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Review

Pharmacologic Properties of Phenyl *N-tert*-Butylnitrone*

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ABSTRACT

Phenyl *N-tert*-butylnitrone (PBN) is the parent of a family of nitrones used as spin-trapping agents to trap free radicals. PBN's pharmacological effects in animal models are extensive, ranging from protection against death after endotoxin shock, protection from ischemia-reperfusion injury, to increasing the life span of mice. Recent additions to the list include protection from bacterial meningitis, thalidomide-induced teratogenicity, drug-induced diabetogenesis, and choline-deficient hepatocarcinogenesis. Because PBN reacts with oxygen radicals to produce less reactive species, it has been suggested that this is the basis of its pharmacological effects. However, there has been no hard evidence for this notation. Nevertheless, many investigators have used the presence of PBN's pharmacologic effect as evidence for free radical involvement in their models. Mechanistic studies on the PBN's antisepsis action revealed that PBN inhibits expression of various pro-inflammatory genes, suggesting that the protective action involves more than a straightforward free radical-scavenging mechanism. Previous and recent developments in the investigations on the pharmacologic properties of PBN are described in this review. Antiox. Redox Signal. 1, 481–499.

INTRODUCTION

IT WAS DISCOVERED IN THE LATE 1960S that specific nitrones would react with free radicals to form stable nitroxide compounds (Fig. 1). Use of this reaction for detection and characterization of free radicals was developed by Janzen and co-workers (Janzen 1971, 1984; Janzen and Haire 1990). This method was termed spin trapping, because the stable nitroxide radical product (spin adduct) made it possible to characterize the free radical (R· in Fig. 1) using electron paramagnetic resonance (EPR) spectroscopy. Because many free radicals are unstable, it is very difficult to prove their presence or to characterize them. Spin trapping has now become a frequently used technique in free radical biology. In biological systems, some investigators have gone one step further and interpreted this free radical capability as a means of detoxifying free radicals, and thus have explored pharmaceutical or therapeutic capacities of nitrone spin traps.

Phenyl *N-tert*-butylnitrone (PBN) is the parent of a family of nitrones used as spin-trapping agents (Fig. 2). Perhaps the earliest report on PBN's pharmacologic effect was by Hill and Thornalley regarding the inhibition of phenylhydrazine-induced hemolysis in human erythrocytes *in vitro* (Hill and Thornalley, 1983). Later, PBN's pharmacological effects in animal models were shown to be extensive, ranging from protection against lethal endotoxin shock and stroke brain damage to anti-aging. Recent

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*Alternate names for phenyl *N-tert*-butylnitrone that have been used in the published literature include: α-phenyl *N-tert*-butylnitrone, C-phenyl *N-tert*-butylnitrone, and *N-tert*-butyl α-phenylnitrone. These four names have also combined with different ways of spacing, hyphenating, and italicizing.

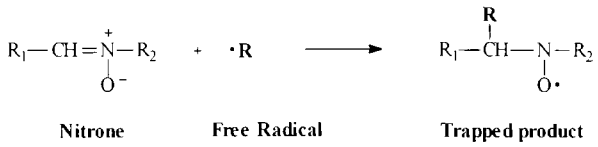
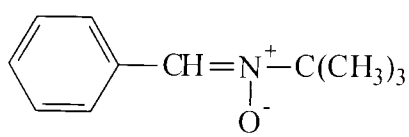


FIG. 1. Spin-trapping reaction scheme with nitrone compound.

additions to the list include protection from bacterial meningitis, drug-induced diabetogenesis, thalidomide-induced teratogenicity, and choline-deficient hepato-carcinogenesis. Novelli *et al.* were the first to show that PBN has a protective capacity against lethality from shock, by demonstrating that PBN protected rats against lethal traumatic shock (Novelli *et al.*, 1985). Since then, PBN has been tested in numerous disease models and has shown therapeutic effects in most cases. In addition, PBN has been tested in cellular systems in various experimental settings to investigate the mechanism of pharmacologic activities.

