\bullet}$ more reactive than $O_2^{\bullet-}$, indicating the partial electrophilic character of radical addition to nitrones. As shown in Table 2 [118-120], this electronic property of the radicals determines their redox properties. Therefore, both charge and spin density distribution on the attacking atom govern radical reactivity to nitrones.

The thermodynamic trend in radical reactivity to nitrones parallel that of the apparent rate constants. For example, experimental rate constants for the reaction of DMPO with radicals are ($HO^{\bullet}$) [121]: 1.93×10^9 M^{-1} s^{-1}; ($CH_3{}^{\bullet}CHOH$) [122]: 1.1×10^6 M^{-1} s^{-1}; (glutathiyl radical $GS^{\bullet}$) [123]: 2.6×10^8 M^{-1} s^{-1}; and ($O_2^{\bullet-}$) [124,125]: 10–50 M^{-1} s^{-1}. The rate constant for the trapping reaction of BocMPO with $O_2^{\bullet-}$ at pH 7.0 was reported to be 75.0 ± 10.5 $M^{-1}s^{-1}$, while at pH 5.0, when $HO_2^{\bullet}$ is predominant, the rate constant is 239.2 ± 10.5 M^{-1} s^{-1} [126].

Effect of nitronyl-C (or C-2) charge density on its reactivity to superoxide

The nucleophilic nature of $O_2^{\bullet-}$ reaction to nitrones (Figure 8) is demonstrated in Figure 9 where the charge density on C-2 for various C-5-substituted nitrones was correlated to the calculated kinetics and thermodynamics of $O_2^{\bullet-}$ addition to these nitrones [127].

The formation of $O_2^{\bullet-}$ adducts from the mono-substituted *N*-methylamide nitrones (AMPO, MAMPO and EMAPO), and that of TFMPO are the most kinetically favored at the mPW1K level of theory, while DEPMPO gave the slowest rate constant, followed by DMPO, DiMAMPO and CPPO. Intermediate rate constants were predicted for the alkoxycarbonyl-substituted nitrones, EMPO and DEPO, and for the lactone CPCOMPO and the lactam TAMPO. The favorable formation of $O_2^{\bullet-}$ adducts in *N*-monoalkylamide nitrones is due to the relatively high positive charge on the C-2 position, as well as the early formation of hydrogen bonding interaction in the transition state between the amide-H and the $O_2^{\bullet-}$, thereby facilitating the formation of the $O_2^{\bullet-}$ adduct. These data suggest that the use of 5,5-disubstituted nitrones with electron-withdrawing alkoxycarbonyl and monoalkylcarbamoyl substituents could be ideal multifunctional spin traps in which target-specific and adduct-stabilizing moieties can be tethered in the parent nitrone molecule employing ester or amide units as linker groups.

While the common nitrones, DMPO and PBN, are able to trap $O_2^{\bullet-}$, the measured rates of adduct formation are too low. In the case of PBN, the PBN-OOH adduct is not observed due to its instability (half-life <1 min) [124]. The trend in $O_2^{\bullet-}$ reactivity observed with PBN derivatives may not follow that observed with DMPO derivatives due to differences in the electronic property of their respective nitronyl-carbons. Results show that these reactions do not correlate with the calculated charge densities on the nitronyl-carbon for PBN derivatives. It was assumed that due to the higher observed electron density on the nitronyl-carbon in PBN derivatives compared with DMPO derivatives, the nature of $O_2^{\bullet-}$ addition to PBN may be considered as a borderline case (i.e., partly electrophilic), which explains the observed non-correlation between the rate constants and charge densities for the PBN derivatives [128].

Effect of the presence of hydrogen bond donors on the nitrone reactivity to superoxide

The spin and charge density distribution on the $O_2^{\bullet-}$ and $HO_2^{\bullet}$, and their short O-O bond distances of 1.33 Å, are characteristics of a π-radical. By virtue of symmetry, the charge and spin density distribution on the two oxygen atoms of $O_2^{\bullet-}$ are equivalent; but addition of a proton to one of the oxygens to form $HO_2^{\bullet}$ perturbs the electronic and charge distribution between the two oxygen atoms, resulting in higher spin density on the terminal oxygen atom

(73%) compared with the oxygen atom (35%) bound to the hydrogen atom (Table 1 & Figure 10). This polarization in the charge and spin density distribution in $HO_2^\bullet$ can have a significant effect on its reactivity, as shown by the difference in the reduction potentials between $O_2^{\bullet-}$ and $HO_2^\bullet$ of $E^{o'}$ = 0.94 and 1.06 V [4], respectively, in which the latter is more oxidizing than the former. Hydrogen bond interaction of $O_2^{\bullet-}$ with hydrogen bond donors such as amides serve as an initial 'anchor' for $O_2^{\bullet-}$ prior to its addition to the nitrone (Figure 10). Superoxide complexation with 1-methyl-1-carbamoylcy-clopentane gave calculated favorable $\Delta G_{rxn,298K,aq}$ and $\Delta H_{rxn,298K,aq}$ of -4.1 and 4.7 kcal/mol, respectively. This interaction has a significant effect on the electronic property of $O_2^{\bullet-}$ in which it can assume a spin and charge density distribution intermediate to that of uncomplexed $O_2^{\bullet-}$ and $HO_2^\bullet$. We hypothesized that the presence of hydrogen bond donors, in general, may affect the rate of $O_2^{\bullet-}$ addition to nitrones in which the $O_2^{\bullet-}$ hydrogen bonding interaction with nitrones can exhibit reactivity close to that of a hydroperoxyl radical ($HO_2^\bullet$) [127].

Increased favorability (both kinetically and thermodynamically) of $O_2^{\bullet-}$ addition to nitrones in the presence of amide substituents compared with methyl, ester or phosphoryl substituents was observed [127]. It was later concluded that factors such as intramolecular hydrogen bonding of $O_2^{\bullet-}$ in the transition state and electrostatic effects facilitate the $O_2^{\bullet-}$ adduct formation [127]. The phenomenon called α-effect plays an important role in increasing $O_2^{\bullet-}$ reactivity upon hydrogen bonding. α-effect is exhibited by a class of nucleophiles that have an electronegative atom (with one or more lone pair of electrons) adjacent to the nucleophilic center called α-nucleophiles [129,130]. α-nucleophiles tend to be very strong electron donors and yet are very weak bases due to the inductive effect of the heteroatom adjacent to it. This high activity is due to the repulsive interactions between the unshared electron pairs on adjacent atoms between the lone pair of electrons on the α-atom and those on the nucleophilic center. This makes an α-nucleophile unstable and, hence, more reactive [129].

Further conjugation of the nitrone spin traps with β-cyclodextrin (CDNMPO [131] and C_{12}CDMPO [132]) or calix[4]pyrrole [133], using amide or ester linker groups, respectively, can further enhance the spin-trapping ability and bioavilability of the nitrones. For example, CDNMPO, C_{12}CDMPO and CalixMPO gave improved rates for spin trapping of $O_2^{\bullet-}$ and longer $O_2^{\bullet-}$-adduct half-lives. The spin-trapping rates have been determined to be 58 (CDNMPO) [131], 221 (C_{12}CDMPO) [132] and 680 (CalixMPO) [133] $M^{-1}s^{-1}$, and improved their respective $O_2^{\bullet-}$ adduct half-lives ($t_{1/2}$), which are CDNMPO: 6 min, C_{12}CDMPO: 9 min and CalixMPO: 25 min in DMSO compared with DMPO alone, which has a rate constant of only 2 $M^{-1}s^{-1}$ and $O_2^{\bullet-}$ adduct half-life of 6 min in DMSO [133]. The high reactivity of $O_2^{\bullet-}$ to CalixMPO was rationalized to be due to a synergistic effect from the polarization of $O_2^{\bullet-}$ through hydrogen bonding with calix[4]pyrrole, enhanced positivity of the nitronyl-C through hydrogen bonding of the nitronyl-O and proximity of the nitrone group to the calix[4]pyrrole via conjugation (Figure 11).

The same behavior was observed for $O_2^{\bullet-}$ with various amino acids, which shows strong hydrogen bond interactions with the amino, carboxylic acid and guanidinium hydrogens [134]. These hydrogen bonding interactions result in significant perturbation of the spin and charge densities of the $O_2^{\bullet-}$. The low endoergicity of the free energies and exoergicity of the

enthalpies of complexation of $O_2^{\bullet-}$ with amino acids may translate to a favorable complex formation in solution. Ramification of $O_2^{\bullet-}$ hydrogen bonding towards facilitation of its addition to tyrosyl radical (TyrO$^\bullet$) to form tyrosyl hydroperoxide had been demonstrated by Winterbourn *et al.* [135], where it was shown that there is an increase in tyrosyl hydroperoxide formation in peptides containing an amino group, such as lysine, which is adjacent to the tyrosyl group. The same enhancement in tyrosyl hydroperoxide formation was observed in the presence of free lysine or ethanolamine [135]. It is, therefore, reasonable to assume that -NH–$O_2^{\bullet-}$ complex formation may play an important role in the facilitation and selectivity of $O_2^{\bullet-}$ addition to TyrO$^\bullet$ in protein systems, especially when these hydrogen bond donors are in close proximity to the TyrO$^\bullet$ group.

Effect of pH on the nitrone reactivity to superoxide

The original research on the pH dependence of rate of $O_2^{\bullet-}$ trapping was first investigated by Finkelstein *et al.* [124]. The mechanism of nitrone antioxidant activity is intriguing, since at neutral pH, the reactivity of $O_2^{\bullet-}$ to DMPO is slow, with a second-order rate constant of only 2.0 M^{-1} s^{-1}; however, at acidic pH the reactivity is approximately 27 M^{-1} s^{-1} or 10^3 M^{-1} s^{-1} at pH 6.2 [136] and 5.0 [124], respectively. The high reactivity of $O_2^{\bullet-}$ to DMPO in acidic pH was proposed to be due to the protonation of $O_2^{\bullet-}$ to form hydroperoxyl radical ($HO_2^\bullet$), since the pK_a for $O_2^{\bullet-}$ and $HO_2^\bullet$ is 4.8 [137] and 4.4 [138], respectively, and that $HO_2^\bullet$ is a stronger oxidizer than $O_2^{\bullet-}$ ($E^{o'}$ = 1.06 and 0.94 V, respectively) [4]. The higher reactivity of HO_2• to DMPO compared with $O_2^{\bullet-}$ was theoretically rationalized and the predicted rate constants in aqueous phase for the $O_2^{\bullet-}$ and $HO_2^\bullet$ addition to DMPO were found to be 5.9×10^{-5} M^{-1} s^{-1} and 285 M^{-1} s^{-1}, respectively, at the PCM/B3LYP/6–31+G**//B3LYP/6–31G* level of theory [127,139]. At the same level of theory, the calculated thermodynamic data in aqueous phase also show a more facile addition of $HO_2^\bullet$ to DMPO compared with $O_2^{\bullet-}$ with ΔG_{rxn} of -4.6 and 11.9 kcal/mol, respectively [139].

Three possible mechanisms for the DMPO-OOH formation have been proposed (Figure 12). At neutral pH, the addition of $O_2^{\bullet-}$ to DMPO in aqueous solution at pH 7.2 is thermodynamically endoergic (ΔG_{298K} = 11.9 kcal/mol) with an experimental rate constant of 1.3×10^{-3} M^{-1} s^{-1} (Mechanism A) [127]. While the established pK_a for $O_2^{\bullet-}$ is 4.8, the pK_a for DMPO was unknown. The pK_a of the conjugate acid of DMPO was determined to be 6.0 using potentiometric, spectrophotometric, ^{1}H- and ^{13}C-NMR, as well as computational methods. An alternative mechanism for the spin trapping of $O_2^{\bullet-}$ in mildly acidic pH was, therefore, proposed [140], which indicates that in slightly acidic medium, DMPO can be protonated first (Mechanism B) and can compete with the alternate mechanism where protonation of $O_2^{\bullet-}$ leads to the addition of $HO_2^\bullet$ to DMPO (Mechanism C), which is more favorable than the addition of $O_2^{\bullet-}$ (Figure 12). A tenfold increase in the charge density of C-2 was observed upon protonation of the nitronyl-O, therefore, increasing the electrophilicity of the nitrone towards $O_2^{\bullet-}$ [140]. Reactivity of DMPO in mildly acidic pH (5.0–7.0) (Mechanism C) can have biological relevance. Mild acidity is characteristic of acidosis and has been observed in hypoxic systems (e.g., ischemic and cancer cells). Since acidosis plays a critical role in the initiation of brain [141,142] or heart damage [143,144] during ischemia, and that production of $O_2^{\bullet-}$ is ubiquitous during these pathophysiological events, spin trapping of $O_2^{\bullet-}$ by nitrones in mildly acidic environment is therefore relevant.

Reactivity of nitrones to other radicals & nucleophiles

Reactivity with $CO_2^{\bullet-}$ & $CO_3^{\bullet-}$

Nitrone addition to $CO_2^{\bullet-}$ and $CO_3^{\bullet-}$ can minimize the effects of these radicals in causing oxidative damage [145,146]. $CO_2^{\bullet-}$ is formed in acute sodium formate poisoning [147] and is also generated as a metabolic product of CCl_4 in rat liver microsomes [148,149]. $CO_2^{\bullet-}$ anion is also a key intermediate in both decarboxylase and oxidase biochemistry and has been detected as an oxalate-derived free radical using spin trapping [150]. The carbonic acid–bicarbonate ion equilibrium plays an important role in maintaining pH in blood plasma [151,152]. It has been shown that bicarbonate anion (HCO_3^-) enhances the peroxidase activity of copper–zinc SOD (SOD1) and that this effect involves $CO_3^{\bullet-}$ formation as induced by cysteine [153] or H_2O_2 [154]. Moreover, $CO_3^{\bullet-}$ has been found to be produced from a copper–zinc SOD/H_2O_2/HCO_3^- system [155] or as a decomposition product of the reaction of $ONOO^-$ and CO_2 (i.e., $ONOOCO_2^-$) [156]. EPR spin trapping of $CO_2^{\bullet-}$ and $CO_3^{\bullet-}$ using DMPO shows formation of C-centered carboxylate (DMPO-CO_2^-) and O-centered carbonate (DMPO-OCO_2^-) adducts. The fate of DMPO-OCO_2^- in solution was computationally and experimentally rationalized, and results show that the major decomposition pathway for DMPO-OCO_2 in solution is via ring-opening of the pyrrolidine ring with subsequent elimination of nitroxyl ($^3NO^-$) to form nitrite (NO_2^-) [146].

Reactivity with reactive sulfur species

Sulfite ($SO_3^{\bullet-}$) is an important reactive intermediate and has been shown to be enzymatically generated from eosinophil peroxidase [157] or xanthe oxidase/H_2O_2-catalyzed [158] oxidation of sulfite; therefore, $SO_3^{\bullet-}$ may be implicated in the initiation of oxidative damage [159,160]. Generation of $SO_3^{\bullet-}$ [161,162] in the presence of spin traps has been shown to give EPR spectra using DMPO. Conflicting reports still arise into the nature of the sulfite adduct formation; for example, Khramstov and co-workers [110] suggest a nonradical pathway via nucleophilic addition reaction and subsequent oxidation; while Ranguelova and Mason proposed direct $SO_3^{\bullet-}$ addition to DMPO from a one-electron oxidation of HSO_3^- as catalyzed by a horseradish peroxidase/H_2O_2 system [109,161]. For the nucleophilic addition reaction mechanism (Figure 13), the equilibrium constant for the formation of the product of DEPMPO with *S*-centered nucleophiles decreases in the following order: sulfite > thioglycolic acid > cysteine > glutathione [163]. Reaction of thiols with nitrones can affect local redox states in biological systems and can have implications in cell-signaling events.

