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Review

# Nitrones, their value as therapeutics and probes to understand aging

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**Abstract**

The nitrone-based free radical traps have significant potential in the treatment of neurodegenerative diseases as well as in the prolongation of life span. The mass action free radical trapping activity of these compounds is the property, which first brought them to the attention of the scientific community. Nevertheless extensive research has demonstrated that these reactions are not responsible for their therapeutic mechanistic basis of activity. Rather the mechanism of action in the case of their neuroprotective activity appears to involve the inhibition of enhanced signal transduction processes that mediate the upregulation of genes, which produce neurotoxic products. The most widely used compound in this series, α-phenyl-*tert*-butyl-nitrone (PBN), has been shown to extend life span in three published studies, i.e. two mouse models and one rat model. Significant prolongation of life span was noted in all three studies. We report the summary of a recent study with a novel nitrone, CPI-1429, which demonstrated the ability to extend life span even though administration of the compound was begun in older animals. Despite these promising studies, much more rigorous research examining the anti-aging activity of the nitrones needs to be conducted. It is not known exactly why the nitrones possess anti-aging activity. They have been shown to quell enhanced signal transduction processes associated with enhanced pro-inflammatory cytokine mediated events. The nitrones interfere in some unknown steps preventing receptor triggered MAP kinase phosphorylation cascades. Stabilization of phosphorylation networks associated with checkpoint proteins could slow cell cycle processes and this could be the basis of the nitrones anti-senescent activity. 

*Keywords:* Neuro-inflammation; Anti-aging nitrones; α-phenyl-*tert*-butylnitrone; Reactive oxygen species; Oxygen free radicals

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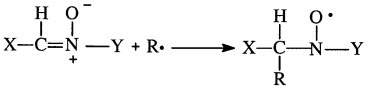
## 1. Introduction

Free radicals have been implicated as important in the etiology and pathology of many of the diseases associated with aging, as well as in the aging process per se. Two very recent publications (Migliaccio et al., 1999; Melov et al., 2000) reemphasize the importance of free radical processes and the effect of their abatement on enhancing life span. Accordingly, it is expected that agents as well as paradigms which blunt free radical processes should influence (i.e. mitigate) the occurrence and severity of the diseases of aging, as well as the aging process. In so far as these notions have been tested, the experimental data collected over the years have proven that these simple ideas have a basis in truth. Additionally, and more importantly, the experimental results have also revealed that the processes involved, as well as the action of free radicals in biological systems are much more complicated than originally conceived. Experiments set up to trap and characterize the free radicals involved in the oxidative damage to brain that occurs in stroke led to the surprise discovery that the nitrone-based free radical traps exhibit neuroprotective activity (Floyd, 1990). Further studies have shown that these compounds have more extensive therapeutic pharmacological properties. Other studies clearly implicate that these compounds appear to have the potential to prolong life span. A brief summary of the pharmacological potential of the nitrones is presented, as well as a review of the studies illustrating their potential activity to delay aging. The exact mechanistic basis of their activity is not known. However, it is clear that their action involves quenching signal transduction processes important in inflammation associated with the diseases of aging. Their potential anti-aging mechanism of action is not known. However, interference in cell cycle processes may be important. The free radical trapping reaction of nitrones is illustrated by the reaction below where the free radical $R^{\bullet}$ adds to the nitrone to yield a more stable free radical adduct. It is highly unlikely that the free radical trapping activity of the nitrones represents the primary therapeutic action of these compounds. PBN (α-phenyl-*tert*-butylnitrone) is the nitrone that has been used in many of the studies discussed. In the case of PBN, X is a phenyl group and Y is a tertiary butyl group.