Because PBN retains well-defined free radical-trapping capabilities, all pharmacologic effects have been explicitly or implicitly attributed to this activity. Many investigators have suggested that beneficial effects of PBN provide evidence for free radical involvement in a pathological process. There have been attempts to detect the trapped product (spin adduct) in the disease model where PBN showed a preventive effect as an evidence that free radicals were produced and scavenged during the event. However, it was not conclusive whether the trapped product could explain the preventive effect. Because most investigators tested PBN with a presumptive notion that it must scavenge free radicals in biological systems, mechanistic studies to reveal how PBN carries out a protection action have been scarce. Pogrebniak *et al.* were the first to show that PBN's protection against endotoxin-mediated death in mice was associated with time-dependent down-regulation of cytokines



PBN

FIG. 2. Formula structure of PBN.

(Pogrebniak *et al.*, 1992). This study suggested that PBN was more than a simple free radical scavenger that protects tissues from direct damage from free radical attack. Later, in the same endotoxin shock model, PBN was shown to inhibit gene induction of inducible nitric oxide synthase (NOS) and subsequent nitric oxide (NO) formation (Miyajima and Kotake 1995, 1997). More recently, PBN has shown inhibition against the activation of the transcription factor NF-κB, the expression of multiple cytokine genes, and multiple apoptosis-associated genes (Kotake *et al.*, 1998; Sang *et al.*, 1999; Stewart *et al.*, 1999). Because the activation of NF-κB is suggested to be redox-sensitive or free radical-mediated, it is possible that PBN's free radical trapping action does function in inhibiting NF-κB activation. The present review summarizes the previous studies on PBN's pharmacologic activities, including recent development of mechanistic studies.

CHEMICAL AND PHYSICAL PROPERTIES, PHARMACOKINETICS, TOXICITY, AND METABOLITES OR PBN

Pure PBN (formula weight 177.2) is a colorless crystalline substance at ambient conditions with a melting point of 73–74°C. Old PBN develops a benzaldehyde-like smell because it is believed to decompose gradually into benzaldehyde and *tert*-butylhydroxyl amine under moist air. The solubility in water is approximately 110 m*M* (19 mg/ml) (Janzen *et al.*, 1995a), but the solubilization rate is slow for crystals. It readily dissolves in ethanol or other lower alcohols, but the solubility in physiologic saline is considerably lower than in water. The octanol/water partition ratio of PBN is 15/1; therefore, PBN is classified as a hydrophobic (lipophilic) agent (Janzen *et al.*, 1995a). A typical hydrophilic nitrone, DMPO (5,5′-dimethylpyrroline-*N*-oxide; Fig. 3) has an octanol/water partition ratio of 1/10 (Janzen *et al.*, 1995a). PBN is commercially available from several sources, but one report showed that commercially available PBN gave an artifactual EPR signal (Dikalov *et al.*, 1999). Although it is not known whether PBN impurities affect the pharmacological properties, many studies have used

