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Review

Diagnostic and therapeutic applications of azulenyl nitrone spin traps

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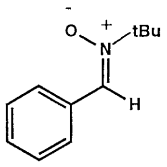
Abstract. Azulenyl nitrones have been recently demonstrated to constitute a new class of nitrone-based spin traps with the unprecedented capacity to tag free radicals by yielding characteristically colored and highly visible diamagnetic (and paramagnetic) spin adducts. In addition, a comparison of the oxidation potentials of azulenyl nitrones such as **1** and congeners to those of conventional nitrone spin traps previously investigated as potential antioxidant therapeutics such as *N*-*tert*-butyl-α-phenylnitrone and its related ortho-sodium sulfonate reveals that the azulene-derived spin traps are far more readily oxidized. These special features render azulenyl nitrones of interest with regard to both their distinct ability to engender the convenient use of colorimetric detection to monitor free radical-mediated oxidative stress in biological systems, and to their potentially enhanced efficacy as neuroprotective antioxidants vs. those conventional nitrone spin traps earlier examined as such. Herein is reported an overview of recent developments pertaining to the use of azulenyl nitrones in the detection of oxidative stress in animal models of amyotrophic lateral sclerosis and stroke, and to their neuroprotective activity in animal models of Parkinson's disease, stroke and neurodegeneration within the retina.

Key words. Azulenyl nitrones; azulenes; nitrones; AZN; PBN; S-PBN; free radicals; spin traps; antioxidants; neuroprotection; oxidative stress; assay; amyotrophic lateral sclerosis; FALS; superoxide dismutase; transgenic mice; stroke; ischemia/reperfusion; Parkinson's disease; MPTP; axotomy, neurodegeneration.

Introduction

Since the elegant structural elucidation of the blue sesquiterpenoid hydrocarbon guaiazulene by Pfau and Plattner in Switzerland in 1936 [1], the chemistry of the azulenes, largely through the many contributions of Hafner and Nozoe, has developed into a mature and integral branch of the field of nonbenzenoid aromaticity. The biology of the azulenes, while sparking limited interest, has been comparatively much less explored. Meanwhile, an important discipline within the life sciences, namely that concerning the harmful role of free radicals in biology, has been evolving steadily since the groundbreaking work of Gerschman, Harman and Fridovich. Free radicals are now widely known to be reactive intermediates in a range of detrimental (as well as beneficial) biological processes [2]. Over the last 3 decades, nitrones as a class have emerged as a powerful tool for investigation of free-radical reactions via the spin-trapping method [3]. More recently, stemming from the work of Novelli et al. in 1986 [4], nitrones have been receiving increasing attention as potential therapeutics for the treatment of pathological conditions in which free radical-driven oxidative stress is suspected to operate as a major etiological component [5]. Nitrones are attractive candidates as chain-breaking antioxidants

due to the remarkable stability of the nitroxides that form after their capture of reactive paramagnetic species. Accordingly, Thomas et al. have recently examined the therapeutic potential of a series of cyclic nitrones such as the 3,4-dihydroisoquinoline N-oxide derivative MDL-101,002 which displays longer-lived nitroxide adducts than