## 2. Pharmacologic potential of the nitrones

The historical observations which foretold the pharmacologic potential of the nitrones have been reviewed before (Floyd, 1997; Floyd and Hensley, 2000; Kotake, 1999). The early studies of Novelli et al. (1985) that demonstrated the protective action of PBN in traumatic shock were refined in the studies by McKechnie et al. (1986), Hamburger and McCay (1989), Pogrebniak et al. (1992) who demonstrated that PBN protected rats from death caused by LPS-induced septic shock. We were the first to show that PBN had broad neuroprotective activity (Floyd, 1990; Carney et al., 1991). The effectiveness of the post-administration of PBN in the global stroked gerbil brain was soon confirmed by Phillis and Clough-Helfman (1990) and later by Mori et al. (1997). Further extending the results, Phillis' group also demonstrated that PBN was neuroprotective in the permanent occlusion focal stroke model in rats (Cao and Phillis, 1994). Siesjo's group demonstrated that PBN was an effective neuroprotective compound if delivered up to 2 h after reperfusion in the middle cerebral artery occlusion model of stroke in rats (Zhao et al., 1994). However, they showed that PBN was not effective in the rat forebrain ischemic model (Pahlmark and Siesjo, 1996). The 2,5 disulfonate derivative of PBN has been shown to be very effective, even at 0.3 mg/kg per h, by I.V. injection in the transient middle cerebral artery occlusion model in rats. PBN and its simple derivatives have broader neuroprotective activity as implicated by several observations. Epileptic seizures induced by kainate are considerably depressed by PBN if it is given up to 90 min after the kainate (Floyd et al., 2000). In contrast, PBN given before kainate increases

the mortality of the rats. This result is interpreted to indicate that PBN interferes with kainate metabolism, perhaps to a non-toxic compound. Bacterial meningitis caused by group B streptococci is prevented by PBN in an infant rat model (Leib et al., 1996). Hearing loss in rats caused by exposure to carbon monoxide combined with loud noise is largely prevented by pre-administration of PBN (Fechter et al., 1997). PBN and related nitrones show pharmacological potency in models other than those involving CNS. These include its ability to prevent the onset of streptozotocin-induced diabetes (Tabatabaie et al., 1997), thalidomide-induced birth defects (Parman et al., 1999), ischemia-reperfusion induced acute renal failure (Pedraza-Chaverri et al., 1992) and choline-deficiency induced liver carcinogenesis (Nakae et al., 1998).

## 3. Primary pharmacologic activity of nitrones is not due to free radical trapping

As noted earlier, it is highly unlikely that the primary pharmacologic action of the nitrones is by trapping free radicals. This is despite the fact that 'spin-trapping' is the property which brought them to the attention of the scientific community (Janzen and Blackburn, 1969). The rationale to support this assertion resides in many different observations. The most obvious invalidation of the 'spin-trapping' notion resides in a close look at the activity of nitrones in experimental stroke. The nitrones protect in stroke even if they are administered up to 2 h after the start of reperfusion (Zhao et al., 1994). Reperfusion is accompanied by an early large flux of free radicals (Cao et al., 1988). The large free radical flux quells within about 30 min after the reperfusion has begun (Floyd and Carney, 1991) yet PBN is active if administered after this time (Clough-Helfman and Phillis, 1991; Zhao et al., 1994; Mori et al., 1997). Another important demonstration which argues against free radical trapping as a primary mode of action is the fact that in 'spin-trapping' studies conducted in chemical systems require very large levels ( $\sim$ 50 mM or higher) of the nitrone to trap a significant amount of the total free radicals generated (Janzen, 1971). This is because the rate constant for the nitrone trapping reaction with free radicals usually is much lower than diffusion limited i.e. on the order of $10^5$–$10^7$ depending on the specific free radicals trapped (Janzen, 1971). Additionally, it has been demonstrated in rat liver microsomal lipid peroxidation systems where crucial lipid free radical intermediates are involved, the amount of PBN required to significantly diminish lipid peroxidation is about 5 mM. In the same system, trolox and butylated hydroxy toluene (BHT) will achieve similar inhibition about one thousand-fold more effectively i.e. requiring about 1–5 μM to cause a 50% decrease in lipid peroxidation (Janzen et al., 1994). With these caveats in mind, it should be noted that in most if not all of the biological systems where the nitrones are active the amount of compound present in the affected tissue is an amount about 1000-fold less than the concentration normally used in mass action free radical trapping experiments. Additional demonstrations providing rationale against the mass action free radical trapping mechanism can also be demonstrated by another study with aging gerbils. We showed that the normally very susceptible old animals were more resistant to a stroke after they were chronically administered PBN for 14 days (Floyd and Carney, 1996). Their enhanced resistance to a stroke remained several days (3 or more) after ceasing chronic administration of PBN (Floyd and Carney, 1996). The half-life of PBN is 132 min (Chen et al., 1990), so it is expected that very little, if any, of the compound is present 3 days after cessation of PBN administration. From all of the above examples it is clear that classical mass action free radical trapping cannot possibly explain the primary pharmacologic action of the nitrones.