Reactivity with reactive nitrogen species

Nitrones have been used for the detection of RNS [164] and have been shown to trap decomposition products and tertiary radicals formed from $ONOO^-$ [165]. Aside from the nitrone reactivity with $ONOO^-$, nitrones have been shown to react with a variety of inorganic radicals and nonradical nucleophiles such as azidyl radicals [166,167], cyanidyl radical [168] and nitrogen dioxide radical [164]. Nitrones are not reactive to NO; instead, nitronyl nitroxide (although not purely a nitrone) [169] and Fe(II)-thiocarbamates have been used as probes for NO detection in a variety of experimental conditions [170,171].

Reactivity with protein radicals

Existence of one-electron oxidized forms of amino acid residues in proteins had been proven, and they play crucial roles in the normal function of an enzyme. Tyrosyl, cysteinyl, glycyl and tryptophanyl radicals have been identified as either important cofactors or involved in turnover by enzyme catalytic action [172]. However, oxidative modification of proteins also accounted for their inactivation. Antioxidants may, therefore, act in either preventing enzyme inactivation via reversing the oxidation state of the active sites; preventing protein unfolding through competitive reaction between the antioxidant and the protein radical for the oxidant; or direct reaction of the antioxidant with the protein radical. Formation of protein radicals specifically through cysteine residues can have significant implications in redox signaling and inflammatory responses, which can have a role in the development of pathophysiological conditions. DMPO had been shown to form adducts with glutathiyl, hemoglobin-cysteinyl and hemoglobin-tyrosyl radicals from $ONOO^-$-mediated oxidation of oxyhemoglobin [173]. A variety of proteins, as well as DNA radicals, have been shown to react with DMPO to form the macromolecule– DMPO adducts. These adducts were detected using the immune spin-trapping technique [174,175]; for example, DMPO adducts of CPB1 [176], myoglobin [177] and mitochondrial cytochrome C [178] via DMPO binding to the tyrosyl and cysteine residues.

Reactivity with lipids

Antioxidant termination of lipid peroxidation chain reaction processes is important in preserving cellular membrane integrity. Peroxyl radicals of polyunsaturated fatty acids, such as linoleic, arachidonic and linolenic acids, play crucial roles in lipid peroxidation and have been shown to form alkoxyl adducts with DMPO [179,180]. Formation of alkoxyl radical adducts was observed from polyunsaturated fatty acid hydroperoxides in the presence of Fe^{2+} and in intact cells [179]. Nitrones have also been shown to form adducts with the radicals of arachidonic acid, dihomo-γ-linolenic acid or docosahexaenoic acid via cyclooxygenase-mediated radical formation [181,182], metal-catalyzed oxidation of diacyl-glycerophosphatidylcholines [183] or reactions with soybean lypoxygenase [184].

Decomposition of superoxide adduct

The potential therapeutic property of a nitrone does not only rely on its reactivity to radicals and nucleophiles, but the decomposition of the aminoxyl-based adducts formed after spin trapping could also further exhibit therapeutic properties. As mentioned above, tetra-alkyl-β-substituted aminoxyl compounds such as TEMPO and PROXYL have been shown to impart therapeutic properties and have found clinical use. However, their radical-scavenging property *in vivo* may not compete with SOD unless in conditions of extreme oxidation stress, such as in the presence of ionizing radiation. Nevertheless, TEMPO shows SOD mimetic properties, where it catalytically transforms $O_2^{\bullet -}$ to H_2O_2 at physiological pH at rate constants 10^4–10^5 M^{-1} s^{-1}, which is four to five orders of magnitude lower than SOD [185]. SOD was also found to reduce nitroxides to their corresponding hydroxylamine in the presence of thiols [186]. However, the main structural difference between tetraalkyl-β-substituted aminoxyls and that of the adducts formed with cyclic nitrones is the presence of

β-H. The absence of β-H in TEMPO and PROXYL is the basis of the reversibility of their redox reactions (Figure 14).

Using a density functional theory approach, the redox properties of the $O_2^{\bullet-}$ adducts of nitrones were investigated [187]. Unlike TEMPO and PROXYL nitroxides, nitrone-radical adducts have β-H that make them susceptible to degradation, hence their redox reactions are not reversible. Figure 15 shows the one-electron oxidation and reduction of $O_2^{\bullet-}$ adducts leading to the formation of various products. The thermodynamically preferred decomposition pathway for the one-electron reduction of DMPO-OOH is via formation of the hydroxylamine and subsequent ring opening to form the aldehyde and elimination of HNO_2. For the one-electron oxidation of DMPO-OOH, the preferred decomposition is the formation of the hydroxamic acid via nucleophilic addition of water. IN addition, the formation of C-nitroso aldehyde was shown to be thermodynamically favorable upon DMPO-OOH oxidation (Figure 15). The redox reactions of $O_2^{\bullet-}$ adduct leads to the formation of NO, H_2O_2 and hydroxamic acid. Hydroxamic acid, for example, is widely employed as an iron chelator and enzyme inhibitor [188-190]. The redox activity of nitrones and their spin adducts, as well as the formation of biologically active byproducts, could play crucial roles in regulating cellular redox states, and may explain the pharmacological activities of nitrones against oxidative stress.

By virtue of the oxidation state of $O_2^{\bullet-}$, it can serve as both a reducing and oxidizing agent leading to the formation of O_2 and peroxide (O_2^{2-}), respectively. Unlike TEMPO or PROXYL, DMPO-OOH does not act as an SOD mimetic and its reaction to $O_2^{\bullet-}$ can lead to decomposition products (Figure 16). Compared with DMPO alone, DMPO-OOH gave higher electron affinity, making the nitroxides more susceptible to reduction than nitrones. The ionization potentials of DMPO and DMPO-OOH are not significantly different, but in general are higher compared with the more stable nitroxides such as TEMPO, PROXYL, DMPO-OH and DMPO-CH_3 adducts, indicating a more facile oxidation for these latter class of nitroxides [187]. In buffer solution, kinetic analysis for the decomposition of various nitrone-OOH yields pseudo-first-order rate constants in the range of $0.6–9.0 \times 10^{-3}$ s^{-1} corresponding to half-lives of approximately 1–20 min [191]. The short half-lives of the $O_2^{\bullet-}$ adducts can be accounted for by the fact that the $O_2^{\bullet-}$ self-dismutation, whose rate is in the order of 105 M-1 s^{-1}, can compete with the nitroxide oxidation by $O_2^{\bullet-}$, whose rate is also in the same order of magnitude [185,192,193]. Base-catalyzed decomposition of DMPO-OOH can also yield C-nitroso aldehydes, which explains the instability of such species in basic medium (Figure 17) [194].

Disproportionation of the radical adduct to yield the nitrone and hydroxylamine bearing the radical moiety can have implication for further reaction with ROS. Although this disproportionation to form stable nitrone-OOH and hydroxylamine-OOH diamagnetic products seems unlikely for $O_2^{\bullet-}$ adducts due to the lability of the O–O bond; formation of nitrone-SO_3^- and hydroxylamine-SO_3^- were stable enough to be observed in solution using NMR [110]. Moreover, trapping of C-centered radicals can also yield stable adducts that can disproportionate to nitrone-R and hydroxylamine-R species. The implication of the formation of hydroxylamine and nitrone from the spin adduct can further enhance the radical scavenging property of nitrones. For example, the observed rate constant for the reaction of

$O_2^{\bullet-}$ with hydroxylamine to form the nitroxide and H_2O_2 is in the order of 10^3 M^{-1} s^{-1} [195] and 10^9 M^{-1} s^{-1} for $HO^{\bullet}$ reaction with hydroxylamine [196]. In fact, hydroxylamine had been shown to confer protection in cardiomyocytes against oxidative stress [197].

Nitric oxide production from DMPO-OOH decomposition

One-electron reduction (Figure 15) and base-catalyzed decomposition (Figure 17) of DMPO-OOH are some of the pathways leading to the formation of C-nitroso aldehyde. However, unimolecular decomposition via homolytic O-O bond cleavage is one of the main pathways for DMPO-OOH decomposition, as shown in Figure 18. The unimolecular decomposition of the $O_2^{\bullet-}$ adduct of DMPO, DEPMPO and EMPO was experimentally investigated and shows first-order decay kinetics during the whole course of adduct decomposition [198]. Table 3 [199-201] shows the experimental first-order half-life of $O_2^{\bullet-}$ adduct of various nitrones in aqueous solution. The overall calculated $\Delta G_{rxn,aq}$ of the $O_2^{\bullet-}$ adducts of AMPO, EMPO, DEPMPO and DMPO decomposition to their respective C-nitroso aldehydes are exoergic, ranging from -2 to -13 kcal/mol, with DMPO-OOH being the most favorable. Less exoergic $\Delta G_{rxn,aq}$ of decomposition were observed for the adducts with electron-withdrawing group substitution at C-5, which qualitatively follows the experimentally observed half-lives with DMPO-OOH having the shortest half-life [202].

Decomposition of $O_2^{\bullet-}$ adducts of DMPO, EMPO and DEPMPO in aqueous solution lead to the formation of NO as detected by EPR spin trapping using Fe(II) *N*-methyl-D-glucamine dithiocarbamate. NO release was observed from the $O_2^{\bullet-}$ adduct formed from hypoxanthine/xanthine oxidase, phorbolmyristate acetate-activated human neutrophils, and DMSO solution of KO_2 (Figure 19). However, NO formation was not observed from the independently generated hydroxyl radical adduct. Formation of NO was also indirectly detected as nitrite (NO_2^-) utilizing the Griess assay. Nitrite concentration increases with increasing $O_2^{\bullet-}$ concentration at constant DMPO concentration, while NO_2^- formation is suppressed at anaerobic conditions [61]. This decomposition chemistry of DMPO-OOH can have implication for controlled delivery of NO through radical scavenging. It can be considered that nitrones are both radical scavenging ROS and NO donors (Figure 20). Most of the NO donor drugs are limited by their stability, controlled delivery and targeting properties [203]. In contrast, nitrones can both scavenge ROS and exhibit controlled release of NO as a consequence [61,202] and are, therefore, more desirable and unique among current NO-donating drugs.

Intracellular compartmentalization properties

The reported partition coefficients for cyclic nitrones (K_p = [nitrone]$_{n\text{-octanol}}$/[nitrone]$_{water}$) are generally low; that is, $K_p < 0.2$, unlike the PBN-type nitrones that can have K_p of up to 30 [204-206]. Recently, various lipid-soluble spin traps derived from EMPO and DEPMPO have been synthesized [205,206]. One of the major limitations of spin traps is their inability to specifically detect radicals at the site of radical formation. Therefore, conjugating spin traps with substituents that are known to compartmentalize in the membrane, cytosol, extracellular matrix and mitochondria could overcome such limitations and can provide optimal radical scavenging, since radicals could be targeted at the site of their production.

Although nitrones such as DMPO, PBN and NXY-059 have been employed as pharmacological agents, they are limited by their lack of intracellular target specificity.

Cell membrane

Radical-induced oxidative damage to the cell membrane primarily occurs through lipid peroxidation process. Membrane-bound radical generators such as NADPH oxidases [207,208] and uncoupled eNOS [209], as well as exogenously produced ROS or ionizing radiation [210], are potential targets for antioxidant therapy. Since the structural organization of the cell membrane bilayer is comprised mainly of phospholipids and sterols, employing cholesterol and long-chain hydrophobic groups as target ligands for membrane compartmentalization is promising [211]. Figure 21 shows some examples of lipophilic nitrones with conjugation to cholesterol (5-ChEPMPO) [212], cyclic-phophoryl group (CyDEPMPO) [213], adamantane [214] and intermediate chain length aliphatic hydrocarbons, BBPO [215], have been previously synthesized but their biological activity has not been explored.

Cytosol—ROS-producing cytosolic enzymes as mediated by CYP450 [216,217] and non-P450 enzymes, such as aldehyde oxidase [218,219] and xanthine oxidase [98,220], have been implicated in pathological conditions, such as in I/R injury, and play an important role in the biotransformation of drugs and xenobiotic [221-223]. Some of the NO synthase (NOS) isoforms are also found mainly in the cytosol and catalyze NO (or $O_2^{\bullet-}$) production [224,225]. Esterification is the most common method employed in the delivery of probes or drugs inside the cell [226]. Rosen *et al.* employed the use of acetoxymethyl ester-derivatized nitroxides and had shown high intracellular retention in Jurkat lymphocytes due to efficient hydrolysis of the ester by the cytosolic esterases [227,228]. Several ester derivatives of nitrones have also been synthesized. Examples of esterified nitrones that can potentially compartmentalize intracellularly are DEEPN [204], DEPO [229], EMAPO [132] and BMPO [230] (Figure 22).

Mitochondria—Mitochondrial respiration accounts for approximately 90% of the oxygen consumed in the cell and produces high levels of ROS, and has been implicated in a variety of pathophysiological conditions [231-233]. The mitochondrial respiratory chain is a major source of $O_2^{\bullet-}$ and is central to activating apoptosis. Oxidative damage due to unregulated production of $O_2^{\bullet-}$ can lead to cell death. Antioxidants have been successfully delivered to the mitochondrion through conjugation with a lipophilic triphenylphosphonium cation (TPP) [234-236]. Mitochondrial uptake of TPP occurs through permeation in the lipid bilayers and accumulation within the mitochondria by several hundred-fold due to the large mitochondrial membrane potential of -150 to -170 mV versus the plasma membrane potential (-30 to -60 mV) [237]. Studies show that TPP can also concentrate from the extracellular fluid into isolated cells from which they further concentrate within the mitochondria by approximately 90%. Selective target delivery to the mitochondria in principle can be achieved through conjugation of TPP with spin traps such as in the case of MitoPBN [237], MitoDEPMPO [238] and MitoSpin [239], as shown in Figure 23. Moreover, some positively charged *N*-aryl [240] and *N*-alkyl [241,242] pyridinium nitrones have also been synthesized to investigate $O_2^{\bullet-}$ production from mitochondria, and were

shown to successfully trap $O_2^{\bullet-}$ produced from intact mitochondria, albeit not from intact cells.

Extracellular & amphiphilic nitrones—Application of spin traps that can exclusively accumulate outside the cell is important for the investigation of free radicals produced by exogenous agents such as ionizing radiation, particulates, cell culture medium, or through diffusion of H_2O_2 (and, hence, $HO^{\bullet}$ via Fenton-type reaction) to the extracellular matrix [243,244]. Due to the high impermeability of lipid bilayers to polar molecules [245], substituents that contain terminal ionic groups such as carboxylates can be potential extracellular spin traps, such as in the case of CMPO (Figure 24), although its ability to trap $O_2^{\bullet-}$ is limited [246], perhaps due to the repulsive effect of the carboxylate group.