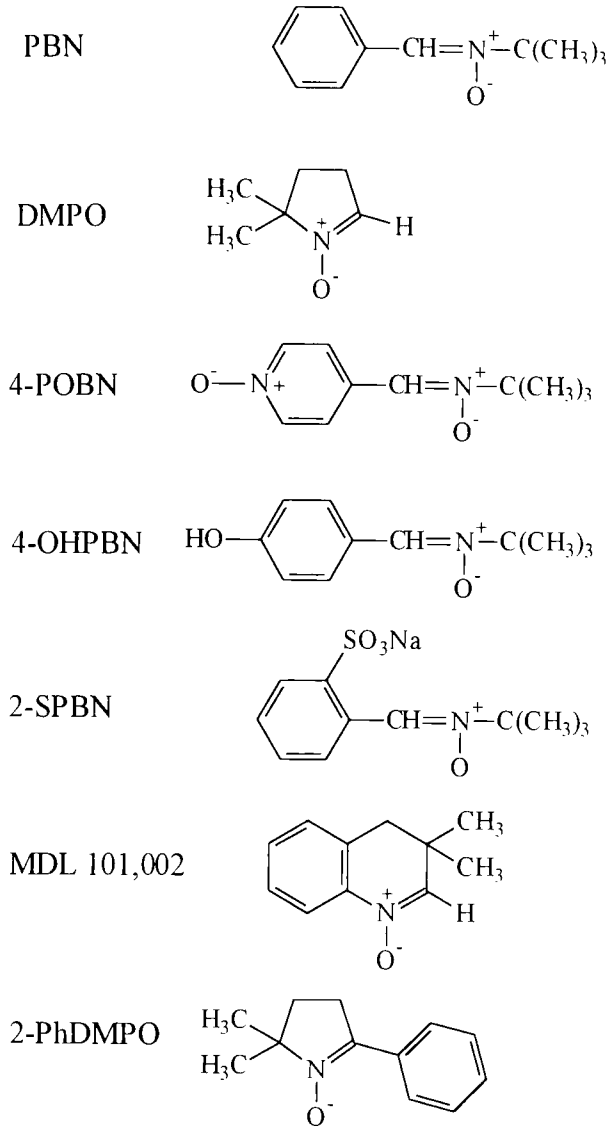


FIG. 3. Formula structures of nitrone compounds used for pharmacologic studies.

commercially available PBN without further purification. PBN can be synthesized using a one-step reaction between benzaldehyde and 2-methyl 2-nitropropane (Huie and Cherry, 1985), or by a condensation reaction with benzaldehyde and *tert*-butylhydroxyl amine. It can be purified either with recrystallization or vacuum sublimation. PBN has been shown to be a source of NO *in vivo* and *in vitro* following decomposition of its hydroxy radical adduct (Chamulitrat *et al.*, 1993, 1995; Saito *et al.*, 1998). However, the relationship between PBN's pharmacologic activities and NO produced from PBN has not been studied.

The pharmacokinetics of PBN have been studied in rats. PBN was administered intraperitoneally (i.p.) and the concentration of the spin trap in various organs was determined by high-performance liquid chromatography (HPLC) (UV detection) (Chen *et al.*, 1990a). The concentration of PBN in plasma peaked at 15 min, whereas the maximum in all organs tested (liver, brain, heart, and kidney) occurred at 30 min. PBN was detected in the urine for as long as 24 hr after injection of the compound. The use of ^{14}C-radiolabeled PBN (C-14 at nitronyl carbon) combined with HPLC yielded similar results (Chen *et al.*, 1990b). An *in vivo* microdialysis study in rats indicated that PBN distributes preferentially to the brain; about 15-fold better than the water-soluble PBN analog, 4-pyridyl-*N*-oxide *N*-*tert*-butylnitrone (4-POBN; Fig 3) (Cheng *et al.*, 1993). Radiolabeled PBN was also used to identify metabolites of PBN *in vivo* and *in vitro*. Rat liver microsomal dispersions metabolized PBN (Chen *et al.*, 1991), but the metabolite was not identified. Later, it was identified as 4-hydroxyphenyl *N*-*tert*-butylnitrone (4-OHPBN; Fig. 3) on the basis of co-chromatography with authentic 4-OHPBN (Reinke *et al.*, 1999). Using gas chromatography/mass spectrometry (GC/MS) techniques with Langendorff rat hearts, the perfused PBN was shown to be mainly taken up by nuclei and mitochondria (Cova *et al.*, 1992).

The lethal ip dose of PBN to male Sprague-Dawley rats was estimated to be 1 g/kg, using corn oil/buffer suspension as a vehicle (Janzen *et al.*, 1995b). Other water-soluble nitrones such as DMPO was less toxic (Schaefer *et al.*, 1996). In mice (Pogrebniak *et al.*, 1992), 300 mg/kg PBN administered intravenously (i.v.) was lethal (Pogrebniak *et al.*, 1992). But, when mice were given 300 mg/kg PBN i.p. or subcutaneously (s.c.), the animals were sedate, with huddled posture and mild piloerection for 1 hr after the injection. In contrast, animals that received in excess of 400 mg/kg i.p. displayed seizure activity. The maximum tolerated i.v. dose of PBN was 150 mg/kg, and these mice immediately had transient seizures but survived without sequellae. PBN showed a low potential of acute skin intolerance in the guinea pig (Fuchs *et al.*, 1998). The cytotoxicity of spin-trapping compounds has been evaluated in bovine aortic endothelial cells, using a neutral red absorption assay (Haseloff *et al.*, 1997). IC_{50} for PBN, the concentration that kills 50% of cell

population was 9.4 m*M*, while the IC_{50} for DMPO was 138 m*M*. Mouse peritoneal macrophages appeared more resistant to PBN, *i.e.*, IC_{50} was approximately 15 m*M* (Kotake *et al.*, 1998).