## 4. Nitrones act to quell exacerbated signal transduction processes

The results of several studies have shown that nitrones act to inhibit some redox-sensitive signal transduction processes. Quelling of exacerbated signal transduction processes can explain most, if

not all, the neuroprotective activity of PBN observed in the experimental animal models. A rational explanation for the neuroprotective activity of nitrones in stroke can be deduced by careful evaluation of the following facts. It has been shown that inducible nitric oxide synthase (iNOS) becomes upregulated in stroke (Iadecola et al., 1995a, 1996). It has also been shown that catalytic inhibitors of iNOS protect in experimental models of stroke (Iadecola et al., 1995b) and that transgenic animals lacking iNOS are more resistant to stroke than are comparable control animals (Iadecola et al., 1997). It is also known that nitric oxide is produced in large amounts by iNOS (Dawson et al., 1993). It is known that nitric oxide is much more toxic to neurons than it is to glia, the cells in which iNOS is upregulated and, therefore, responsible for its synthesis (Ding et al., 1997; Dawson et al., 1993). It has also been shown that PBN inhibits the synthesis of nitric oxide in activated astrocytes and very effectively prevents the formation of nitric oxide mediated 3-nitro-tyrosine adducts in activated astrocytes (Hensley et al., 1997). From these series of observations it is possible to construct a simplified model to explain the neuroprotective activity of nitrones in brain injury. In the case of stroke this large insult causes the upregulation of many genes, including iNOS and possibly other genes that produce large levels of neurotoxins, such as nitric oxide, which are involved in the mediation of brain injury. This in essence is the rudimentary notion underlying the neuroinflammation concept (Floyd, 1999). We postulate that nitrones act by suppressing the upregulation of certain genes that produces neurotoxic species. The neuroinflammatory concept is now being evoked to explain brain injury and dementia associated with Alzheimer's disease (Floyd, 1999; McGeer and McGeer, 1999) and Parkinson's disease (Langston et al., 1999). There is strong experimental support for this concept not only in stroke as noted above, but notably in Alzheimer's disease (Hensley et al., 1998a, 1999; Floyd, 1999; McGeer and McGeer, 1999) and in Parkinson's disease (Langston et al., 1999).

As more research into the neurodegenerative diseases continues, the central importance of neuroinflammatory processes as a mechanistic basis for the dementia and brain damage associated with these diseases becomes more evident (Floyd, 1999). Fig. 1 presents a simplified scheme to illustrate the neuroinflammatory concept and the action of nitrones to inhibit the activation of genes that produce products toxic to neurons, especially. Perhaps the best experimental animal model study thus far illustrating the importance of neuroinflammatory events in brain injury and the concurrent demonstration that nitrones can quell these processes and preserve brain function is the study we recently conducted using the kainate-mediated brain injury in rats (Floyd et al., 2000). Kainate administration to rats mediates injury to the hippocampus causing the animals to suffer recurrent convulsive seizures and apoptotic neuron loss in the CA1 and CA3 regions (Floyd et al., 2000). The lesions produced provide a good experimental model of epilepsy. Fig. 2 illustrates that PBN administered at 150 mg/kg as a bolus 90 min after the kainate dosing mediated protection from the seizures as well as prevented mortality