Amphiphilic nitrones, composed of a hydrophilic polar head and a lipophilic group, were shown to efficiently inhibit oxidative stressmediated damages with a high potency [247-249]. Amphiphilic compounds possessing both hydrophilic and lipophilic groups may exhibit improved cellular permeability [247,250,251]. The hydrophilic polar head of the amphiphiles provides water solubility while the hydrophobic tail ensures sufficient lipophilicity to pass through cell membranes. Durand *et al.* reported the synthesis of novel amphiphilic amide nitrones, comprising a lactobionic acid based polar head group and an alkyl chain, both linked via an amide bond to the *N-tert*-butyl group and the phenyl ring of the PBN moiety (LPBNAH and LPBNH15) were found to improve the mitochondrial bioenergetics and confer protection against dopamine-induced mitochondrial dysfunction [252]. Glycosylated amphiphilic linear and cyclic nitrones, (FAPBN and FAMPO) [247] and lectin directed [253] ones have also been synthesized, however their application for the detection of $O_2^{\bullet-}$ in cells has yet to be evaluated. Co-conjugation of long chain hydrocarbons to β-cyclodextrin nitrone conjugate (CDNMPO) forming C_{12}CDMPO [132] also showed amphiphilic property (Figure 24).

Peptidic nitrones—Previously, a PBN-type nitrone with an iodoacetamide group was recently synthesized by Liu *et al.* and was used to conjugate with GSH (to form GS-PBN or GS-PPN) (Figure 25) or bovine serum albumin (BSA) [254,255]. The PBN-peptide conjugate was successfully used to trap C-centered radicals in water and $O_2^{\bullet-}$ in the case of GS-PPN. The synthesis of the biotinylated analogue of DMPO (Bio-SS-DMPO) could allow facile conjugation of targeted peptidic ligands to nitrones [256].

Biconjugation—Through bi-functionalization of the nitrones, one would be able to integrate most of the desired spin-trapping properties in one molecular design such as high reactivity to $O_2^{\bullet-}$, improved bioavailability and target specificity. Only a limited number of disubstituted nitrones, however, have been synthesized over the past years, such as C-5 diesterified nitrone (DECPO) [257], *N*-hydroxysuccinimide-, biotin-, alkylphosphonium-, Me-β-cyclodextrin-conjugated DEPMPO at C-4 (as NHS-DEPMPO, biotin-DEPMPO, mito-DEPMPO and CD-DEPMPO, respectively) [238,258], amphiphilic PBN derivative (LPBNAF) [259] and the C-5 disubstituted cyclic nitrone with a long-chain hydrocarbon and β-cyclodextrin (C_{12} CDMPO) (Figure 24) [132]. There are three popular linker groups that have been employed for molecular tethering of cyclic nitrones, that is, ester, amide and phosphoryl groups. The main advantage of these groups is that due to their electron-

withdrawing properties, they can increase the electrophilic character of C-2 through inductive effect and, in the case of amide substitution, α-effect can facilitate $O_2^{\bullet-}$ addition to nitrones. The biconjugation of the nitrone, such as in the case of C_{12} CDMPO, can provide opportunities for a nitrone design that includes target specificity to cellular compartments.

Recently, it has been predicted through a computational approach the unusually high exoergic $\Delta G_{298K,aq}$'s for $O_2^{\bullet-}$ adduct formation for (2*S*,4*S*)-2-methyl-2,4-*bis*(methylcarbamoyl)-1-pyrroline *N*-oxide (Figure 26) and HOO adduct formation for (2*R*, 3*R*)-2-(dimethoxyphosphoryl)-3-(ethoxycarbonyl)-1-pyrroline *N*-oxide with $\Delta G_{298K,aq}$ = -3.3 and -9.4 kcal/mol, respectively. These energetics are the most exoergic $\Delta G_{298K,aq}$ observed thus far for any nitrones at the level of theory employed in this study [260].

Stability, cytotoxicity & cytoprotective properties

Nitrone stability

The reported reduction potentials for nitrones are mostly >-2.0 V [261] while GSH and ascorbate have standard reduction potentials of E^o_{red} = 0.92 V and 0.28 V [118], respectively. Therefore, nitrones in the presence of ascorbate and GSH are stable. The oxidation potential of DMPO, for example, ($E^o O_x$ = 1.63 V) [262] is significantly higher than any of those generated in biological systems in which the only conceivable oxidants are the radicals themselves [109]. In general, nitrones with electron-withdrawing group substitution at the C-5 position result in higher calculated electron affinities and ionization potentials, making these nitrones more susceptible to reduction, but they are more difficult to oxidize compared with DMPO. Reduction of nitrones yielded elongated N-C_2 and N-O bonds but shorter C_5-N bonds, while their oxidation only results in a slight elongation of the N-C_2 bond. Figure 27 shows the mesomeric forms for the oxidized and reduced nitrones showing electron delocalization on the nitronyl-N and -O for the reduction and oxidation forms, respectively [187].

Cytotoxicity

Nitrone toxicity on bovine aortic endothelial cells was assessed on various spin traps and nitroso compounds after 24 h of incubation. The lowest toxicity was observed for DMPO (IC_{50} ~140 mM), but toxicity was highest for the nitroso compounds (IC_{50} ~0.1 mM) [263]. The more lipophilic nitrone, PBN, gave higher toxicity (IC_{50} ~9 mM) than DMPO. In a separate study, cytotoxicity of DMPO, CMPO, EMPO, BMPO and DEPMPO were compared using Chinese hamster ovary cells and results indicate negligible toxicity within the concentration range of 2.5–50 mM of spin trap concentrations, except for BMPO, which gave significant toxicity at 50 mM. Effect of the spin traps on the number of colonies formed in Chinese hamster ovary cells and 9L-tumor cells was found to be cell-type dependent [264]. Cytotoxicity of various derivatives of EMPO on different carcinoma cell lines also show BMPO to be the most toxic (IC_{50} ~5–6 mM) for all cell lines [265], with DEPMPO and *i*-PrMPO as the least toxic (IC_{50} ~100–300 mM), but are cell-line dependent. Conjugation of an amphiphilic carrier to PBN and DMPO to form FAPBN and FAMPO, respectively, only affects FAMPO toxicity but not FAPBN. FAMPO showed 20% and 50% decrease in cell viability at 0.5 and 1 mM, respectively, while the carrier alone gave cell

toxicity of 5–25% at 0.025–1.0 mM [247]. These results indicate that cellular permeability plays an important role on nitrone toxicity.

Cytoprotective properties

The cytoprotective property of nitrones is dependent on nitrone cellular permeability and oxidant compartmentalization properties. Cytoprotection of fluorinated amphiphilic carrier (FA) conjugates of cyclic (FAMPO) and linear (FAPBN) nitrones against hydrogen peroxide (H_2O_2), $ONOO^-$ (via SIN-1) and 4-hydroxynonenal-induced cell death using BAEC was investigated [247]. FAPBN gave the most robust protection against H_2O_2 while FAMPO was found to be most protective against SIN-1, and none of the spin traps were protective against 4-hydroxynonenal. HPLC analyses revealed that FAMPO and FAPBN localize extra- and intra-cellularly, respectively, only during the first 2 h of incubation. It is expected that H_2O_2 (pK_a >15) can diffuse inside the cell while ONOOH (pK_a = 6.8) is mostly present as anion in neutral pH, and therefore should be extracellular. Our unpublished results show that C_{12}CDMPO was relatively more protective than the nonconjugated cyclodextrin and β-CD-nitrone conjugate, CDNMPO [Villamena F, Unpublished data]. However, the nontarget-specific nitrones, AMPO, a carbamoyl-substituted nitrone and DMPO gave the highest protection compared with C_{12}CDMPO, indicating the varying cellular localization of the nitrones affects their cytoprotective properties.

Therapeutic applications of nitrones

Since the seminal work of Novelli *et al.* [45,266], nitrone spin traps have been widely used as protective agents in several biological models, such as I/R injury to the heart [53,267,268], brain [269,270] and lung [271], stroke [47,55,272,273] neurodegenerative diseases [274-276], cancer [54,56,277,278], ageing [248,279], visual impairment [49,280] and loss of hearing function [281-283]. In two review articles published by Floyd *et al.* [51,55] the protective properties of the PBN nitrones against stroke, neurodegenerative disorders, hearing impairment, aging and cancer have been elaborately discussed. In this section, we have included only the most recent developments in nitrone therapeutics for both PBN and DMPO against ROS-related disorders, with special emphasis on their mechanism of action.

Cardioprotection

The heart is one of the major organs affected by uncontrolled ROS production. Recent evidence suggests that oxidative stress is a common denominator in many aspects of cardiovascular diseases. Mitochondrial dysfunction, which is characterized by inactivation of the ETC, is an important source of ROS during ischemia [107,284]. There are several reports of ROS from mitochondria [285], NADPH oxidases [286], xanthine oxidase [287] and NOS [288,289] being key mediators of cardiac damage during I/R injury. A reperfusion-associated burst of $O_2^{\bullet-}$ generation has been shown to occur when isolated hearts are subjected to ischemia and reperfusion [104,107]. The radical-scavenging properties of nitrones have enhanced their applications as cardioprotective agents in the last two decades due to the critical roles free radicals play in the phathogenesis of I/R-induced myocardial dysfunction. In the early 1990s, there were reports of applications of PBN and DMPO

against cardiac injuries [290-293]. DMPO was shown to confer protection against I/R-induced oxidative damage in isolated rat and guinea pig hearts. Recently, Tanguy *et al.* reported that LPNAH, an amphiphilic derivative of PBN, exhibits cardioprotective properties in isolated rat hearts [249]. Aside from the conventional nitrones, PBN and DMPO, a novel second-generation azulenyl nitrone known as stilbazulenyl nitrone was also reported to confer protection against myocardial I/R injury [267]. While the mechanism of cardioprotective action for nitrones is not well understood, it is becoming apparent there is more to the radical-scavenging property of nitrones to explain their cardioprotective properties. Zuo *et al.* showed that DMPO confers protection against cardiac I/R injury by salvaging the mitochondrial ETC complexes [53]. This report adds a new insight to the mechanism of cardio-protection by nitrones. It is well established that inactivation of the mitochondrial ETC is an important event for ROS generation [285,294]. It was observed that DMPO treatment reduces ROS formation, improves the recovery of left ventricular function and coronary flow in the post-ischemic heart models and decreases the infarct size. The infarct size was found to be decreased significantly from 23.0 ± 3.0% in the untreated control hearts to 14.4 ± 2.6% in those treated with 1 mM DMPO (Figure 28). The enzymatic activities of the mitochondrial ETC complexes were also found to be recovered in DMPO-treated hearts. Upregulation of the mitochondrial proteins associated with ETC, such as FMN-binding subunit of complex I, FAD-binding subunit of complex II, and subunits I and Vb of complex IV was observed in post-ischemic hearts treated with DMPO (Figure 29). eNOS-derived NO is known to play an important role in mitochondrial biogenesis [295,296], but it is not very clear whether DMPO itself modulates eNOS activity or if NO is originating as a metabolite product from decomposition of the nitrone [61]. Hence, future studies are needed to further characterize the precise molecular mechanisms of DMPO-induced myocardial protection.

Retinal protection

Retinal dystrophies in humans comprises a group of inherited ocular disorders characterized by the loss of visual functions including night blindness, narrowing of the visual field, reduced central vision and increasing sensitivity to glare. Exposure to bright light enhances the progression and severity of the age-related macular degeneration and some forms of retinitis pigmentosa [297,298], usually observed in older adults resulting in blindness and visual impairment. Apoptosis of photoreceptors and retinal pigment epithelial cells due to acute light exposure has been reported as the key events that lead to age-related macular degeneration and retinitis pigmentosa [299,300]. Oxidative stress is known to be an early event in light-induced retinal damage and has been detected within minutes of light exposure [301]. Generation of ROS due to the retinal exposure to intense light has been implicated as a key mediator of photoreceptor death in multiple ocular disorders [302-304]. It has also been reported that exposure of the cultured rod cells to blue light leads to ROS generation via impairment of the mitochondrial ETC [305], whereas the cultured retinal ganglion cells were reported to exhibit mitochondrial-mediated oxidation and apoptosis as a consequence of light-induced oxidative stress [306].

Various natural and synthetic antioxidants, such as coenzyme Q [307], melatonin [308], sulforaphane [309], α-tocopherol [310], CAT [311] and antioxidant gene therapy [312] have

been reported to either directly or indirectly prevent the retinal degeneration. Due to its radicalscavenging property, PBN was administered to prevent the light-induced ocular dysfunction. Therapeutic application of this spin trap against light-induced retinal damage was first reported by Ranchon *et al.* [313]. According to their study, direct injection of PBN into retina of the albino rats confers protection against light-induced retinal degeneration. Over the past decade, several studies have been performed to elucidate the exact mechanism of protection by PBN against light-induced visual damage. Intraperitoneal administration of PBN (50 mg/kg) to the rats 30 min prior to light exposure (2700-lux intensity of fluorescent light) was found to inhibit the induction of apoptosis in the treated animals compared with control rats [314].

Activation of the transcription factor AP-1, which mainly consists of a c-Fos/junD heterodimer [300,315], is known to be a crucial event in light-induced apoptosis of the retinal cells [314,315]. In the c-Fos-depleted mice (*c-fos*$^{-/-}$), the normal retinal function and morphology was found to be similar to that of the wild-type, but the *c-fos*$^{-/-}$ mice were found to be highly resistant to light damage compared with the wildtype mice [316]. Furthermore, it was reported that deletion of JunD or c-Jun has no effect on retinal protection [317,318], which confirms the importance of c-Fos in AP-1-mediated apoptosis of the retinal cells. Tomita *et al.* reported that pretreatment of Sprague-Dawley rats with PBN significantly inhibited both c-Fos expression and AP-1 activation [314]. But the mechanism by which AP-1/c-Fos activation is inhibited by PBN is not very well understood. It has been reported that expression of c-Fos in mouse retina depends on the light–dark cycle (visual cycle) [319]. In *Rpe65*$^{-/-}$ mice depleted for Rpe65 (an important enzyme participating in the visual cycle), c-Fos expression was significantly decreased compared with the wild-type [320]. The gene *rpe65* codes for a 65 kD enzyme, which catalyzes the hydrolysisisomerization of all-*trans*-retinyl esters to 11-*cis*-retinol, an important step of the visual cycle [321-323]. The 11-*cis*-retinol after being oxidized to 11-*cis*-retinal binds covalently to the protein opsin to form the visual pigment rhodopsin [323]. Thus, 11-*cis*-retinal acts as a chromophore of rhodopsin, and since rhodopsin bleaching is reported as the 'trigger' for retinal light damage [324,325], prevention of rhodopsin regeneration within the visual cycle may lead to the protection from light-induced retinal damage. This hypothesis was strongly supported by the knockout animal studies, where it was shown that the *Rho*$^{-/-}$ (depleted for rhodopsin) and *Rpe65*$^{-/-}$ mice had acquired resistance to light damage [320]. Mandal *et al.* reported that the pretreatment, but not the post-treatment, of Albino Sprague-Dawley rats with PBN resulted in the inhibition of light-induced retinal damage, indicating that PBN might interfere with the initial events that trigger the light-induced damage [49]. Interestingly, they observed that the isomerohydrolase activity of Rpe65 is inhibited by PBN and this is accompanied by the suppression of rhodopsin regeneration [49]. This report adds up a new insight in explaining the molecular mechanism of PBN-induced ocular protection. Since it is known that a full visual cycle is required for c-Fos expression [319], inhibition of the visual cycle by PBN may be the mechanism by which c-Fos expression is modulated in PBN-treated retinal cells.