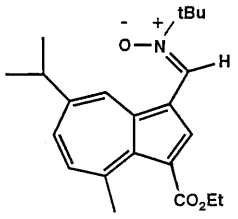
PBN

and enhanced inhibition of lipid peroxidation [6–10]. In theory, cyclic nitrones, in the presence of a suitable bioreductant, may catalytically deactivate hydroxyl and alkoxyl radicals in a more efficient manner than acyclic nitrones, since the tether connecting any ensuing carbonyl and hydroxylamine moieties should entropically promote regeneration of the cyclic nitrone by a simple dehydration, whereas diffusion is expected to impair such turnover in the case of acyclic nitrones. Interestingly, along with its antioxidant properties, MDL-101,002 produces side effects resembling sedation and bears some structural similarities to the drug librium-a nitrone-containing benzodiazepine hypnotic agent. In addition to their putative role as scavengers of harmful oxygen radicals in vivo [11], several other hypotheses have emerged concerning the mechanism by which nitrones may exert their therapeutic effects, such as by the lowering of peroxynitrite levels via inhibition of the oxidant-promoted expression of inducible nitric oxide synthase [12], or by vasodilation caused by small quantities of nitric oxide arising via a multistep oxidative breakdown of the nitrones themselves [13]. This review summarizes the recent applications of azulenyl nitrones, a new class of nitrones first prepared in our laboratory in 1995 [14], to the detection of oxidative stress in animal models of stroke and amyotrophic lateral sclerosis (ALS), and to their provision of neuroprotection in animal models of Parkinson's disease, stroke and axotomy-induced neurodegeneration in the retina.

Diagnostic applications

Stroke model: azulenyl nitrones in gerbils subjected to transient forebrain ischemia/reperfusion

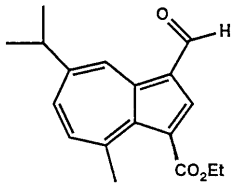
Recent collaborative work with the CNS Diseases Research Division of the Pharmacia and Upjohn Company (Kalamazoo, MI, USA) has provided evidence supporting the utility of azulenyl nitrone



AZN (1)

in detecting free-radical activity in the hippocampus of test animals undergoing bilateral carotid occlusion (BCO) with subsequent reperfusion [15]. Importantly, AZN provides neuroprotection in this model (vide supra) that is concomitant with the observed oxidative stress, suggesting that free-radical damage is linked to the neuronal death which accompanies the procedure.

The success of AZN in detecting oxidative stress in this and other models of neurodegeneration hinges upon both its oxyradical-mediated conversion to the corresponding aldehyde chromophore



AZA

[14] and on its high lipophilicity, which renders it readily able to cross the blood-brain barrier. Azulenyl nitrones such as AZN represent the only chromotropic nitrone spin traps yet to be reported. The strongly absorbing green chromophore of AZN and the red chromophore of AZA enable their facile colorimetric detection, which can be conveniently accomplished in certain applications with the naked eye (e.g. in monitoring air-induced lipid peroxidation in corn oil [14]), or by employing high performance liquid chromatography (HPLC) with ultraviolet-visible (UV-VIS) detection at their common maxima of 390 nm [15]. This wavelength constitutes a useful window for observation of free-radical activity in vivo, since there are few components in a biological matrix that can be extracted into water-immiscible organic solvents (e.g. EtOAc) and which also absorb strongly in this region of the spectrum.

The results of the ischemia/reperfusion studies with AZN in gerbils are in accord with previous experimental data that suggest that oxygen-radical production increases in the brains of the animals subjected to the BCO stroke model protocol. Thus, compared with sham animals, there is a near threefold increase in the conversion of AZN to AZA in the hippocampus of the ischemic animals (fig. 1) [15]. Importantly, this increase is detected only in hippocampal tissue and not in blood, thereby bolstering the assertion that the pathological

consequences are a ramification of changes within tissue rather than of those hematogenic in character. Furthermore, since the frequently employed salicylate assay cannot detect peroxyl radicals, employs a hydrophilic spin trap and often gives rise to analyte artifacts due to enzymatic hydroxylation within blood, the AZN assay provides a useful and potentially more discriminating diagnosis of oxyradical activity within the brain.