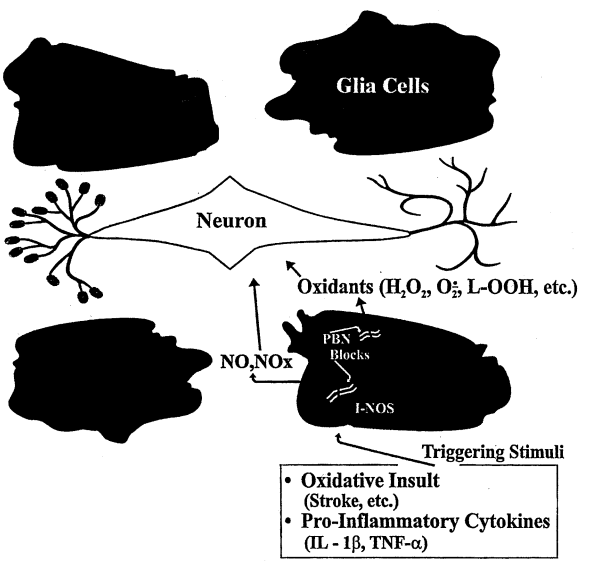



Fig. 1. Model illustrating the basic tenets of the neuroinflammatory concept and the action of nitrones (PBN in the case presented) in suppressing the production of neurotoxic products. PBN is shown inhibiting the induction of genes, such as iNOS, which are responsible for production of the neurotoxic products. Many stimuli are capable of triggering the activation of glia leading to the upregulation of genes that produce the neurotoxic products (Floyd and Hensley, 2000).

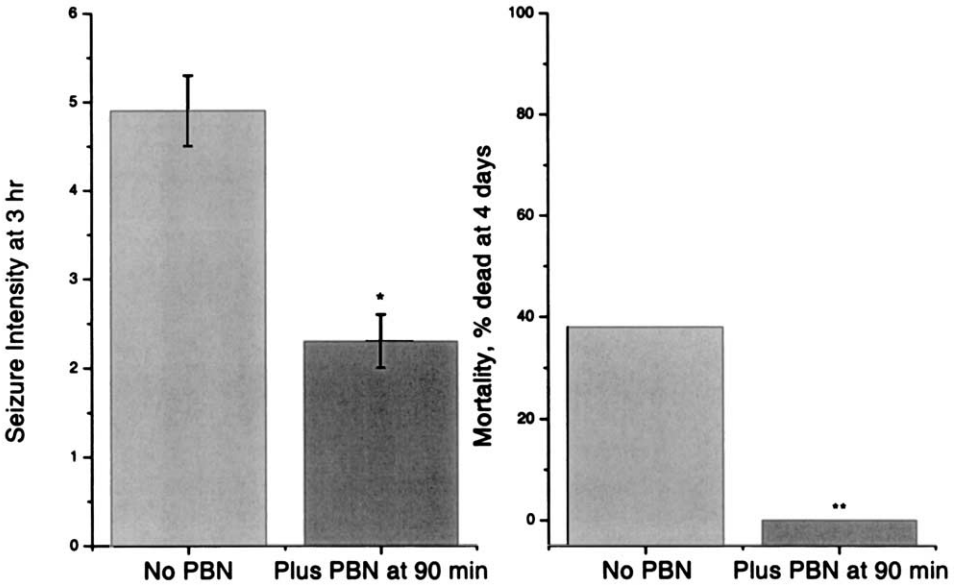



Fig. 2. Seizure rating (at 3 h) and mortality of rats (at 4 days) administered kainate that were then either given PBN at 150 mg/kg I.P. at 90 min or the saline carrier. Graphs were reconstructed from the published data (Floyd et al., 2000).

caused by the toxin. Immunohistochemically we showed that kainate mediated p38 MAP kinase activation in the hippocampus 3 h after the toxin was administered. PBN given 90 min after the toxin suppressed p38 activation. Additionally we observed immunohistochemically that kainate activated the NF-κB transcription factor in the hippocampus and that PBN administration prevented its activation. Electromobility shift analysis of NF-κB activation as analysis of apoptosis-associated caspase expression showed that kainate administration caused their activation but that PBN significantly suppressed their activation, see Table 1.

A large amount of research has now shown that nitrones act to suppress proinflammatory cytokine (and other stressors such as $H_2O_2$) mediated induction of genes in a wide range of biological systems. For example, PBN inhibits the cytokine-mediated upregulation of iNOS in rat insulinoma cells (Tabatabaie et al., 2000). It has also been shown that PBN effectively prevents the activation of p38 in astrocytes (Robinson et al., 1999). p38 is a MAK kinase involved in the signal transduction cascade mediating the upregulation of iNOS (Bhat et al., 1998; Da Silva et al., 1997). In a macrophage cell culture, PBN has been shown to inhibit LPS-mediated nuclear factor kappa B (NF-κB) binding to nuclear DNA and to