Photobleaching of the visual pigment rhodopsin is the key event that triggers light-induced damage [323]. The rate of rhodopsin regeneration is an important factor in modulating the light sensitivity of the retina. Rapid regeneration of rhodopsin after photobleaching increases

the retinal susceptibility to light exposure, whereas reversing this process enhances the resistance of the photoreceptors to light-induced damage. Although it was reported that PBN treatment resulted in the significant inhibition of the visual cycle enzyme Rpe65, the mechanism of inhibition required further discussion. One hypothesis put forward in the same article stated that the isomerohydrolase reaction catalyzed by Rpe65 involved the formation of an intermediate retinoid radical cation and PBN might trap this radical to inhibit the progression of the reaction [49]. It has been reported that PBN can form stable adducts with the carotenoid radicals [326] and, furthermore, it was observed that PBN did not bind to free Rpe65 enzyme but rather binds to the enzyme–substrate complex to inhibit Rpe65 activity in an uncompetitive fashion [49]. This observation strengthens the idea that PBN may bind to the retinoid radical intermediate that forms a complex with the enzyme at its active site. Due to its hydrophobicity, PBN can readily enter the active site of the enzyme and can trap the retinoid radical to inhibit the reaction. S-PBN, a relatively hydrophilic derivative of PBN, is reported to be unsuccessful in inhibiting Rpe65 activity, and simultaneously does not confer protection to retina against lightinduced damage. In one study, Poliakov *et al.* had reported that more hydrophilic spin traps, such as DMPO, S-PBN, α-(4-pyridyl 1-oxide)-*N*-*tert*-butylnitrone and NXY059, did not inhibit Rpe65 activity up to 400 μM [327], which clearly suggests the SAR behind the antagonistic roles of nitrones against light-induced retinal damage. In spite of the progress made in the elucidation of nitrone protection against ocular degeneration, the mechanism of action of nitrones is still not well understood and warrants further investigation.

Anticancer properties—Based on the fact that ROS play a crucial role in carcinogenesis, PBN and its derivatives, such as 4-OHPBN or 3-OHPBN and OKN-007, have been administered against several carcinoma models [58,277,328-330]. In reviews by Floyd *et al.*, the anticancer role of PBN and PBN-derived compounds against hepatocellular carcinoma and glioma models had been elaborately discussed [54,55]. In this article, we have tried to focus on the recent developments of nitrone therapeutics in anticancer research and also highlight the mechanisms behind the anti-tumorigenic properties of nitrones, although the exact mode of nitrone action is still far from being understood.

The anticancer property of PBN nitrones was first reported by Nakae *et al.* on choline-deficient L-amino acid deficient (CDAA) rats with hepatocarcinoma [331]. Recently, anti-tumorigenic properties of PBN and OKN-007 were further investigated in glioblastomas, which represent the most common form of adult brain tumors. It was reported that application of PBN compounds inhibited the rate and extent of tumor progression and angiogenesis in C6 rat glioma models [58,328]. PBN was administered to the rats in drinking water (75 mg/kg/day) either 5 days prior to implantation with C6 glioma cells (pretreatment group) or approximately 14 days after the glioma implantation (post-treatment groups). Parameters associated with tumor progression and angiogenesis were assessed by MRI and magnetic resonance angiography, respectively. It was observed that administration of PBN resulted in the significant inhibition of tumor growth and progression in both pre- and post-treatment conditions, while the survival of the implanted rats had increased considerably (up to 40%) in PBN-treated groups [328]. The PBN-derivative OKN-007 was also found to induce tumor regression and enhance the survival of C6 glioma rats when

administered in post-tumor stage. Treatment with OKN-007 resulted in the drastic reduction of tumor volume (~threefold), a decrease in apparent diffusion coefficients (~20%) and a simultaneous increase in the tissue perforation rates compared with the untreated rats [58].

After the successful application of PBN nitrones against liver and brain cancer models, they were challenged against colon cancers. The anticancer potential of nitrones was investigated in $APC^{min/+}$ mice models for colorectal cancer [60]. APC is a multifunctional protein that can trigger the activation of β-catenin and induction of WNT signaling [332,333]. In $APC^{min/+}$ mice, the adenomas are developed in the early stages of infancy and they are subjected to death at 16–20 weeks of tumor progression due to intestinal hemorrhage [334]. When these tumor-bearing mice were administered with PBN in drinking water (100 mg/kg/day), a significant reduction in the tumor volume and associated intestinal adenomas were observed [60].

Although PBN-derived nitrones were found to be effective against various tumor models, the mechanism by which they induce tumor regression is not very clear. Unlike the popularly used anticancer drugs – curcumin, resveratrol, cisplatin and doxorubicin, to name a few – nitrones do not possess cytotoxic potential against cancer cells. It is reported that PBN had no detrimental effect on cancer cells [60]. But the anti-inflammatory properties of nitrones may be the key to their effectiveness against tumor progression as inflammation and cancer development are intertwined. There is a long-standing relationship between cancer and inflammation, and the modulators of inflammation, such as NF-κB, Cox-2 and iNOS, are known to play important roles in carcinogenesis [335-337]. It has been reported that PBN treatment resulted in downregulation of the expression of these inflammatory genes in liver carcinogenesis [331] and macrophages [338]. NO plays an important role in tumor growth and angiogenesis, and the hepatocytes isolated from CDAA rats were found to produce high levels of NO compared with those isolated from the rats fed with normal diet. Similarly, the bile flowing in the liver of the CDAA rat showed enhanced NO production compared with normal rats, confirming the importance of NO in liver carcinogenesis [54]. The cyclic nitrone, DMPO, was found to inhibit LPS-induced oxidative and inflammatory stresses in cultured macrophages. DMPO protected cells from LPS-induced toxicity via downregulation of iNOS expression after 24 h of nitrone incubation. Early genes were also investigated within 16–60 min and showed DMPO inhibition of MAPKs, Akt and IκBα phosphorylation, and reduced the NF-κB p65 translocation [339].

Beside the anti-inflammatory activity of nitrones, PBN compounds were also reported to modulate tumor metabolism in glioma models [58]. In a very recent report by He *et al.* it was shown that treatment of C6 glioma rats by PBN and OKN-007 resulted in the alteration of the levels of metabolites associated with brain cancer [58]. MRI results revealed that levels of the major metabolites such as choline, creatine, *N*-acetyl aspartate and lipids (lip 1.3 and lip 0.9) were affected by nitrone treatment throughout the course of tumor progression. Thus, the levels of *N*-acetyl aspartate and creatine were found to decrease in the untreated C6 rats, but in the presence of PBN and OKN-007 their levels had increased significantly. Alterations in lipid levels have been previously demonstrated to be a marker of tumor malignancy and indication of response to treatment [340,341]. When C6 rats were treated with nitrones, drastic alterations in lipid levels were observed at different phases of

tumor progression. Thus, it may be concluded that nitrones can induce tumor regression by inhibiting the onset of inflammation and also by modulating tumor metabolism.

Conclusion

By exploiting the intermolecular and intramolecular hydrogen bonding interaction from an amide linker group and substitutents with the nitrone moeity and $O_2^{\bullet-}$, $O_2^{\bullet-}$ addition reaction to nitrones can be greatly facilitated. For targeting $O_2^{\bullet-}$, employing anion recognition using synthetic receptors, which has been gaining attention in the field of host–guest chemistry due to their potential biological and environmental applications [342], could be a very promising strategy in increasing $O_2^{\bullet-}$ reactivity to nitrones. Synthetic anion receptors employ hydrogen bond donors using amide, urea, thiourea, sulfonamides or hydroxyl groups mimicking naturally occurring anion-binding sites. Results (both computational and experimental) indicate extraordinary enhancement of $O_2^{\bullet-}$ addition reaction to nitrone via conjugation of the nitrone to calix[4]pyrrole and protection of the spin adduct from $O_2^{\bullet-}$ itself, thereby significantly increasing the adduct $t_{1/2}$ [133]. Synthesis of nitrone–anion-receptor conjugates can potentially extraordinarily enhance the rate constants for $O_2^{\bullet-}$ addition to nitrones, increase biostability of the nitrones and adducts by making them less susceptible to bioreduction/oxidation, and improve cellular permeability through biconjugation to target specific groups.

The mechanism by which nitrones impart their antioxidant properties in cells or animal models is not clear cut, where their spin-trapping property is not solely responsible for their therapeutic property. However, what is clear is that nitrones can act in a variety of pathways that can alter the redox state of the system via ROS/RNS scavenging and NO donation – all of which have significant implications in the control of signaling transduction and gene induction. The lack of multifunctional antioxidants that can ameliorate cardiovascular disease is a key factor limiting the application of antioxidants as therapeutic agents. Synthetic antioxidants commonly function as direct radical scavengers (e.g., SOD mimetics, mitochondria-targeted nitroxides, quinones and vitamin E) or indirectly by bolstering antioxidant defense mechanisms (e.g., ebselen derivatives and αLA), but nitrones offer promising therapeutic potential by scavenging ROS/NOS, increasing NO bioavailability, bolstering antioxidant defenses and by inhibiting pro-apoptotic pathways. Nitrone therapeutics as applied to the treatment of diseases, with the goal of ameliorating, reversing or preventing the effect of oxidative injury, have yet to be fully explored due to lack of innovative nitrone designs that can increase bioavailability and target the specific subcellular effector compartments. Given the number and the variety of antioxidant-based therapeutic strategies, few antioxidants have found clinical application, such as *N*-acetylcysteine, α-LA and some flavonoids [343]. Nitrones offer potential as therapeutic agents due to the rich chemistries and biological activities they impart. However, the unsuccessful application of NXY-059 to clinical use, as discussed by Firuzi *et al.* [343] showed a need for developing antioxidants that are more disease-specific, organelle- or cellular-targeted, and bioavailable.

Nitrone therapeutics can be mechanism specific. For example, in the pharmacology of cardiovascular disease affecting contractile function of the heart, uncoupling of the mitochondrial ETC and NOSs or ROS/RNS-mediated alteration in intracellular calcium

handling, are reasonable targets. Compounds that can target these mechanisms all at once by preventing, minimizing or ameliorating the effects of ROS can be a powerful therapeutic strategy. ROS generation can increase under conditions of hyperoxia and post-I/R and can cause mitochondrial dysfunction via inactivation of the ETC complexes. Therefore, therapeutic drugs that are targeted to the mitochondria may provide better pharmacological effects against I/R injury. Moreover, abnormalities in calcium handling have been implicated in many forms of cardiac disease. An increase in intracellular calcium can result in further release of calcium from the sarcoplasmic reticulum (SR) via the ryanodine receptor, and this increases free intracellular calcium concentration by several-fold. Evidence is also mounting that neuronal NOS enhances contractile function through regulation of SR calcium cycling [344]. Altered regulation of $O_2^{\bullet-}$ and NO occurs in various cardiomyopathies (i.e., I/R injury and heart failure), making NOS an ideal target for therapeutic study. I/R is characterized by upregulated overproduction $O_2^{\bullet-}$ and, therefore, NO bioavailability can be compromised due to the high rate of $O_2^{\bullet-}$ reaction with NO approaching diffusion controlled limit to form $ONOO^-$. In conditions of oxidative stress, eNOS can switch from producing NO to $O_2^{\bullet-}$, a process called eNOS uncoupling. This uncoupling is presumed to be primarily caused by oxidation of the cofactor BH_4 by $ONOO^-$ [345]. An increase in ROS, such as $O_2^{\bullet-}$, together with decreased NO bioavailability contribute to endothelial dysfunction, which can lead to cardiovascular disease. Therefore, therapeutic agents that can target cytosolic matrix and affect kinase-mediated phosphorylation processes in the cell may be able to upregulate eNOS. Nitrones being both $O_2^{\bullet-}$ scavengers and NO donors [61] may exhibit pharmacological activity against cardiac mechanical dysfunction caused by I/R injury. Recently, lipophilic NO-donor and lipophilic antioxidants, when introduced as hybrid molecules, not as a mixture, can exhibit cardiac protection from I/R through significant reduction of infarct size with improved recovery of cardiac function [346]. Moreover, magnesium lithospermate B has been shown to protect endothelium from hyperglycemia-induced dysfunction via eNOS phosphorylation and induction of phase II enzymes by Nrf-2 nuclear translocation, while α-LA was claimed to be unable to cause these effects [347]. Recently, our laboratory had shown that nitrones can inhibit ROS-induced apoptosis in endothelial cells via increased phase II enzyme activity through Nrf-2 nuclear translocation and suppression of mitochondrial-dependent pro-apoptotic signaling [348].

Future perspective

Our hypothesis centers on how nitrones can regulate *S*-glutathionylation. *S*-glutathionylation [349] has been gaining attention as an important mechanism in the induction of signal-transduction processes as regulated by the cellular redox status. Glutathionylation is a post-translational oxidative protein modification that involves cysteine coupling with the tripeptide glutathione, an abundant low-molecular-weight molecule. It has been shown that glutathionylation is involved in modulating Nrf-2 transcription and in the induction of antioxidant genes expression. Moreover, glutathionylation of cysteine proteins under oxidative stress conditions has been implicated in altered Ca^{2+} signaling [350,351], mitochondrial damage [352] and eNOS uncoupling [353], the three major biochemical events that have the most profound effect on I/R. Therefore, determination of the main location of oxidant production relevant to glutathionylation is important. We have

preliminary evidence showing that DMPO and some of its analogues salvage mitochondrial ETC dysfunction during I/R, reverse eNOS uncoupling and improve Ca^{2+} handling, which could explain the cardioprotective property of DMPO in isolated rat heart during I/R [53]. Considering that eNOS is mostly localized near the cell membrane and that mitochondria and SR are mostly cytosolic, the use of nitrones that can specifically target these compartments can provide opportunities to pinpoint the most effective cellular sites for the prevention of *S*-gluthionylation since nitrones such as DMPO and DEPMPO have been shown to reversibly add to glutathiyl radicals [354,355]. By identifying which targeted cellular event/s contributes the greatest to I/R injury, and which target-specific nitrone (or combinations thereof) will exhibit the most robust protection, can give valuable insights into the mechanisms of nitrone protection. Also, by correlating the degree of *S*-glutathiolation with the ability of spin trap to reverse eNOS uncoupling, salvage mitochondrial dysfunction and improve contractility, will be helpful in determining the main cause of oxidative stress.