ALS model: azulenyl nitrones in FALS transgenic mice expressing G93A mutant human Cu,Zn superoxide dismutase

ALS (Lou Gehrig's disease) is a debilitating condition involving degeneration of neurons in the spinal cord, brain stem and cortex. The recent discovery [16, 17] of a subset of familial ALS patients (FALS) possessing autosomal dominant mutations in the SOD1 gene corresponding to Cu,Zn superoxide dismutase has fostered intense research aimed at elucidating the relationship of such mutations to the etiology of the disease. Studies reported by Gurney et al. in 1994 [18] established the first animal model of FALS in which a line of transgenic mice expressing a G93A mutant human Cu,Zn superoxide dismutase was created and was found to exhibit pathology paralleling that of the human disease. Namely, SOD1 G93A transgenic mice display progressive paralysis in association with motor neuron loss that leads to death. By virtue of experiments showing that transgenic mice expressing wild-type human Cu,Zn SOD develop without any evidence of motor neuron dysfunction, it has been postulated that the pathology can be attributed to gain-of-function mutations in the SOD1 gene [18]. Along these lines, the research groups of Valentine and Stadtman have reported that, in vitro, SOD1 mutations lead to the formation of damaging hydroxyl radicals from hydrogen peroxide [19, 20], which is the normal product of the disproportionation reaction of superoxide effected by both the wild and the G93A mutant forms of SOD. Subsequent studies have now shown that while there is no significant difference in hydroxyl radical production between the wild and the G93A mutant enzymes in their Cu,Zn form [21], the respective Cu,Cu forms of these proteins do indeed exhibit higher rates of deactivation in the presence of hydrogen peroxide [22]. Thus, one recent hypothesis is that the mutant SOD in FALS may display erroneous metal loading such that the normal binding site for zinc becomes filled instead with copper, which, in turn, leads to increased formation of the hydroxyl radical [22]. The putative hydroxyl radical generation would take place via a Fenton reaction involving one of the active site coppers of the mutant Cu,Cu enzyme.

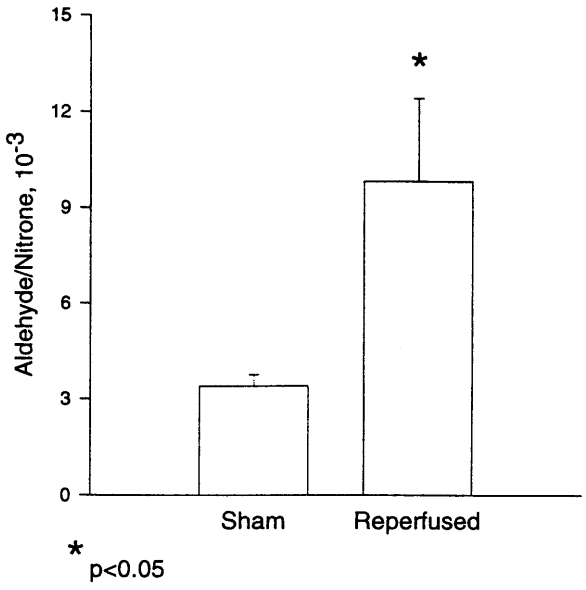


Figure 1. The ratio of AZA to AZN (AZA/AZN) was measured in hippocampus from gerbils that were subjected on average to 7 min of BCO and 7 min of reperfusion. Gerbils were injected intraperitoneally with 100 mg/kg of AZN dissolved in intralipid. Data are means and SEM for six animals per group. The increase in AZA/AZN was significant at $P < .05$ by ANOVA. The absolute tissue values of sham-treated animals were 2.0 ± 0.3 μmol/g for the nitrone and 0.0073 ± 0.0016 μmol/g for the aldehyde. The absolute tissue values of reperfused animals were 2.5 ± 0.2 μmol/g for the nitrone and 0.020 ± 0.004 μmol/g for the aldehyde [15].