PHARMACOLOGIC EFFECTS OF PBN IN ANIMAL MODELS

Endotoxin shock, traumatic shock, and bacterial meningitis

The first published study on PBN's pharmacologic effect *in vivo* was on rat models of traumatic shock (Novelli *et al.*, 1985). In this model, rats were administered PBN (50–150 mg/kg, i.p.) 10 min before being submitted to 100% lethal whole-body trauma (rotating drum); their survival, pathology, acid-base status, and hematocrit level were evaluated. PBN administration was highly effective both in the prevention and reversion of these parameters of traumatic shock in rats. For example, when PBN (100 mg/kg i.p.) was administered 10 min before the shock episode, all animals survived 24 hr ($n = 14$), whereas no control animals survived ($n = 13$). PBN treatment 1 hr after the end of trauma was still highly protective against lethality (100% survival). The authors suggested that this effect was related to the PBN's free-radical scavenging capability but did not exclude other possibilities. Later in the same model, the temporal changes of interstitial glycerol, lactate, and glucose were observed (Lewen and Hillered, 1998), and PBN pretreatment (30 mg/kg, i.v.) significantly attenuated the interstitial accumulation of glycerol and lactate. But in a recent study with a rat traumatic head injury model, free radicals were not spin-trapped by PBN (did not show EPR signals) in whole brain homogenate 1 hr after the shock episode (Awasthi *et al.*, 1997).

Preadministration of PBN protected rats from lethal endotoxin shock (McKechnie *et al.*, 1986; Novelli *et al.*, 1986). Conscious rats were treated with PBN (250 mg/kg, i.p.) 20 min before lipopolysaccharide (LPS) infusion (10 mg/kg i.v., given over 4 hr) (McKechnie *et al.*, 1986). The survival rate for PBN-treated rats 96 hr later was 58% ($n = 12$) as compared to 13% ($n = 23$) for control animals. In the same study, multiple pretreatment with DL-α-tocopherol (100 mg/kg s.c., 3 days) also increased the survival to 54% ($n = 15$), but superoxide dismutase (SOD) pretreatment failed to increase survival. Other indicators of endotoxin shock severity in PBN-treated animals were closer to normal animals (McKechnie *et al.*, 1986; Novelli *et al.*, 1989). This protective effect was attributed to its free-radical scavenging activity, a fact that had been well established in chemical systems. PBN's protective effect on rat endotoxin shock lethality was confirmed later, and other nitrones such as DMPO and 4-POBN also have been shown to exhibit positive but weaker protective effects than PBN (Hamburger and McCay, 1989). Later, a dramatic protection by PBN was demonstrated in a mouse endotoxin shock model. For example, s.c. administration of PBN (250 mg/kg) to C_3H/HEN mice 15 min before a lethal dose (40 mg/kg, i.v.) of LPS completely protected the animals from death ($n = 30$, 7 days) (Pogrebniak *et al.*, 1992). Without PBN administration, 75% of control animals ($n = 30$) died in 2 days. I.p. injection of PBN resulted in a similar level of protection (Miyajima and Kotake, 1995; Pogrebniak *et al.*, 1992). Nitrones with cyclic structure were synthesized and tested for protection against endotoxin lethality in rats (French *et al.*, 1994). Some of the new compounds provided effective protection at a lower dose than PBN. Moreover, these compounds showed higher trapping efficiency against hydroxy radicals than PBN, suggesting a possible relationship between the nitrone's free-radical trapping capability and antishock activity. Treatment with a PBN analog, 2-sulfoxyphenyl *N-tert*-butylnitrone (2-SPBN; Fig. 3) of sublethal endotoxin shock in horses were reported to be effective in normalizing heart and respiratory rates (Harkins *et al.*, 1997).

In an infant rat model of bacterial meningitis, PBN treatment (100 mg/kg, i.p.) before group B streptococci infusion significantly reduced the parameters indicative of the formation of reactive oxygen species (ROS) in the brain and improved pathological conditions (Leib *et al.*, 1996). For example, PBN restored cerebral cortical perfusion to 72% ($p = 0.05$),

which had been lowered to 37.5% from non-treated animals. This study used PBN's effect to support the hypothesis that free radicals are involved in bacterial meningitis.