Successes in nitrone therapeutics can perhaps be accomplished by also focusing on the organelle specificity of drug delivery. Targeting organelles where mechanisms of oxidative stress mentioned above mostly occur can be an effective strategy in minimizing oxidative stress. For example, Golgi apparatus [356] have been gaining attention as an important organelle implicated in oxidative stress since it is a vital organelle responsible for protein misfolding, resulting in many pathophysiological conditions such as neurodegeneration and cardiovascular diseases [357]. Endoplasmic reticulum (ER) stress has also been implicated in various pathophysiological conditions. The ER is an organelle responsible for protein folding, calcium homeostasis and lipid synthesis, and deviation from its normal function can lead to the accumulation of unfolded or incorrectly folded proteins resulting in ROS production [358,359]. The activity of eNOS is highly regulated by the post-translational myristoylation and palmatoylation of the enzyme, which results in its translocation from Golgi to the cholesterol-rich domains of the plasma membrane including the calveolae and lipid rafts [360]. Therefore, targeting post-translational regulation of NOS activity in specific organelles, such as the plasma membrane, SR/ER or Golgi, may salvage or reverse eNOS uncoupling where it is most susceptible. Conjugating Golgi-specific lipids such as C5-ceramide and C6-sphingomyelin or ER-targeting carbocyanine moieties to spin traps will facilitate their better compartmentalization in the cellular milieu. Thus, the application of these novel conjugated nitrones to elucidate mechanisms of NOS uncoupling in each of the subcellular compartments may enhance the scope of nitrone therapeutics in the treatment of ROS-related disorders.

Nitrones can be applied as potential therapeutic agents in the treatment of chronic metabolic disorders comprised of inter-related pathophysiological conditions such as hypertension, diabetes, chronic kidney disease and hypercholesterolemia. Nitrones have potential in modulating insulin signaling and resistance by minimizing the effects of ER stress, which play a crucial role in the regulation of several metabolic pathways that can lead to chronic metabolic disorders. Perhaps one effective therapeutic strategy is a multidrug delivery approach, in which various organelle-specific nitrones can be introduced *in vivo*, which may provide a more effective way of sequestering ROS at different locations simultaneously.

Looking into the future of nitrone therapeutics, the diagnostic ability of nitrone to detect free radicals can be concurrently exploited and employed as theranostic agents for future applications similar to that of nanoparticles [361], since multiconjugation of the nitrone is now possible. Co-conjugation of nitrones with other probes, drugs, delivery vectors and/or stabilizing agents can further enhance nitrone theranostic ability. The biomedical values of developing new spin traps include understanding the origin of radical production, toxicology of xenobiotics and antioxidant activity of some compounds, as well as improving the accuracy of radical identification, which could potentially open up new grounds Looking into the future of nitrone therapeutics, the diagnostic ability of nitrone to detect free radicals can be concurrently exploited and employed as theranostic agents for future applications similar to that of nanoparticles [361], since multiconjugation of the nitrone is now possible. Co-conjugation of nitrones with other probes, drugs, delivery vectors and/or stabilizing agents can further enhance nitrone theranostic ability. The biomedical values of developing new spin traps include understanding the origin of radical production, toxicology of xenobiotics and antioxidant activity of some compounds, as well as improving the accuracy of radical identification, which could potentially open up new grounds in spin-trapping applications from cellular level to whole animals. However, this would entail big leaps in the development of spin traps with extraordinary stable spin adducts *in vivo* and the development of spin traps with platforms different to the parent DMPO, coupled with advances in imaging technology either by nuclear medicine, magnetic resonance or optical imaging.

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Glossary

Reactive oxygen species/reactive nitrogen species

Examples of radical reactive oxygen species are $O_2^{\bullet-}/HO_2^{\bullet}$, $HO^{\bullet}$, $RO_2^{\bullet}$, $RO^{\bullet}$, $CO_3^{\bullet-}$ and $CO_2^{\bullet-}$; nonradicals include: H_2O_2, HOCl, O_3, $O_2{}^1\Delta g$ and ROOH; while examples of reactive nitrogen species are NO, $ONOO^-$, $^{\bullet}NO_2$ or ROONO.

Oxidative stress

Biological imbalance between the pro-oxidant generation and antioxidant repair mechanisms in favor of the former.

Antioxidant therapy

Pharmacological strategy to suppress minimize or reverse oxidative stress through direct sequestration of reactive species, induction of antioxidant enzymes expression or activity, suppression of signal transduction or gene induction leading to apoptosis, and reversal or suppression of pro-oxidative enzyme activity, among others.

NXY-059

Disulfonyl derivative of α-phenyl-*tert*-butyl nitrone. Was under development for the treatment of acute stroke by AstraZeneca and was the first nitrone-based compound to reach the SAINT-1 Phase III clinical trials.

DMPO

First synthesized by Sir Alexander Todd, 1957 Nobel laureate in chemistry, as a model compound to investigate the corrin nucleus of vitamin B12. Iwamura and Inamoto in 1967 first demonstrated the spin trapping of C-centered radicals by 5,5-dimethyl-1-pyrroline *N*-oxide, and Janzen coined the word spin trapping in 1968.

Executive summary

Innovative spin-trap design

- Spin-trap development requires incorporation into one molecular design of the three major required qualities that are still lacking from the currently used nitrones; that is, high rate constant for $O_2^{\bullet-}$ trapping, biostable spin adduct and target specific.
- Using computational chemistry, rational design of spin traps can be achieved. Advancements in spin-trap development over the past few years had focused on increasing spin trap reactivity to $O_2^{\bullet-}$ by exploiting the electron-withdrawing ability of amide substituents, as well as its interaction with the $O_2^{\bullet-}$ via α-effect.
- Spin adducts with extraordinary stability can be achieved through nitrone conjugation with cyclodextrin or anion receptors such as calixpyrroles.
- Subcellular specificity of nitrones can be realized via bi-conjugation with target-specific groups.

Nitrone chemical properties

- Nitrones react with a variety of reactive oxygen, nitrogen and sulfur species, as well as nucleophiles that are implicated in the initiation of oxidative damage to key biomolecular systems.
- Nitrones (conjugate acid $pK_a = 6.0$ versus pK_a for $HO_2^{\bullet}/O_2^{\bullet-} = 4.8$) react more efficiently under slight acidic pH than in neutral pH to $O_2^{\bullet-}$, which is relevant in acidosis condition such as in ischemic and cancer cells.
- Cyclic nitrones (e.g., 5,5-dimethyl-1-pyrroline *N*-oxide) react to $O_2^{\bullet-}$ and $HO_2^{\bullet}$ faster than the linear nitrones such as α-phenyl-tert-butyl nitrone.
- The first example of an antioxidant that can convert reactive oxygen species to more beneficial radicals such as NO. Nitrone $O_2^{\bullet-}$ adducts decompose to yield NO, thereby making them controlled delivery systems for NO as a function of reactive oxygen species generation.

Nitrone biological properties

- Nitrones have been applied in both *in vitro* and *in vivo* systems as therapeutic agents against cardiovascular disease, neurodegeneration (which includes ocular damage) and

cancer. Other than their spin-trapping ability, the exact mechanisms of their protection is not clear.

- *In vitro* studies show that nitrone protection against various types of oxidant is dependent on nitrone structure.
- Nitrone pharmacological activity was found to be due partly to salvaging the mitochondrial electron transport chain, increasing mitochondrial biogenesis, activation of transcription factors and downregulation of modulators of inflammation such as NF-κB, Cox-2 and *i*NOS.
- Preliminary evidence in our laboratory shows that nitrones can bolster antioxidant enzymes activity (e.g., SOD, catalase, heme oxygenase and glutathione reductase) via Nrf-2 nuclear translocation, increase glutathione levels, improve contractile function of the *nNOS*$^{-/-}$ knockout myocytes, inhibit caspase-3 activation and reverse eNOS inactivation.

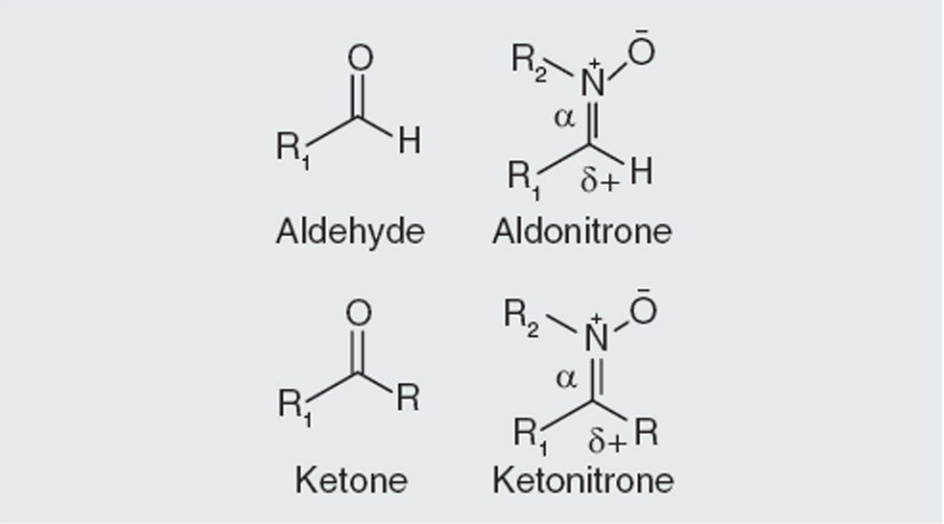

Figure 1. Analogy between carbonyl compounds and nitrones showing the electropositive α-carbon

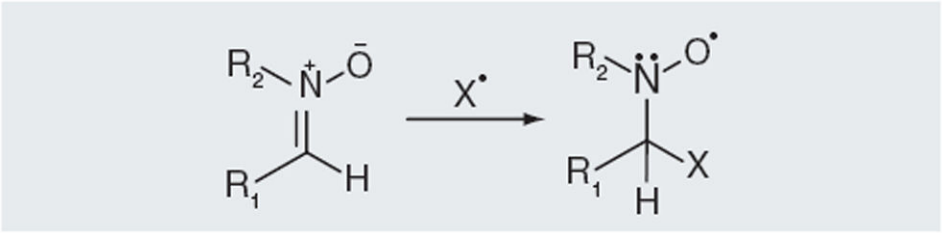

Figure 2. Formation of aminoxyl spin adduct from nitrones

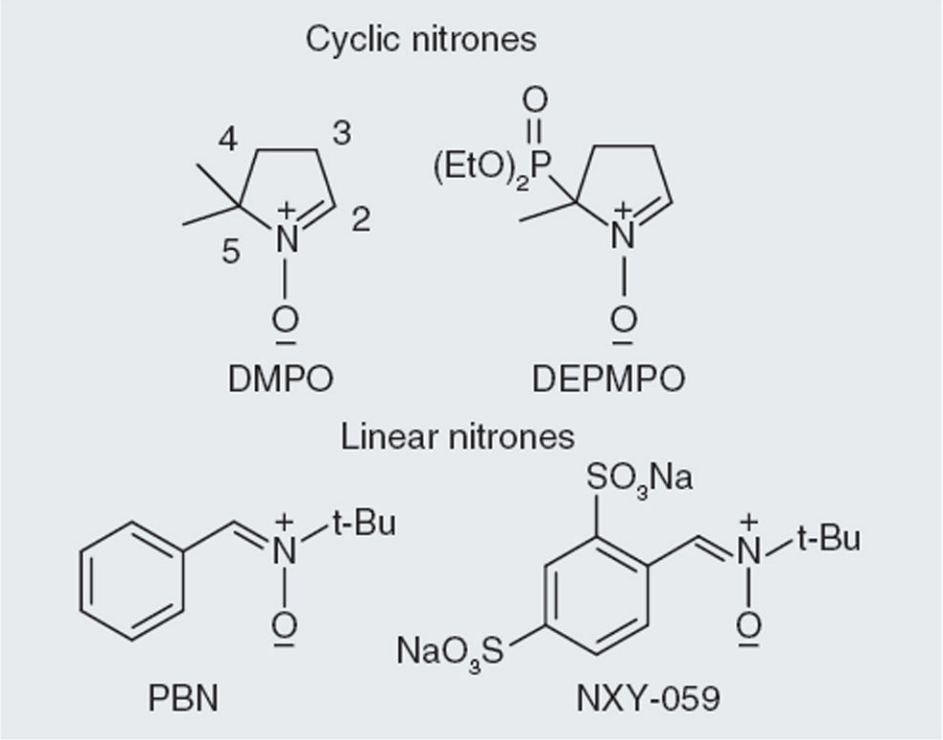

Figure 3. Common cyclic and linear nitrones

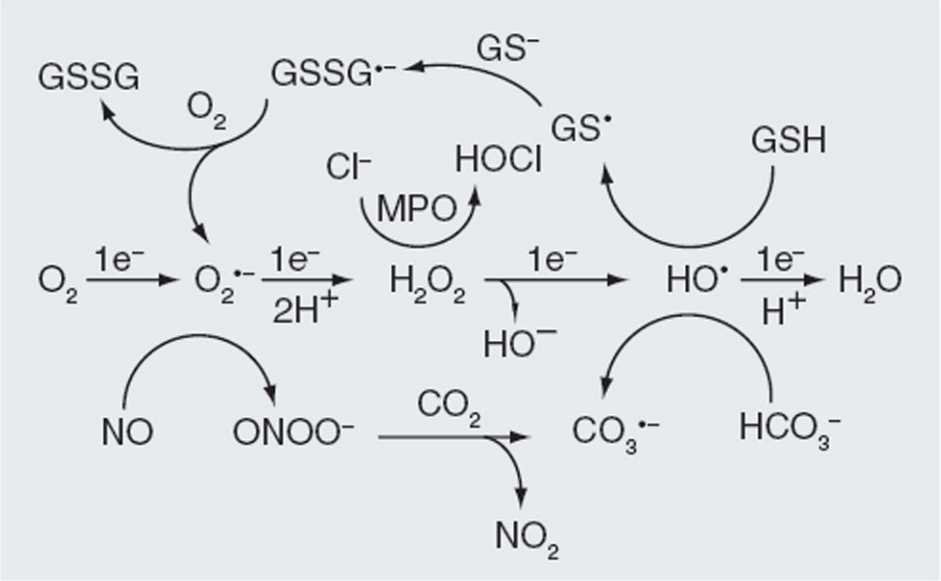

Figure 4. Formation of reactive species from $O_2^{\bullet -}$

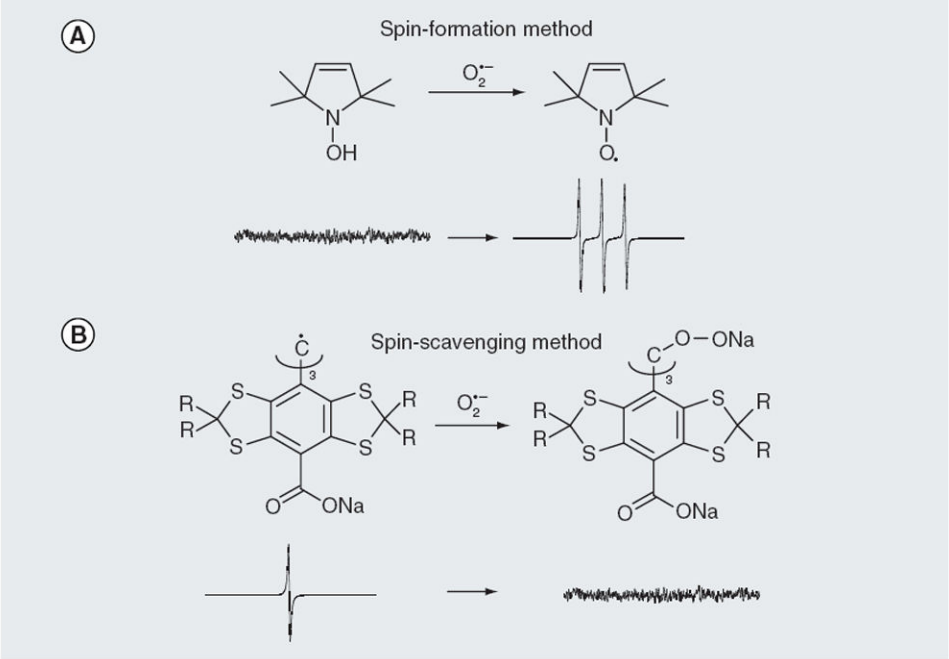

Figure 5. Electron paramagnetic resonance spectroscopic techniques for the detection of $O_2^{\bullet-}$

Detection of $O_2^{\bullet-}$ using **(A)** spin-formation and **(B)** spin-scavenging methods.