A recent collaboration with Gurney and his co-workers at Pharmacia and Upjohn has demonstrated the utility of AZN in providing in vivo evidence of radical-driven oxidative stress in the SOD1 G93A transgenic mouse model of FALS [23–25]. In order to confirm that AZN could provide meaningful data on the in vivo level of oxidative stress in the FALS transgenic mice, it was first deemed appropriate to measure whether AZN could detect any in vitro differences between radical production of spinal cord extracts of wild vs. mutant SOD1 transgenic mice in the presence of hydrogen peroxide. Determination of the dismutase activity toward superoxide showed that the extracts from both the mutant TgN(SOD1G93A)1Gur and the wild TgN(SOD1)2Gur lines had comparable ability to catalyze the conversion of superoxide to hydrogen peroxide. The conversion of AZN to AZA increased linearly in the mutant transgenic spinal cord extracts with increasing hydrogen peroxide concentration to at least 30 mM and increased linearly at 30 mM hydrogen peroxide in the presence of increasing enzyme concentration. However, in the presence of AZN and hydrogen peroxide, the spinal cord extracts from the mutant transgenic mice consistently showed a significant increase in the conversion of AZN to AZA relative to that in the case of the wild transgenic and nontransgenic mice (fig. 2). Preincubation for

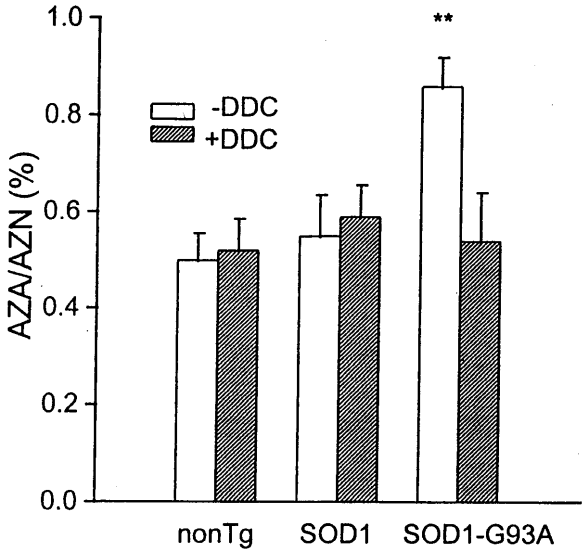


Figure 2. Detection of free-radical production by spinal cord extraxts prepared from 90 day-old mice on incubation with hydrogen peroxide (troplicate assays, ** $P < 0.001$). The enhanced production of free radicals catalyzed by SOD-G93A spinal cord extracts is blocked by preincubation with DDC, a specific inhibitor of Cu,Zn SOD (triplicate assays, $P < 0.0001$) [23].

20 minutes with 1 mM diethyl dithiocarbamate (a specific inhibitor of Cu,Zn SOD which acts by chelating the active-site copper), which was independently verified to abrogate the enzyme-mediated conversion of superoxide to hydrogen peroxide in both the wild transgenic and mutant transgenic cord extracts, reduced the AZN to AZA conversion of the mutant transgenic cord extracts to the baseline value observed in wild transgenic or nontransgenic spinal cord extracts (fig. 2). These experiments show that mouse spinal cord extracts containing the G93A mutant form of human Cu,Zn SOD catalyze excess production of free radicals from hydrogen peroxide and that this excess production can be obviated by an active-site modification of the enzyme.

In view of the successful outcome of the in vitro experiments, the next phase of the investigation was commenced. Thus, two spin traps, AZN and salicylate, were studied with respect to their capacity to report on whether the level of free-radical activity in the spinal cord of SOD1-G93A mice was elevated in vivo. The spinal cord extracts of three lines of mice (nontransgenic, SOD1 transgenic and SOD1-G93A transgenic) were evaluated from animals 90 days of age. Significant elevation of free-radical production occurred only in SOD1-G93A transgenic mice, and this was detected only in AZN-administered mice. Specifically, conversion of AZN to AZA was found to be 4.5% in the SOD1-G93A mice spinal cords vs. 0.9% in both the nontransgenic and wild-type transgenic mice (fig. 3). Importantly, this fivefold increase represents the first in vivo evidence for increased oxidative stress in FALS. It is also important to note that salicylate was unable to detect any differences in radical production in any of the three lines of mice studied. Unlike AZN, which is converted to AZA in the presence of hydroxyl, peroxyl and alkoxyl radicals, salicylate does not react with oxyradicals other than hydroxyl radicals. The failure of salicylate to report the increased oxidative stress detected with AZN may be attributed to a number of possible scenarios. First, although hydroxyl radicals may be the primary radicals to form via the interaction of the mutant enzyme with hydrogen peroxide, the high promiscuity of the hydroxyl radical may lead to the rapid formation of secondary radicals such as peroxyl radicals at a faster rate than that by which salicylate captures hydroxyl radicals. Lipid peroxidation precipitated by hydroxyl radicals via the intermediacy of peroxyl radicals would be registered by AZN but not salicylate. Alternatively, it is possible that the damaging hydroxyl radicals are generated in relatively lipophilic subcellular compartments where AZN but not salicylate may localize.

