Nitrones as Neuroprotectants and Antiaging Drugs

ROBERT A. FLOYD,[a,b] KENNETH HENSLEY,[a] MICHAEL J. FORSTER,[c] JUDITH A. KELLEHER-ANDERSON,[d] AND PAUL L. WOOD[d]

[a]*Free Radical Biology and Aging Research Program, Oklahoma Medical Research Foundation, Oklahoma City, Oklahoma 73104, USA*

[b]*Department of Biochemistry and Molecular Biology, University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma 73104, USA*

[c]*University of North Texas, Fort Worth, Texas 76107-2699, USA*

[d]*Centaur Pharmaceuticals, Inc., Santa Clara, California 95050, USA*

ABSTRACT: Specific nitrones have been used for more than 30 years in analytical chemistry and biochemistry to trap and stabilize free radicals for the purpose of their identification and characterization. PBN (α-phenyl-*tert*-butyl nitrone), one of the more widely used nitrones for this purpose, has been shown to have potent pharmacologic activities in models of a number of aging-related diseases, most notably the neurodegenerative diseases of stroke and Alzheimer's disease. Studies in cell and animal models strongly suggest that PBN has potent antiaging activity. A novel nitrone, CPI-1429, has been shown to extend the life span of mice when administration was started in older animals. It has also shown efficacy in the prevention of memory dysfunction associated with normal aging in a mouse model. Mechanistic studies have shown that the neuroprotective activity of nitrones is not due to mass-action free radical–trapping activity, but due to cessation of enhanced signal transduction processes associated with neuroinflammatory processes known to be enhanced in several neurodegenerative conditions. Enhanced neuroinflammatory processes produce higher levels of neurotoxins, which cause death or dysfunction of neurons. Therefore, quelling of these processes is considered to have a beneficial effect allowing proper neuronal functioning. The possible antiaging activity of nitrones may reside in their ability to quell enhanced production of reactive oxygen species associated with age-related conditions. On the basis of novel ideas about the action of secretory products formed by senescent cells on bystander cells, it is postulated that nitrones will mitigate these processes and that this may be the mechanism of their antiaging activity.

KEYWORDS: nitrones; PBN; DMPO; learning; memory

Address for correspondence: Robert Floyd, University of Oklahoma Health Sciences Center, 825 N.E. 13th Street, Oklahoma City, OK 73104. Voice: 405-271-7580; fax: 405-271-1795. Robert-Floyd@omrf.ouhsc.edu

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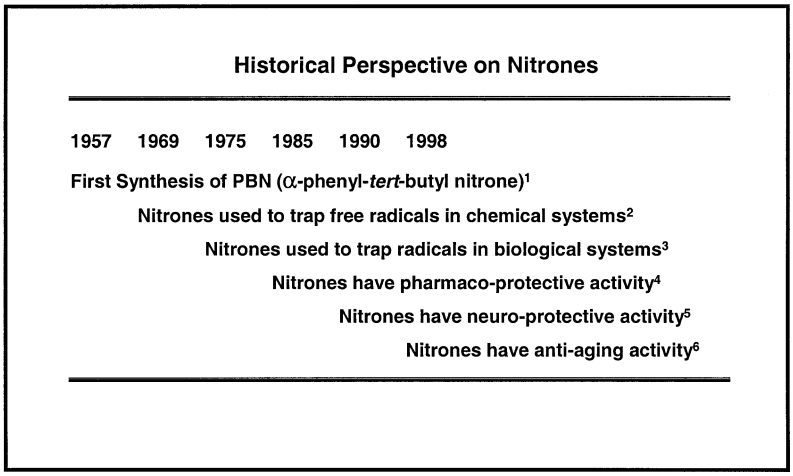
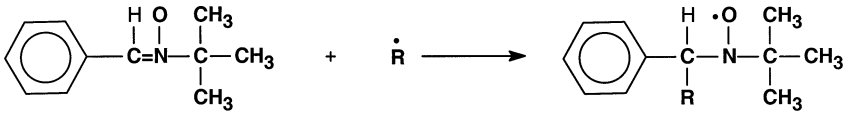


FIGURE 1. Important historical events and discoveries pertinent to the antiaging activity of nitrones. Numbers 1–6 denotes relevant references.

INTRODUCTION AND BACKGROUND

One of the most widely known nitrones is PBN (α-phenyl-*tert*-butyl nitrone). FIGURE 1 presents a time sequence of the significant biomedical research discoveries pertinent to nitrones where studies with PBN played an especially prominent role. The compound was first synthesized in 1957.[1] Its widespread use in analytical chemistry was brought about by the pioneering use of it to trap (spin-trap) rapidly reacting free radicals in chemical systems.[2] The more stable nitroxide free radical adduct formed (see FORMULA 1) allowed the characterization of free radical intermediates in many chemical systems where such intermediates were involved.