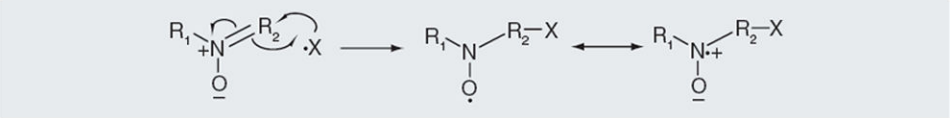

Figure 6. Reaction of a nitrone with a free radical

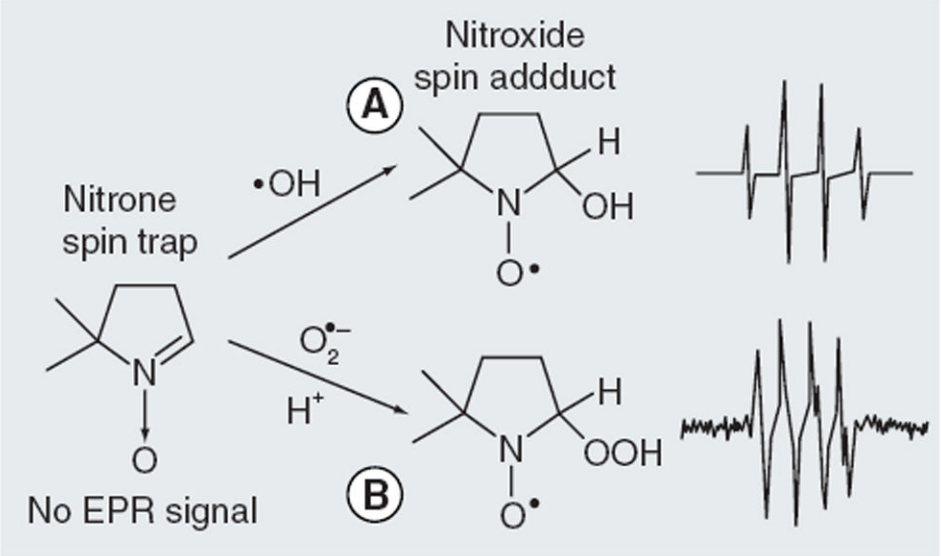

Figure 7. The electron paramagnetic resonance spin-trapping process

Showing the formation of a unique spectral profile of **(A)** the DMPO-OH and **(B)** DMPO-OOH adducts.

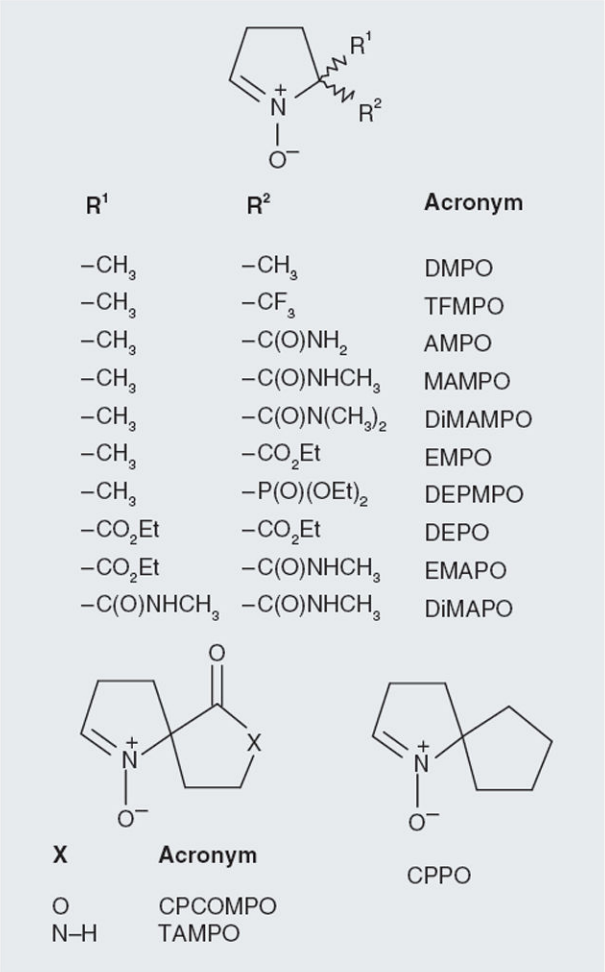

Figure 8. DMPO type nitrones used as models for the theoretical investigation of spin trapping of superoxide radical

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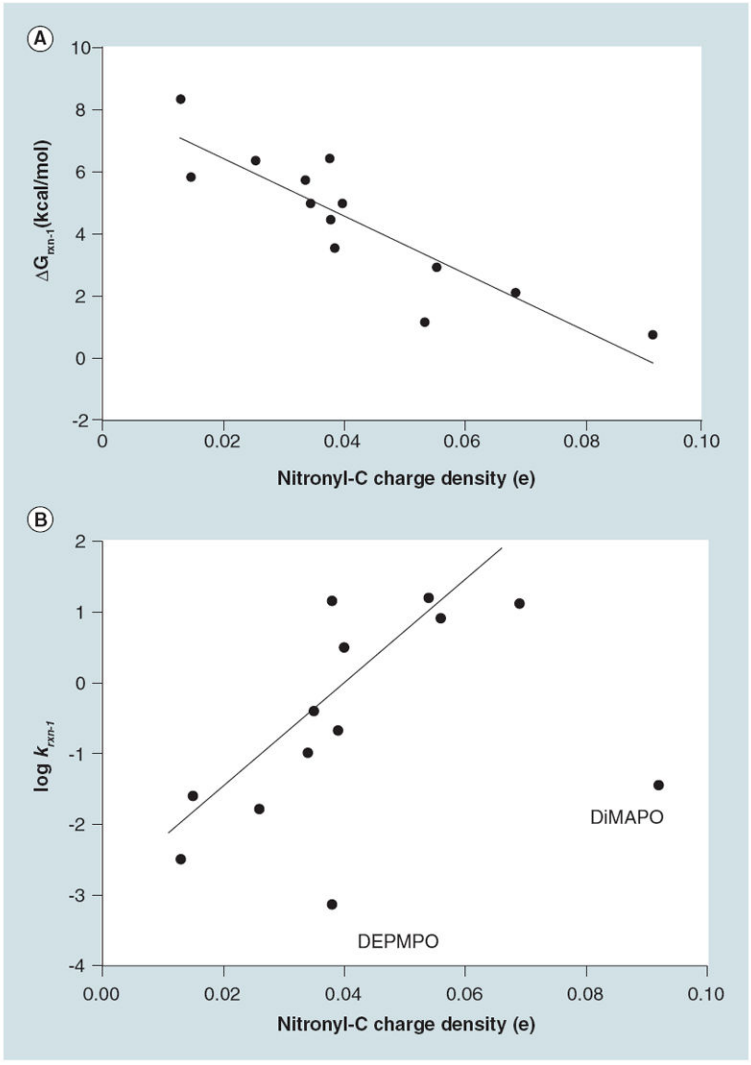

Figure 9. Nitronyl-C (C-2) charge densities

Correlation of the nitronyl-C (C-2) charge densities with that of the **(A)** free energies ($\Delta G_{\text{rxn-1, 298K}'}$ kcal/mol) ($r^2 = 0.78$) and **(B)** rate constants (log $k_{\text{rxn-1}}$) of $O_2^{\bullet-}$ addition to nitrones in the aqueous phase at the Polarizable Continuum Model (water)/mPW1K/6–31+G(d,p) level of theory at 298 K ($r^2 = 0.76$, excluding the outliers DEPMPO and DIMAPO). Shows the degree of correlation between log $k_{\text{rxn-1}}$ and $\Delta G_{\text{rxn-1}}$ at the same level of theory with $r^2 = 0.73$ excluding the outliers DIMAPO and DEPO).

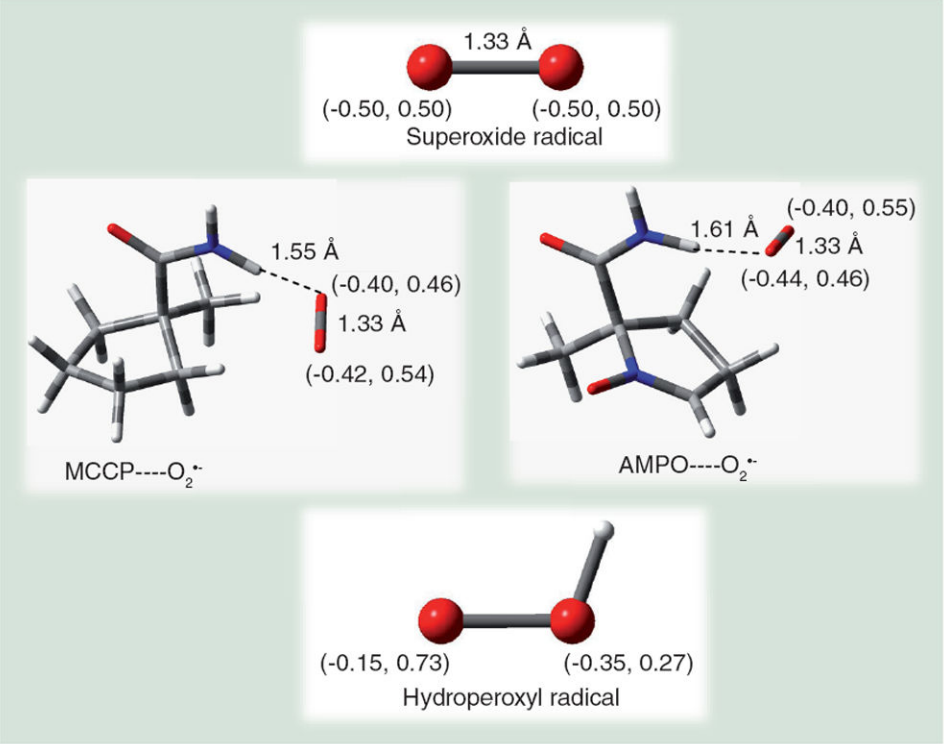

Figure 10. Perturbation on the spin and charge density distribution on the superoxide radical upon hydrogen bonding with MCCP and AMPO showing intermediate electronic distribution between superoxide and hydroperoxyl radicals

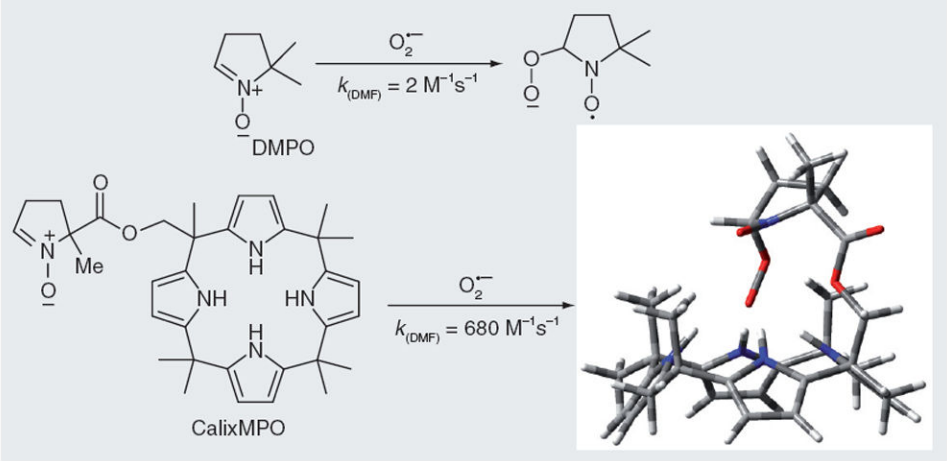

Figure 11. Improved spin trapping of $O_2^{\bullet-}$ using calixpyrrole conjugated to DMPO
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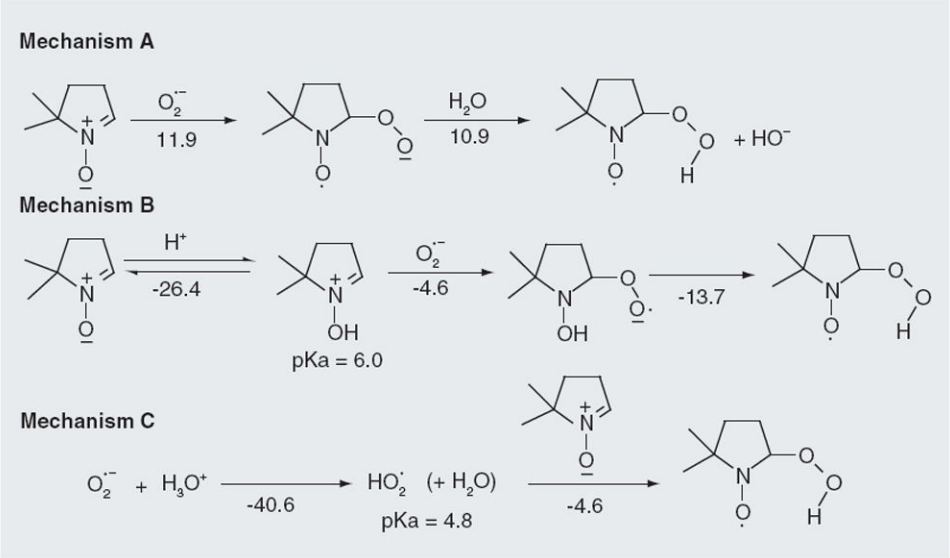

Figure 12. Suggested mechanisms and free energies ($\Delta G_{298K,aq}$ in kcal/mol) of $O_2^{\bullet -}$/$HO_2\bullet$ addition to a nitrone at the Polarizable Continuum Model (water)/B3LYP/6–31+G(d,p)// B3LYP/6–31G(d) level of theory

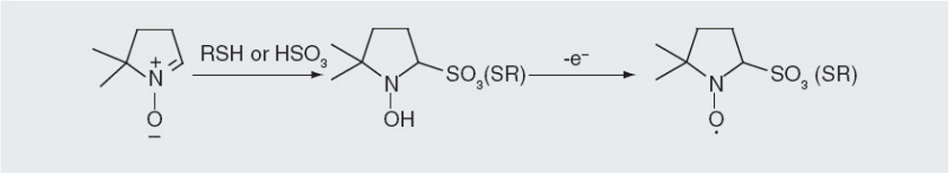

Figure 13. Nucleophilic addition/oxidation (Forrester–Hepburn) mechanism for the formation of radical adduct

SR: Sarcoplasmic reticulum.