Table 1
Influence of PBN administration 90 min after Kainate (KA) injection on NFκB expression, various cytokines as well as various caspases and apoptosis regulator proteins in rat brain

| Parameter | Time after KA | PBN | |
|---|---|---|---|
| | | Without[a] | With[a] |
| NFκB | 3 h | 56 | 29 |
| | 4 days | 51 | 18 |
| IL1α | 3 h | 47 | 5 |
| IL1β | 3 h | 41 | 7 |
| TNFα | 3 h | 15 | 7 |
| YAMA/Caspase 3 | 1 day | 20 | 7 |
| | 4 days | 37 | 17 |
| ICH/Caspase 2 | 1 day | 51 | 36 |
| | 4 days | 60 | 33 |
| BAX | 1 day | 14 | 11 |
| | 4 days | 21 | 8 |
| Bcl-2 | 1 day | 13 | 0 |
| | 4 days | 11 | 0 |

[a] Net increase above controls (no KA-treatment) based on optical density of digitized autoradiograms (arbitrary units).

Table 2
Life span studies conducted to date regarding effect of PBN

| Description | Results | | |
|---|---|---|---|
| | | (50% life span (weeks)) | |
| | | +PBN | −PBN |
| 1 Senescence Accelerated Mice (SAM-P8), Males & Females groups Daily I.P. injections PBN (30 mg/kg) or saline. PBN started at 3 months of age, 12–13 per sex group, life span measured, consumption of food (?), body weights (?) | Males and females | 56 | 42 |
| | Males only | ∼59 | ∼38 |
| | Females only | ∼51 | ∼45 |
| | | +PBN | −PBN |
| 2 C57BK/6J male mice, two groups of 50 each PBN in drinking water (∼37.5 mg/kg per day); started at 24.5 mo. Free access to food and water (monitored) weight monitored (No differences, + or −PBN) | Mean life span (mo.) | 30.1** | 29.0 |
| | Maximum life span (mo.) | 33.3 | 31.7 |
| | Weight (gm) | 34.4 ± 2.9 | 35.0 ± 2.3 |
| | Water consumed | 6.09 ± 1.36* | 7.58 ± 2.72 |
| | | +PBN | −PBN |
| 3 Sprague–Dawley male rats, start at 24 months old, 11–12/group PBN, 32 mg/kg/day, I.P., taken past 33.5 months old free access to water and food, weigh weekly, no difference, Morris Water Maize given at 26 months | ∼50% survivorship (months) | 34.2 | 29.4 |
| | ∼% Surviving at 36.2 months | 45 | 8 |
| | Morris Water Maize | | |
| | ∼% time in home Quadrant | 58* | 40 |

Study 1 conducted by Edamatsu et al., 1995; Study 2 by Saito et al., 1998; and Study 3 by Sack et al., 1996. When ∼ values are given, the author has interpolated the values from the published graphs.
*P < 0.05; **P < 0.005

suppress mRNA expression of cycloxygense II and iNOS (Kotake et al., 1998). Similar results were obtained in an LPS-mediated septic shock model in experimental animals (Sang et al., 1999). In the LPS-induced septic shock mouse model, it has been demonstrated that PBN inhibits the enhanced formation of nitric oxide (Miyajima and Kotake, 1995) but does so by preventing the induction of the gene rather than acting catalytically to inhibit the iNOS enzyme per se. PBN has also been shown to prevent the expression of a broad spectrum of apoptosis-associated genes in the livers of rats treated with LPS (Stewart et al., 1999). The protective activity of PBN in the LPS septic shock model may reside also in its ability to mediate the enhanced expression of the anti-inflammatory cytokine (Kotake et al., 1999) as well as quell the enhanced production of toxic gene products.