FORMULA 1.

Within six years of the beginning of the use of nitrones in analytical chemistry, they were starting to be used in biochemical systems.[3] Extensive use of PBN, and another spin-trap, DMPO (5,5-dimethyl-1-pyroline-1-oxide), became a prominent method in the 1980s to investigate free radical intermediates in biological systems.

It was during this interval that the pharmacological potential of the nitrones was first noted.[4,5] Novelli *et al.*[4] demonstrated that preadministration of PBN to rats protected them from the shock trauma they experienced caused by placing them in a tumbling mill. The potential of nitrones as neuroprotective agents became clear as a result of our discovery, made in 1988, that administration of PBN protected gerbils from a stroke.[5] The first conclusive demonstration that PBN had antiaging activity was made by Cutler's group in 1998.[6] Earlier observations by Packer's group[7] and Arendash's group[8] made on senescence-accelerated mice and Sprague-Dawley rats, respectively, clearly implicated the antiaging activity of PBN. However, these earlier studies suffered from being done on a mouse model that does not have normal aging characteristics[7] and in the latter case because the experiment was terminated early (i.e., before full life span was completed).[8]

MECHANISTIC BASIS OF THE NITRONE ACTIVITY

A large amount of experimental data has been collected on the neuroprotective activity of nitrones. The following generalizations can be drawn from the studies: (*a*) PBN has been shown to be neuroprotective by a number of laboratories in several models. (*b*) From the limited studies available it is clear that not all nitrones are neuroprotective, that is, neuroprotective activity can vary widely and depends on the specific chemical structure. (*c*) The activity does not depend upon a simple mass action type of mechanism, but appears to reside in nitrone's ability to effectively quell certain exacerbated signal transduction events which are associated with enhanced neuroinflammatory processes common to several neurodegenerative diseases such as stroke, Alzheimer's disease, Parkinson's disease, AIDS-related dementia, and others.[9] (*d*) Nitrones, and especially PBN, have been shown to have potent pharmacological activity in model systems of a wide range of pathological conditions and diseases associated with aging.[10–14]

PBN has become a widely used research tool to explore questions regarding antioxidants and oxidative damage in various research problems. PBN does, in general, lower oxidative stress and oxidative damage, as, for example, in lowering protein oxidative damage in brains of chronically treated old gerbils,[15] yet exactly how it does mitigate oxidative stress and oxidative damage is more complex than the simple concepts normally used to explain antioxidant activity. Instant deductive thought concludes that free radical–trapping explains the mechanistic action of PBN. But many observations and careful analysis of the facts do not support this conclusion. *First*, it is widely observed in classical spin-trapping studies that very high levels (on the order of 50 mM or more) of PBN (and other spin traps) are required to trap significant amounts (50% or more) of the total free radicals being produced in several well-known chemical systems. PBN never reaches very high levels in the target tissue. For instance, administered dosages of 75–150 mg/kg yield brain levels of no more than about 500 μM.[16,17] *Second*, PBN has its action after the highest target tissue free radical flux has occurred. That is, PBN shows neuroprotective activity in "stroked" brains when it is given 30 minutes after the stroke in the case of gerbils, [18,19] and even up to 3 hours after the stroke (brain reperfusion) in the case of the middle cerebral arterial occlusion model in rats.[20] *Third,* in an isolated rat liver microsomal system undergoing rapid lipid peroxidation, PBN is only about 0.01% as effective in

ceasing peroxidative activity as is Trolox or butylated hydroxy toluene (BHT) in this system.[21] These three chemicals were tested in head-on comparisons in this system and it required 5 mM of PBN to inhibit 50% of the peroxidation activity but only 40 μM and 6 μM of Trolox and BHT, respectively.

We have concluded that the neuroprotective action of PBN is explained by its ability to prevent the induction of genes, caused by the brain insult brought on by a period of ischemia.[10–12] Some of the induced genes produce products that are toxic to neurons. One such product is nitric oxide and its oxidative products, including peroxynitrite. Brains given a stroke do, within a few hours, show an increase in inducible nitric oxide synthase. In models where strokes are given to transgenic animals lacking this enzyme, or when nitric oxide synthase has been inhibited, significantly less damage was caused by the stroke.[22–25] Microglia and astrocytes, when activated, will mediate the induction of inducible nitric oxide synthase (iNOS), which then produces high levels of nitric oxide, which is much more toxic to neurons than to the glia that produced it. These events represent the basic notions of neuroinflammatory processes and establish a testable notion for examination of the neuroprotective action of PBN and other active nitrones.