It should be emphasized that a significant conversion of AZN to AZA was observed only in the spinal cords of the SOD1-G93A mice, not in the brain and blood of

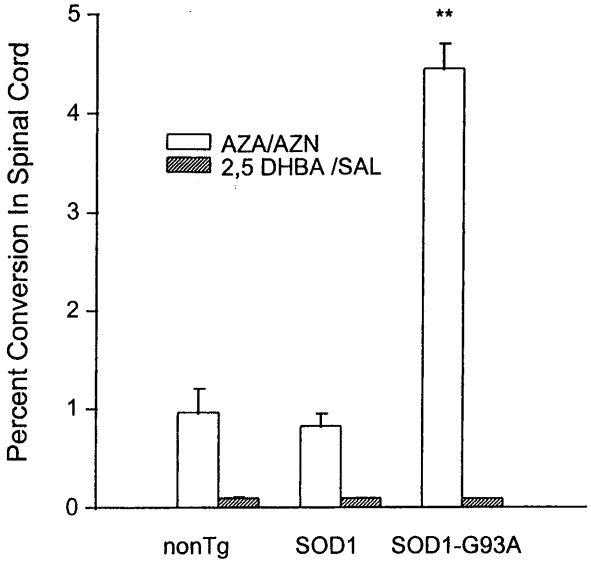


Figure 3. Spinal cord conversion ofAZN or salcylate to AZA or DHBA, respectively, after intravenous injection of nontransgenic, SOD1 and SOD1-G93A transgenic mice (six mice per group, ** $P < 0.001$). Only data for conversion of salicylate to 2,5-DHBA are shown [23].

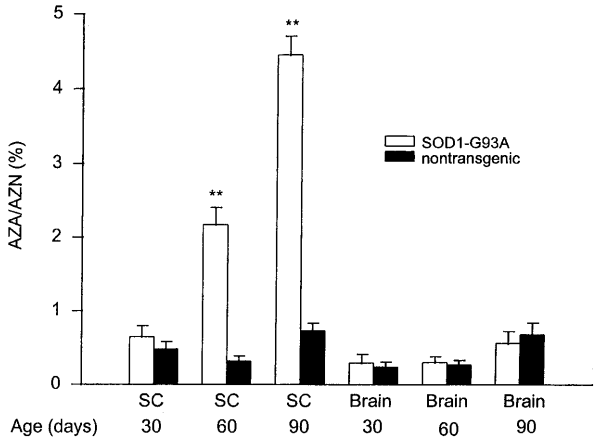


Figure 4. Oxygen-radical content of SOD1-G93A and nontransgenic mouse spinal cord and brain at different ages as assessed by conversion of AZN to AZA after intravenous injection (six mice per group, ** $P > 0.001$) [23].