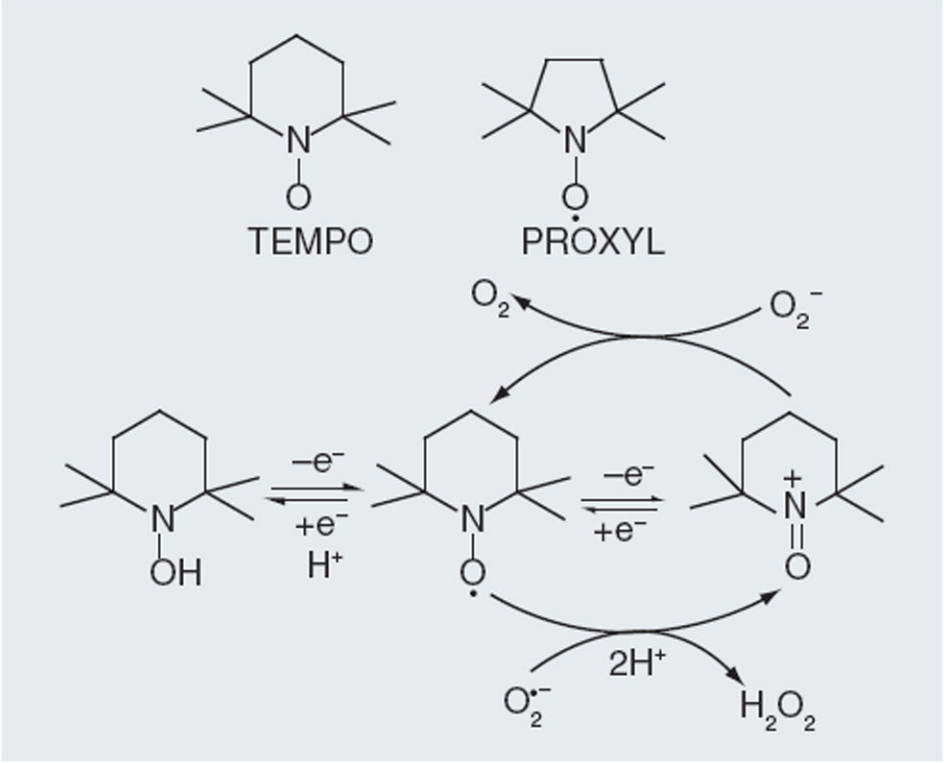

Figure 14. Examples of tetra-alkyl-β-substituted nitroxides, TEMPO and PROXYL, showing the reversibility of their redox behavior and superoxide dismutase mimetic property

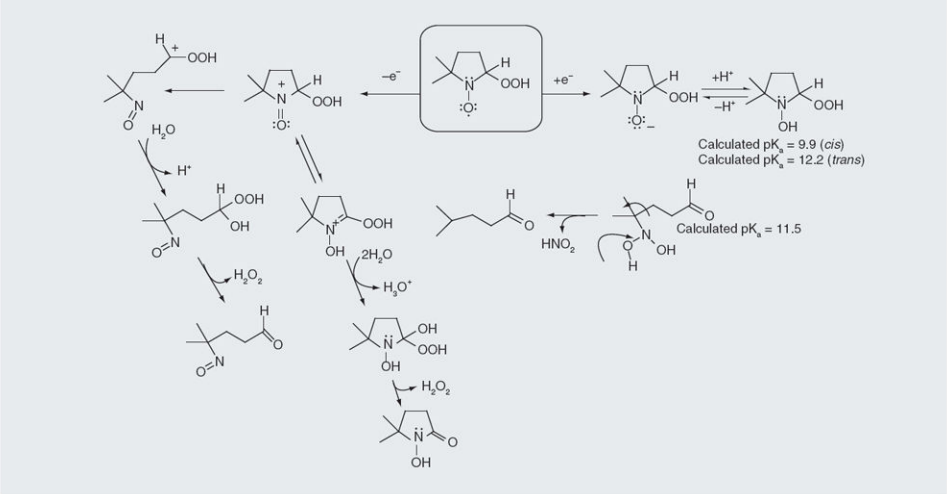

Figure 15. Superoxide radical adduct of DMPO showing the irreversibility of its reduction and oxidation leading to the formation of various biologically relevant by-products

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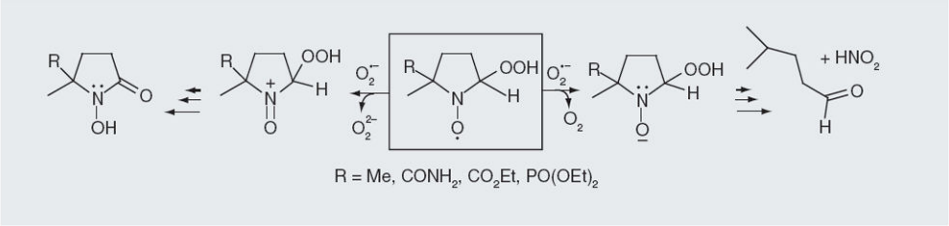

Figure 16. Redox reaction of $O_2^{\bullet -}$ with superoxide radical adduct of DMPO leading to the formation of various products

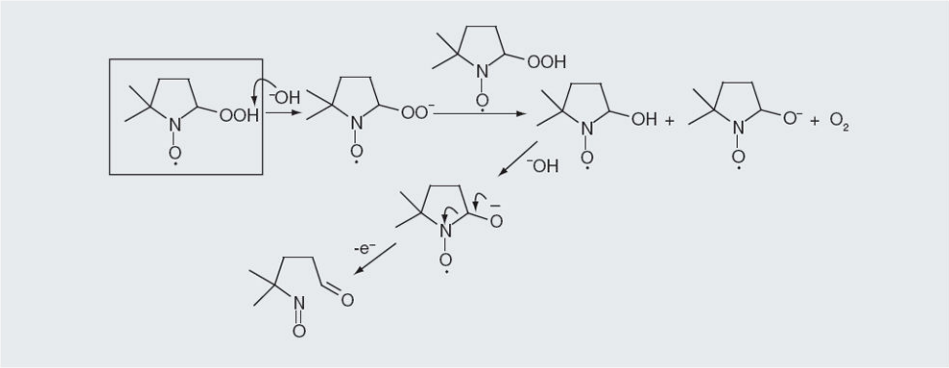

Figure 17. Mechanism of decomposition of superoxide radical adduct of DMPO under basic conditions

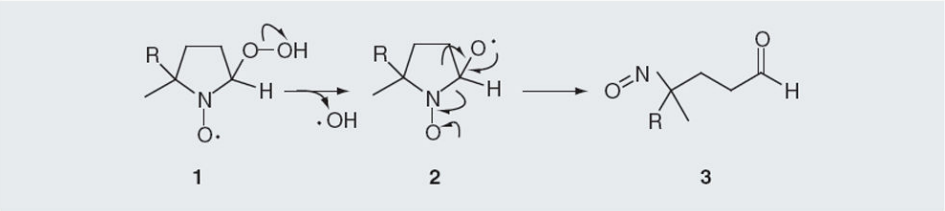

Figure 18. Unimolecular decomposition of the hydroperoxyl adduct of nitrones
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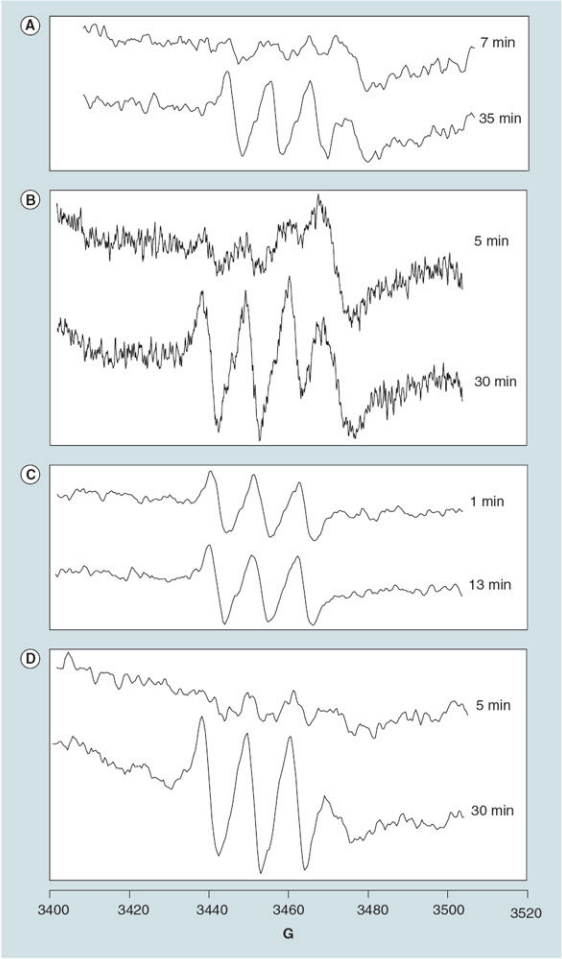

Figure 19. X-band electron paramagnetic resonance spectra of $NOFe(MGD)_2$ complex

Spectra resulting from purging DMPO solution containing **(A)** phorbolmyristate acetate-activated human neutrophils at pH 6.7; **(B)** xanthine/xanthine oxidase; (C) KO_2 in phosphate buffer solution-DMSO at pH 8.1 with argon to a solution of $Fe(MGD)_2$; **(D)** KO_2 in DEPMPO at pH 8.1 with argon to a solution of $Fe(MGD)_2$.

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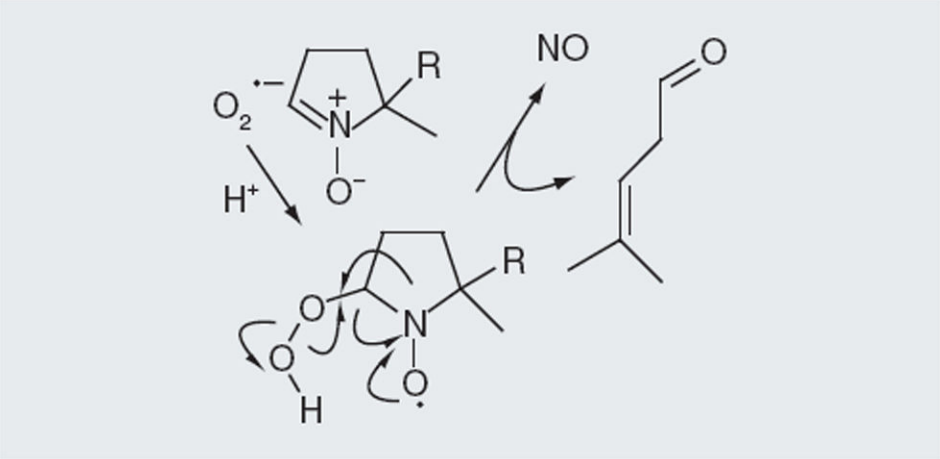

Figure 20. Superoxide scavenging by spin trap and decomposition leads to NO release