The most convincing published research substantiating the notions that the mechanistic action of PBN involves quelling of enhanced signal transduction processes comes from studies in brain cell culture[26,27] and in a few animal model studies.[28] More advanced studies on stroke have shown that certain derivatives of PBN, especially 2,5-disulfonate PBN, (disodium 4-[(*tert*-butylimino)-methyl] benzene-1,3-disulfonate *N*-oxide), protect against brain damage in the rat model of transient occlusive middle cerebral arterial stroke[29] and in the marmoset model of permanent occlusive middle cerebral arterial stroke.[30] In the former model, significant protection was afforded when the drug was administered up to 6 hours after the stroke was initiated.[29] In cell culture studies, signal transduction activation was monitored by p38 map kinase activation in rat brain astrocytes.[26,27] Activation was elicited by hydrogen peroxide or the proinflammation cytokine IL1β. PBN as well as *N*-acetylcysteine at 1 mM suppressed p38 activation by 70–90%. The most direct demonstration of the mechanistic basis of the neuroprotective activity of PBN was shown in kainic acid (KA)–induced epilepsy in rats.[28] PBN given at 150 mg/kg 90 minutes after KA administration significantly protected the rats from seizures and death. Immunohistochemical examination of the CA1 subregion of the hippocampus showed that KA-mediated p38 activation as well as NF-κB activation. Activation of p38 and NF-κB was significantly suppressed by PBN administration 90 minutes after KA was administered.[28] This clearly demonstrates the PBN mediates protection from KA-mediated brain injury, which is closely associated with enhanced signal transduction processes that were significantly suppressed by PBN.

NITRONES IN AGING STUDIES

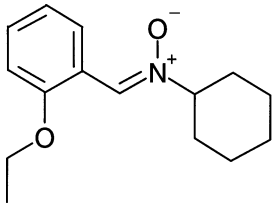
There are three published reports[6–8] in which PBN was used in mice and rats where life span data clearly indicate the antiaging activity of this compound. The most rigorous study was conducted by Saito *et al.*[6] using C57Bk/6J mice. These mice were administered PBN in the drinking water at 32 mg/kg/day starting when

they were 24 months old. PBN significantly increased the mean and maximal life span of these animals from 29.0 to 30.1 months and 31.7 to 33.3 months, respectively.[6] A study by Arendash's group[8] demonstrated that PBN administered 32 mg/kg/day and begun at 24 of months age in Sprague-Dawley rats increased the 50% survivorship from 29.4 to 34.2 months. It also improved memory retention as measured using a Morris water maze.

There are three published reports[31–33] of the effect of PBN on cellular aging under culture conditions. Ames and colleagues demonstrated that PBN caused delay of replicative senescence in cultured human fibroblasts.[31,32] They showed that nitrous-*tert*-butane was a breakdown product of PBN and that it delayed senescence in these cells.[32] Another independent report[33] using human fibroblasts confirmed that PBN increased cellular longevity and decreased rates of telemere shortening.

ANTIAGING ACTIVITY OF A NOVEL NITRONE

A novel nitrone referred to as CPI-1429 (FORMULA 2) has been shown to delay mortality as well as memory impairment in an aging mouse model.[34,35] The results in summary form are presented here.



FORMULA **2.**

In this study we evaluated the potential for CPI-1429 to ameliorate or delay progression of the learning and memory deficits associated with normal aging in C57BL/6 mice. Separate groups of young (4–5 month) and old (23–24 month) mice were treated daily (via gavage) with CPI-1429 or vehicle for two weeks prior to testing on a recent memory task motivated by shock avoidance.[34] The daily treatments were maintained for a period of up to 29 weeks, during which the mice were exposed to learning (during weeks 3–8) and retention (after 20 weeks) phases of testing. During the learning phase, the mice were given a series of up to 25 daily sessions during which they could avoid shock by making a correct turn in a T-maze apparatus. Successful avoidance of shock required recall, after a short delay of 1 minute, of information presented at the first trial of each daily session. The retention phase began approximately 12 weeks after the mice had completed the learning phase. During the retention phase, memory capacity of the mice was challenged by requiring them to remember the safe side of the apparatus over longer periods of up to 90 minutes.