## 5. Nitrones as anti-aging compounds

Several published observations clearly point to the conclusion that nitrones act to delay aging in

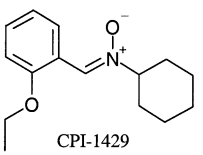
experimental models. Table 2 summarizes the published reports in mice and rats. Two studies in mice both show that PBN prolonged life span. One study (Edamatsu et al., 1995) was conducted with rapidly aging SAM-P8 mice. In this study the animal husbandry conditions were not carefully documented. Therefore, it is difficult to draw rigorous conclusions from this study even though the survival data clearly showed that PBN treated animals lived about 33% longer than the untreated controls. The other mouse study (Saito et al., 1998) was conducted on C57Bk/6J animals. PBN administration (in the drinking water) was begun in older mice (24.5 months) and continued until death. Much higher quality animal husbandry parameters, such as weekly weighing and careful monitoring of water consumption, were reported. Although certain improvements, such as pair feeding perhaps, could have been done to help define the caloric consumption for instance, nevertheless the data clearly show that chronic PBN administration prolonged the mean life span significantly.

The one reported study with rats involved an examination of the action of PBN to improve memory retention in older Sprague–Dawley rats. In this same study, they also monitored survivorship in the animals (Sack et al., 1996). PBN administration (p.o. at 32 mg/kg per day) starting at 24 months of age significantly improved memory retention, measured in a Morris water maze, and also delayed death, i.e. 50% survivorship was delayed from about 29.4 to 34.2 month in the untreated and PBN treated rats, respectively (Sack et al., 1996). The rats had free access to food and water and no differences in weekly weight measurements between the treated and untreated group was reported.

Although none of these three studies was conducted in the most rigorous fashion to critically evaluate the anti-aging activity of PBN per se, nevertheless all three studies clearly showed a large apparent anti-aging effect of the nitrone. There are many issues regarding the potential anti-aging effect that need to be carefully examined. For instance, a careful evaluation of the effect of PBN on caloric intake must be done. Additionally it is not known if PBN prolongs life by interfering with death producing pathologies in the various experimental models. This needs to be carefully evaluated.

## 6. Study of anti-aging activity of CPI-1429

A novel nitrone CPI-1429 has been synthesized. Its chemical structure is provided below. In the course of testing it for possible pharmacological activity in neurodegenerative models, it was discovered to have apparent anti-aging activity. The preliminary results have been reported earlier (Forster et al., 1999).



CPI-1429

CPI-1429 was tested for its possible efficacy for the treatment of learning and memory deficits displayed by C57BL/6 mice during the course of normal aging. Since the learning/memory paradigm employed (Forster and Lal, 1992) required extensive training and testing, the old mice (23–24 month) in these experiments were maintained under chronic treatment with the compound for up to 27 weeks. When survival analyses were performed on the treated and control groups, it was evident that mortality was significantly lower in mice treated with CPI-1429, over a relatively broad dose range. The survival data are presented in Fig. 3. The treatment was without effect on body weight of the mice, and was associated with improved performance in the learning and memory task (Forster et al., 1999). The complete set of learning and memory results in old and young mice are being prepared for publication elsewhere.

## 7. Possible mechanism of anti-aging activity of nitrones

Since very little is known about the ultimate causes of aging and senescence, it is exceedingly difficult to argue for specific mechanisms by

which any pharmacological agent may slow the senescence process. Nevertheless, speculation is possible from a nascent body of evidence that nitrones affect all cycle control on a fundamental level. Ames and colleagues have reported that PBN and one of its oxidative breakdown products, nitroso-*tert*-butane, delay replicative senescence in human fibroblasts (Chen et al., 1995; Atamna et al., 2000). This phenomenon is accompanied by an increased amount of cell cycle progression (Chen et al., 1995). Furthermore, the increase in replicative potential is associated with decreased DNA oxidation, indicating some effect (possibly indirect) of the nitrone on oxidative events linked to cell senescence (Chen et al., 1995). Independently, von Zglinicki and colleagues corroborate PBN's effect on senescence and associate the increased cellular longevity with decreased rates of telomere shortening, a fundamental correlate of cell senescence that decreases with replicative doubling, (von Zglinicki et al., 2000). The anti-senescence effects of PBN in the cell culture system resembles effects produced by culture in reduced oxygen tension (3%) (Chen et al., 1995) or by treatment with superoxide dismutaste mimetics (Melov et al., 2000). Contrastingly, pulse treatment with oxidants can induce a state of premature replicative senescence (Chen, 2000; Dumont et al., 2000). It is, therefore, tempting to speculate that nitrones act, in part, by modulating signal transduction pathways linked to cell cycle control.