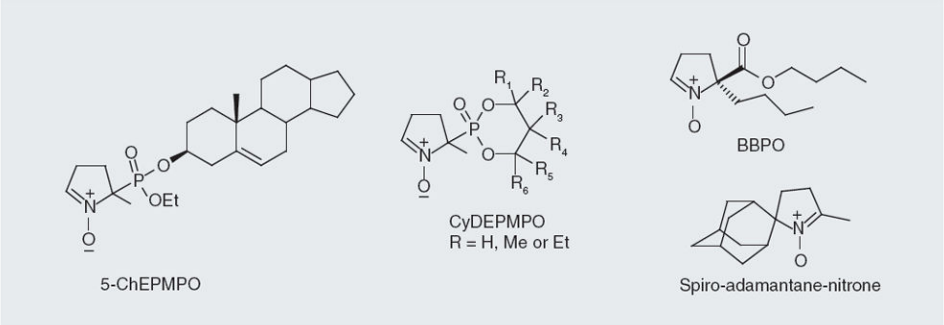

Figure 21. Examples of lipophilic nitrones

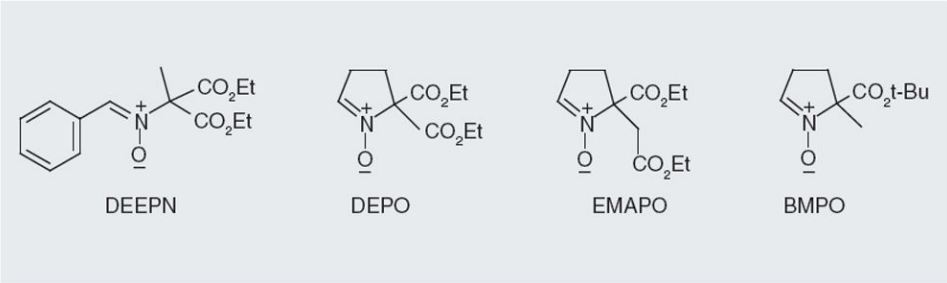

Figure 22. Examples of esterified-nitrones

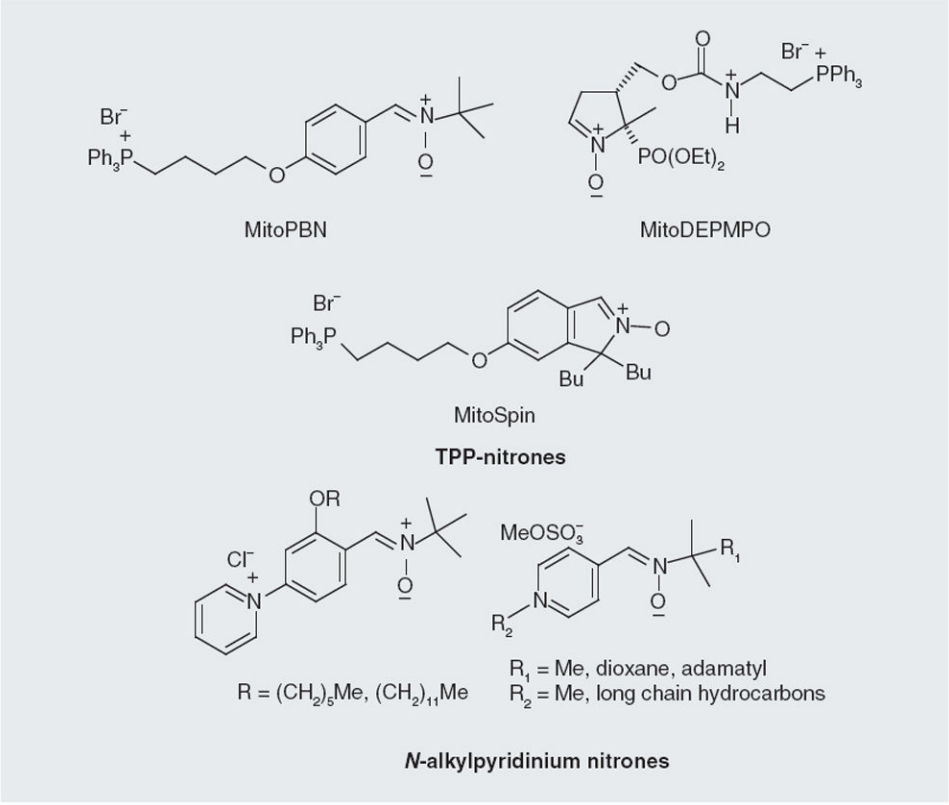

Figure 23. Lipophilic positively charged nitrones

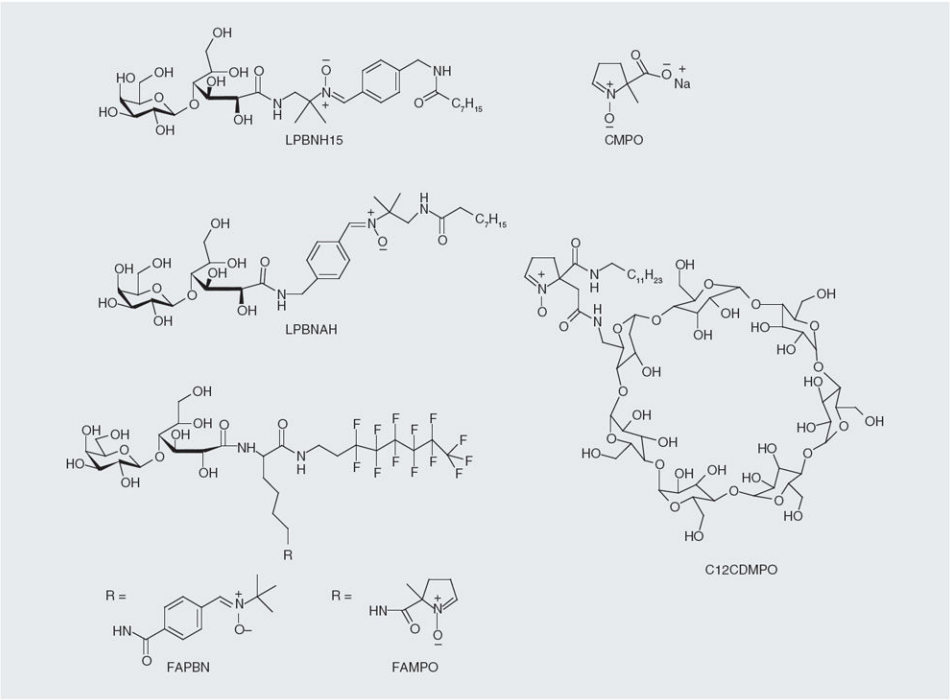

Figure 24. Extracellular (CMPO) and amphiphilic nitrones

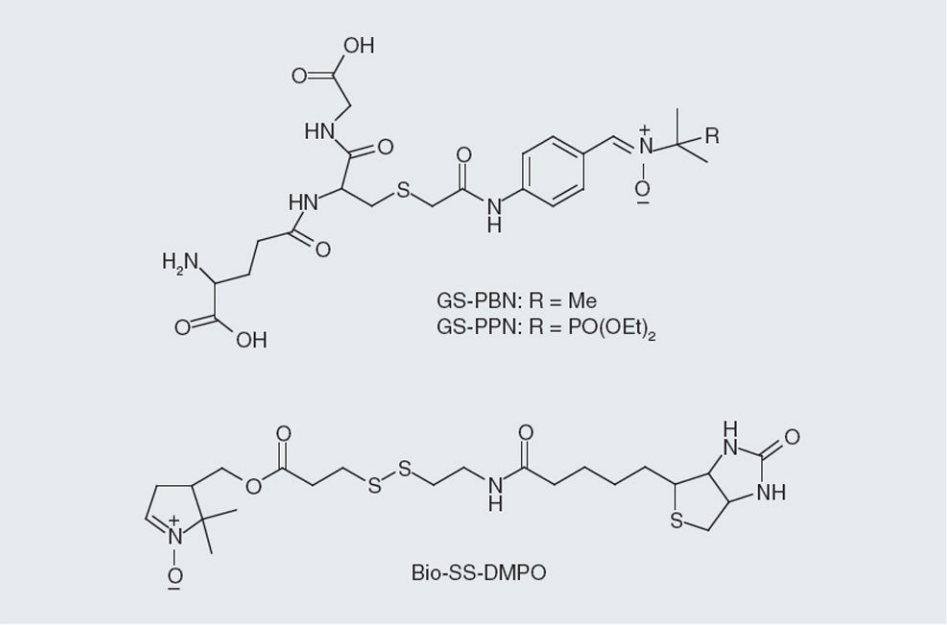

Figure 25. GS-PBN, GS-PPN and bio-SS-DMPO

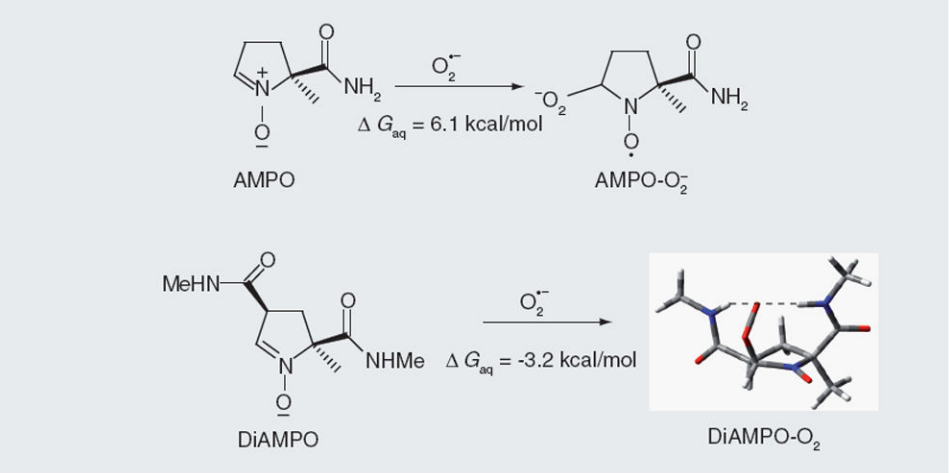

Figure 26. Enhanced reactivity to $O_2^{\bullet -}$ by biconjugated DiAMPO

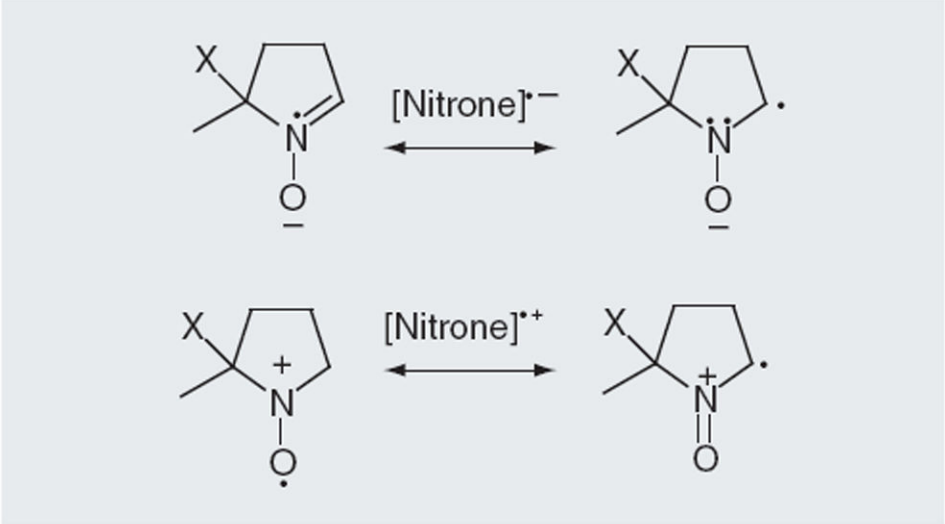

Figure 27. Resonance structures

The radical **(A)** anion and **(B)** cation showing the location of the unpaired electron. Reproduced with permission from [187] © American Chemical Society.

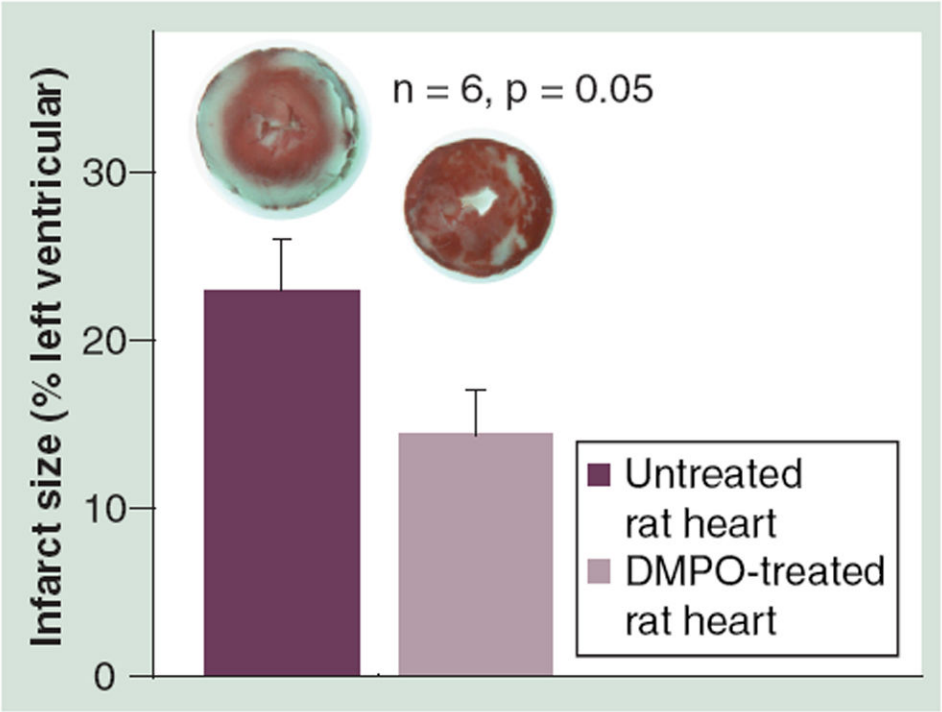

Figure 28. Reduction in the left ventricular infarct size in untreated and DMPO-treated rat heart after ischemia and reperfusion using isolated heart Langendorff preparation

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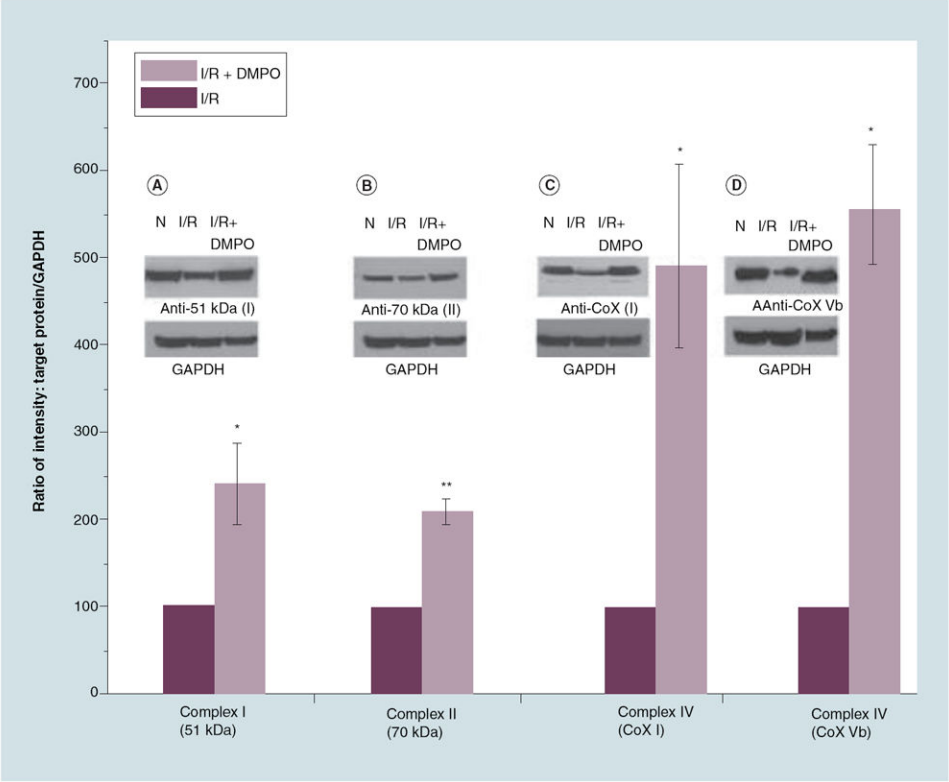

Figure 29. Effect of DMPO treatment on the protein expression of mitochondrial electron transport chain in the postischemic heart

Tissue homogenates of untreated and 1 mM DMPO-treated post-ischemic hearts were subjected to SDS-PAGE and then immunoblotted with antibodies against mitochondrial electron transport complex **(A–D)**. The antibodies used are: anti-51 kDa (FMN-binding subunit) polyclonal antibody for complex I, anti-70 kDa (FAD-binding subunit) polyclonal antibody for complex II, and anti-CoX I and anti-CoX Vb monoclonal antibodies for complex IV.

I/R: Ischemic–reperfusion.

Table 1

Calculated spin and charge densities, and free energies, $\Delta G_{298K,aq}$, of addition to DMPO versus PBN at the Polarizable Continuum Model (water)/ B3LYP/6–31+G(d,p)// B3LYP/6–31G(d) level of theory.

ROS	O(1) (kcal/mol)		O(2) (kcal/mol)		$\Delta G_{298K,aq}$ of addition (kcal/mol)	
	Spin (e)	Charge (e)	Spin (e)	Charge (e)	DMPO	PBN
O-O$^{\bullet-}$	0.50	-0.50	0.50	-0.50	11.9	18.3
HO(1)-O(2)$^{\bullet}$	0.27	-0.35	0.73	-0.15	-4.6	-0.9
HO(1)-O(2)$^{-}$	n/a	-0.68	n/a	-0.83	7.2	29.0
HO$^{\bullet}$	n/a	n/a	1.02	-0.50	-38.9	-31.2
HO^{-}	n/a	n/a	n/a	-1.46	9.0	14.6

DMPO: 5,5-dimethyl-1-pyrroline N-oxide.

Table 2

Average free energy of reaction ΔG_{rxn} of radicals with nitrones at 298.15 K and their corresponding reduction potential Eo′.

Radical	Average ΔG_{rxn} (kcal/mol)	Half reaction	$E^{o'}$, V	Ref.
$HO^{\bullet}$	-43.66	$HO^{\bullet}$, H^+/H_2O	2.31	[118]
$^{\bullet}CH_3$	-32.17	$H_3CH_2C^{\bullet}$, H^+/CH_3CH_3	1.90	[118]
$HS^{\bullet}$	-16.67	$HS^{\bullet}/HS^-$	0.92	[119]
$HO_2^{\bullet}$	-13.92	$HO_2\bullet$, H^+/H_2O_2	1.06	[118]
$O_2^{\bullet -}$	-7.61	$O_2^{\bullet -}$, $2H^+/ H_2O_2$	0.94	[118]
NO	14.57	$NO/^3NO^-$	-0.80	[120]

Table 3

Reported experimental first-order half-lives of the $O_2^{\bullet -}$ adduct of cyclic nitrones.

Adducts	$t_{1/2}$ (min)	Ref.
DMPO-OOH	1.0	[111]
EMPO-OOH	8.6	[199]
DEPMPO-OOH	14	[200]
AMPO-OOH	9.9	[201]