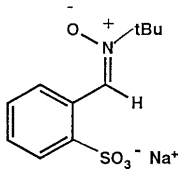
these animals (fig. 4). This is consistent with the observation that, at 90 days of age, SOD1-G93A mice frequently display symptomatic disease with loss of motor neurons in the spinal cord [26]. Thus, the free radical-mediated oxidative stress is detected by the AZN assay precisely in that tissue in which the pathology is most deleterious. The levels of SOD1-G93A in transgenic mice have been found to increase substantially in the spinal cord by 90 days of age, whereas, in brain the levels of expression are about threefold less, and increases over time do not occur. These findings link the generation of free radicals in situ to the tissue content of mutant human Cu,Zn SOD1-G93A transgenic mice, and suggest a role for oxidative stress in the pathogenesis of FALS.

Therapeutic applications

Parkinson's disease model: protection conferred by azulenyl nitrones against dopamine depletion in the murine brain upon exposure to the neurotoxin MPTP

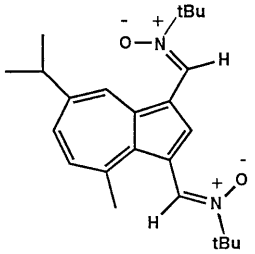
N-Methyl-1,2,3,6-tetrahydro-4-phenylpyridine (MPTP) is known, by virtue of the work of Langston et al. in the 1980s, to induce a Parkinsonian-like condition in humans [27]. The mechanism by which MPTP is believed to exert its neurotoxicity involves inhibition of complex 1 of the electron transport chain within mitochondria by the pyridinium metabolite MPP+, with subsequent impairment of energy production and increased oxidative stress caused by elevated oxyradical production [28]. MPP+ accumulates in dopaminergic neurons such as those of the substantia nigra by binding strongly to the dopamine transporter. Depletions of dopamine levels in the murine brain caused by MPTP administration reflect the extent of neurodegeneration in an animal model of Parkinson's disease.

In collaborative work with Beal and co-workers at Harvard Medical School/Massachusetts General Hospital, AZN and a water-soluble congener (w-AZN) have been shown to protect mice against MPTP toxicity in this Parkinson's disease model [29]. Earlier work by Beal et al. demonstrated that the conventional nitrone spin trap



S-PBN

affords neuroprotection against a mild MPTP insult (resulting in a 30% depletion of dopamine in animals not receiving S-PBN) but not against moderate or severe regimens of MPTP administration [30]. It is interesting to note that AZN outperforms S-PBN by providing superior neuroprotection in this model. Azulenyl nitrones were designed and then synthesized with the expectation that they would outperform conventional nitrone spin traps with regard to antioxidant behavior, due to the much lower oxidation potentials that azulenyl nitrones such as AZN were predicted to possess vs. those of phenylnitrone derivatives such as PBN and S-PBN [31]. Chain-breaking antioxidants within a given class of compounds tend to increase in potency with decreasing oxidation potential [32]. Structural features in AZN are such that the corresponding radical cation is highly stabilized relative to that of PBN, and cyclic voltammetric experiments in collaboration with Echegoyen et al. [33] confirmed that the oxidation potential of AZN (0.84 V vs. SCE) is more than 0.6 V less than that of PBN (1.47 V vs. SCE) [34]. The azulenyl bis-nitrone



w-AZN

has an even lower oxidation potential of 0.63 V vs. SCE [31], which is less than that of the glutathione thiolate anion (0.69 V vs. SCE), a crucial biological antioxidant.

The data in figure 5 indicate that both AZN and w-AZN provided dose-dependent protection against