Cell cycle regulation is a complex interplay of protein kinases and phosphatases, many of which are redox-sensitive, and most of which are linked to cell-surface receptors via multiple interdependent and redundant intermediate components (reviewed in (Shackelford et al., 2000)). As discussed above, PBN can antagonize cytokine-stimulated MAP kinase cascades apparently by suppressing oxidative inactivation of regulatory protein phosphatases (Robinson et al., 1999). Repetitive treatment of cell cultures with small doses of peroxide induces replicative senescence, associated with increased levels of cyclin-dependent kinase inhibitors and reduced ability to phosphorylate the checkpoint protein retinoblastoma (Dumont et al., 2000). It is, therefore, plausible to hypothesize that nitrones act to stabilize phosphorylation networks linked to cell cycle control. This stabilization may occur directly, by some binding interaction with key kinases/phosphatases, or indirectly, through the suppression of oxidant gen-

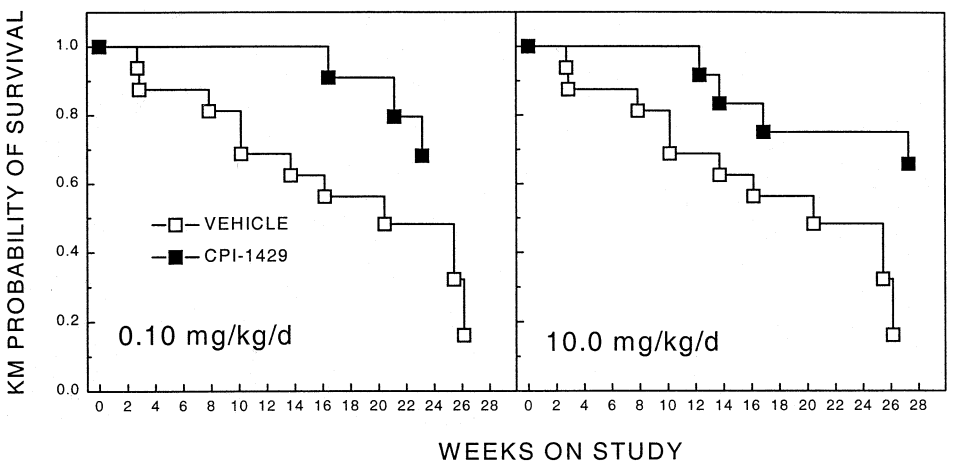



Fig. 3. Survival probability for aging C57BL/6 mice as a function of time and chronic daily dose of CPI-1429. The mice were 23–24 months old when they began daily (p.o.) treatment with the vehicle (buffered saline), or CPI-1429 [0.10 mg/kg (left panel), 10 mg/kg (right panel)]. The data for the control group are duplicated in each panel. Data are shown as Kaplan–Meier probability of survival. A Mantel log-rank test (with vehicle, low dose, and high dose as the strata) indicated a significant effect ($P = 0.029$). All mice in this study were tested on a learning/memory task beginning 2 weeks following initiation of treatment, that was continued for up to 22 weeks (Forster et al., 1999).

eration at the level of mitochondria as proposed elsewhere; see (Hensley et al., 1998b). The validity of this hypothesis remains to be determined in specific research designed to address the interaction of nitrones with cell cycle regulatory elements.

## 8. Summary and conclusions

The results of several studies demonstrate that the nitrone-based free radical traps have potent pharmacological activity. Their pharmacological activity does not depend on their ability to trap free radicals in a mass-action type reaction but appears to reside in their ability to interfere with enhanced signal transduction processes brought into play by stressful insults. Pro-inflammatory cytokine mediated induction of genes that produce neurotoxic products is the basis of neuro-inflammatory processes linked to several neurodegenerative diseases. The activity of nitrones to prevent the induction of genes that produce neurotoxic products is the basis of their activity to protect in neurodegenerative diseases. Several published reports indicate that the nitrones have significant anti-aging potential. These studies were conducted in experimental animals as well as in cultured cells. It is likely that the anti-aging activity of the nitrones depends on their ability to stabilize phosphorylation networks linked to cell cycle control processes. More rigorous research needs to be done on the anti-aging activity of the nitrones.

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