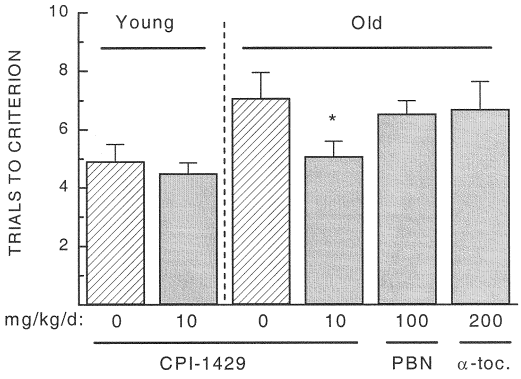


FIGURE 2. Mean number of trials (±SE) for mice to reach a learning criterion of 2 consecutive correct turns to the safe arm under a discriminated avoidance task[36] as a function of age and treatment regimen. Young (4–5 mo) and old (23–24 mo) C57BL/6 mice received daily treatment with the vehicle or 10 mg/kg (po) of CPI-1429 for a period of two weeks prior to testing. Results are compared with data from similar studies of α-phenyl-*tert*-butyl nitrone (PBN, 100 mg/kg/d, i.p.) and alpha-tocopheryl acetate (α-toc., 200 mg/kg/day p.o.). $*P = 0.017$ when compared with the old vehicle-treated control group (individual comparison within 1-way ANOVA).

The results of the behavioral studies indicated that performance of vehicle-treated old mice was impaired relative to the younger mice on both learning and retention phases of the test. By contrast, the rate of learning by the old mice treated with 0.1 or 10 mg/kg/day of CPI-1429 was comparable to that of the young mice receiving chronic treatment with the vehicle. The effects of CPI-1429 were particularly prominent on the second session of the learning phase, which involved a "reversal" from the previous session of the safe arm of the maze (FIG. 2). Data from similar studies of the nitrone prototype PBN and vitamin E failed to indicate any significant effects during the avoidance learning phase (FIG. 2).

During the retention phase, when mice were tested for memory under conditions of high demand (after a delay of 90 minutes), the beneficial effect of CPI-1429 was also notable. As indicated in FIGURE 3, performance was more accurate in old mice treated with 0.1 or 10 mg/kg CPI-1429 than in the old vehicle-treated controls.

The treatment regimens were maintained for a significant portion of the life span of the old mice (seven months) in these studies and it was notable that, in addition to improving learning and memory performance, CPI-1429 treatment also resulted in a significant lengthening in the mortality curve of the mice.[34,35] Overall, this pattern of effect on behavior and mortality rate for CPI-1429 is consistent with effects reported for the nitrone prototype, PBN, and is generally supportive of the hypothesized antiaging actions of some nitrones. Nevertheless, the nature of studies performed with CPI 1429 leaves open several possibilities with regard to the exact nature of the beneficial effects. Given the current data, it appears that CPI-1429 could act via any or all of the following mechanisms: (1) a generalized enhancement of cognitive processes; (2) a specific amelioration of age-related brain dysfunctions;

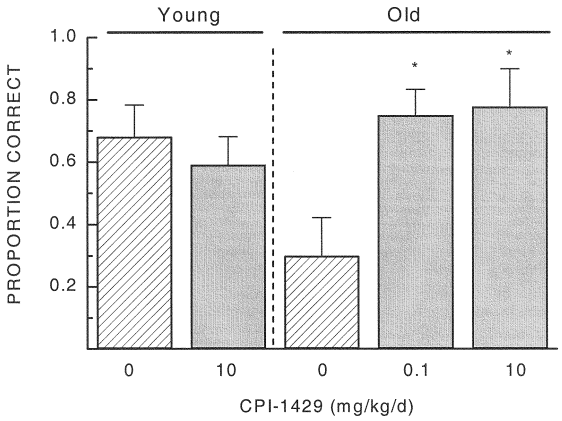


FIGURE 3. Recent memory performance for groups of young and old mice treated for 20 weeks with vehicle or CPI-1429. The figure shows accuracy (mean proportion correct ± SE) over a series of test sessions in which mice could run to the safe side of a T-maze to avoid shock, 90-minutes after the safe side had been first identified.[34] *P <0.025 when compared with the old vehicle-treated control group (individual comparison within 1-way ANOVA).

or (3) an arrest or delay of age-related decline in brain function. That CPI-1429 could enhance maze learning after only two weeks would be difficult to explain in terms of an arrest of age-related decline, but would be consistent with mechanisms 1 or 2. The fact that CPI-1429 dramatically improved performance of the old mice, but was without significant effect on performance of the young mice, suggests an "age-selective" pattern that would tend to support mechanism 2 over mechanism 1. The fact that CPI-1429-treated mice performed in a manner equivalent to young controls after 20 weeks of treatment could have resulted from any of the above mechanisms. Although data are inconclusive, it is much more likely that an arrest of progressive memory decline (3) could have contributed to the superior performance of treated mice after 20 weeks of treatment with CPI-1429.

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