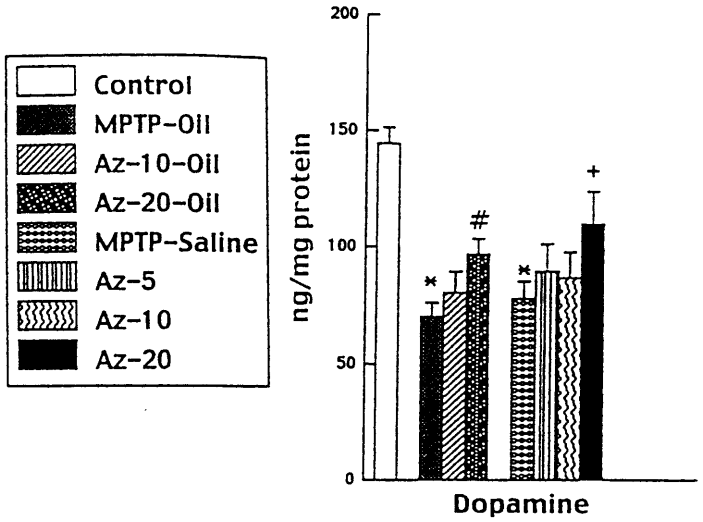


Figure 5. Effects of administration of azulenyl nitrone spin traps (5, 10 or 20 mg/kg) on MPTP-induced depletions of dopamine. *$P < 0.001$ compared with control, #$P < 0.05$ compared with MPTP in peanut oil, +$P < 0.05$ compared with MPTP in saline (Az-Oil represents AZN, Az represents w-AZN) [29].

MPTP-induced dopamine depletion in Swiss-Webster mice. A challenging regimen of MPTP administration (resulting in 50% dopamine depletion in animals not receiving the test nitrone) was employed. MPP+ levels were unchanged in the presence of the nitrones, thus demonstrating that the spin traps do not impair MPTP uptake or the MPTP to MPP+ metabolism. These results therefore show that azulenyl nitrone spin traps are more efficacious than S-PBN, which does not afford protection against the severity of this regimen. AZN and w-AZN provided 35 and 45% protection, respectively. This improved protection afforded by the azulenyl nitrones compared with that of S-PBN ($E1/2 = 1.34$ V vs. SCE) [34] may indeed be due to their far lower oxidation potentials and bodes well for azulenyl nitrones as fortified antioxidants. Synthetic work is now in progress with the aim of preparing new azulenyl nitrones with still lower oxidation potentials in hopes of achieving even greater antioxidant potency. Given these results and continuing interest in nitrones as neuroprotectants within the pharmaceutical industry, the potential therapeutic value of azulenyl nitrones for the treatment of Parkinson's disease is such that further research in this area is warranted.

Stroke model: neuroprotection conferred by azulenyl nitrones in the gerbil transient forebrain ischemia/reperfusion protocol

The fact that the AZN spin trap assay provided data supporting the intermediacy of damaging oxyradicals in the BCO stroke model in gerbils (vide infra) [15] raised the question of whether AZN could afford neuroprotection in this animal model. Nitrone spin traps such as

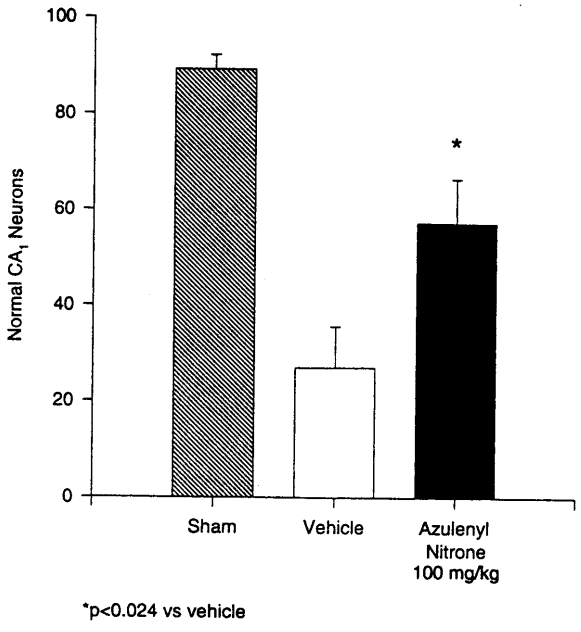


Figure 6. CA1 neuronal counts from gerbils subjected to 7 min of forebrain ischemia followed by 5 days of reperfusion. Data are means and SEM for 14 animals per group. Neuronal counts with AZN were significantly greater than counts with vehicle where $P < 0.024$ by analysis of variance [15].

PBN are known to possess therapeutic utility in stroke models [35], and Centaur Pharmaceuticals in collaboration with Astra is currently engaged in Phase IIa clinical trials of CPI-22 (NXY-059), a proprietary NRT (nitrone related therapeutic), for the treatment of stroke. In the event, pretreatment with AZN (100 mg/kg in an Intralipid vehicle) was found to attenuate the loss of CA1 neurons to 36 vs. 70% loss after 7 min of forebrain ischemia followed by 5 days of reperfusion in the vehicle-treated gerbils (fig. 6) [15]. This level of neuroprotection is comparable to that observed with PBN. Namely, with 5 min of ischemia and 7 days of reperfusion, 75 mg/kg PBN increased the survival of CA1 neurons by 63% [36]. These results indicate that AZN and congeners may have promise as clinical neuroprotectants in ischemic brain injury.

Azulenyl nitrones protect axotomized ganglion cells in the developing retina

Newly completed collaborative research with Clarke et al. at the University of Lausanne has shown that azulenyl nitrones confer neuroprotection in the retina of chick embryos following axotomy [37]. Previous work in Clarke's laboratory documented the neuroprotective activity of PBN in this model [38]. That azulenyl nitrones such as AZN and conventional nitrone spin traps such as PBN possess markedly different pharmacological profiles in this model is evident upon inspection of the data in table 1. Thus, it is clear that AZN and PBN must differ with regard to their mechanisms of action in the presence of the glutathione mimic *N*-acetyl cysteine (NAC), since the combination of NAC and AZN is slightly more protective than administration of AZN alone, whereas the combination of NAC and PBN is less efficacious than sole administration of either of these two antioxidants. Furthermore, while the therapeutic dosage range is narrow for both AZN and PBN, the superior degree of protection is afforded by AZN and at concentrations within the retina far lower than those of PBN. It is also worth noting that AZN provides substantial neuroprotection within the retinas of chick embryos against an oxyradical insult instigated by coinjection of *tert*-butyl hydroperoxide and ferrous sulfate.

Table 1. Effects of antioxidants on pyknotic cell counts at 13 days past the moment of incubation in the ganglion cell layer contralateral to a tectal lesion performed at 12 days past the moment of incubation.

Treatment (nmol/eye)	Number of embryos	Number of pyknotic cells (mean ± SEM)
DMSO	13	108.9 ± 6.4
NaCl	9	103.0 ± 5.0
NAC (500)	8	78.0 ± 10.3
AZN (70)	11	71.4 ± 6.6
PBN (50)	8	74.8 ± 10.3
NAC (500) + AZN (70)	6	51.0 ± 6.5
NAC (500) + PBN (50)	7	98.2 ± 9.6

Various antioxidants were injected intraocularly either separately or in combination. The number of pyknotic cells in NAC- and PBN-injected embryos was compared with NaCl-injected control embryos, whereas AZN-injected embryos were compared with DMSO-treated control embryos. *t* tests showed a difference from the vehicle group: $^{*}P<0.05$, $^{***}P<0.001$; and a difference from NAC alone: § $P<0.05$. At these intraocular dosages (50 nmol PBN/eye and 70 nmol AZN/eye), the concentration of AZN within the retina is far less than that of PBN.

Conclusion

It has been only 3 years since the diagnostic and therapeutic potential of azulenyl nitrone spin traps have been under active investigation. In this short time it is already apparent that these novel nitrones can yield important insight into the role played by free-radical damage in neurodegenerative diseases and that they may constitute a useful new class of palliative antioxidants for such conditions. Much remains to be explored, and further work in our laboratory as well as collaborative work with the research groups of Beal, Gurney and Mullan is now underway to expand our base of knowledge to include the development of diagnostic uses for azulenyl nitrones in other neurodegenerative illnesses such as Alzheimer's disease, and to the design and synthesis of congeners with enhanced therapeutic efficacy.

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