Ischemia/reperfusion injury in a brain-stroke model

The early demonstration of the effect of PBN on a traumatic shock model (Novelli *et al.*, 1985) suggested that PBN may have passed through the blood–brain barrier. Also at that time, studies on the mechanism of ischemia/reperfusion (I/R) injury started to suggest that free radicals were involved in this injury. For example, hydroxylation of salicylate, which was believed to be an indication of hydroxy radical formation, was demonstrated in an I/R injury model in gerbils (Cao *et al.*, 1988). In the same gerbil model, PBN (100 mg/kg, i.p.) administered either 30 min prior to or 30 min after a 5-min period of bilateral carotid occlusion prevented the increase in locomotor activity observed in saline-injected ischemic animals (Phillis and Clough-Helfman, 1990; Clough-Helfman and Phillis, 1991). A neuroprotective effect of PBN was reported when age-related parameters in old gerbils were reversed by chronic administration of PBN. Chronic treatment with PBN (14 days with twice daily dosages of 32 mg/kg i.p.) caused a decrease in the level of oxidized protein and an increase in both glutathione and neutral protease activity in aged gerbils (15–18 months of age). But, if PBN administration was stopped after 2 weeks, the decreased level of oxidized protein and increased glutathinone and neutral protease activities in old gerbils changed back to the levels observed in aged gerbils prior to PBN administration. In addition, older gerbils treated with PBN made fewer errors in a radial arm maze test for temporal and spatial memory than the untreated aged controls (Carney and Floyd, 1991; Carney *et al.*, 1991; Floyd, 1991). Later, some of these observations were reproduced and confirmed (Dubey *et al.*, 1995). Possible reduction of free radicals by PBN was assessed by a spin-trapping method using a hydrophilic spin trap, 4-POBN. A reduction of the spin adduct signal was observed when a protective dose of PBN was administered (Sen and Phillis, 1993; Sen *et al.*, 1994). This result may support the hypothesis that endogenous free radicals were decreased by PBN's trapping activity, thus causing the pharmacologic effect.

In other animal models, it has been shown that PBN protects from stroke if given before (Folbergrova *et al.*, 1995) or after (Zhao *et al.*, 1994) brain reperfusion in rats. It is important to point out that all previously shown effects were observed when PBN was given *before* the animals were subjected to stress. The delayed PBN treatment was also effective in middle cerebral artery occlusion (MCAO) stroke models in rats. In the permanent MCAO model, PBN protected against brain necrosis or loss of neuronal defects, even if given 5–12 hr after the lesion (Cao and Phillis, 1994; Siesjö and Siesjö, 1996). In the transient MCAO model, PBN administration 3 hr after reperfusion was shown to be effective (Carney and Floyd, 1991). In these models, PBN-treated animals showed pronounced recovery of energy state, with ATP and lactate contents in both focus and penumbra approaching normal values (Folbergrova *et al.*, 1995). A later study showed that PBN but not 2-SPBN protected from transient forebrain ischemia in the rat (Pahlmark and Siesjö, 1996). Most recently, the decline in the ability of isoproterenol to augment GABAergic responses in cerebellar Purkinje neurons in rats that had been brought on by norbaric hypoxia was shown to be reversed by PBN treatment (3 days prior to hypoxia, 10 mg/kg i.p., twice daily) (Bickford *et al.*, 1999).

PBN has been shown to possess vasodilating functions. In a perfused rat heart model, PBN showed coronary vasodilation when the perfusate contained PBN higher than 3 m*M* (Konorev *et al.*, 1993). In the same study, other nitrone or nitroso spin traps also showed vasodilating functions with varying potencies. PBN has shown a vasodilating function in preconstricted isolated rat pulmonary artery rings, and this was attributed to reversible calcium channel blockade measured with patch-clamp techniques (Anderson *et al.*, 1993). *In vivo*, PBN has been shown to increase cortical cerebral blood flow in rats. This was ascribed to the PBN's ability to inhibit the breakdown of NO

(Inanami and Kuwabara, 1995). It is possible to speculate that these vasodilating functions of PBN contributed to the effectiveness of delayed treatment in the stroke model.

The cyclic nitrone spin trap MDL 101,002 (Fig. 3) was more protective in model systems of central nervous system (CNS) injury in rats than PBN (Thomas *et al.*, 1994a). In other studies, PBN reduced infarct size and prevents a secondary mitochondrial dysfunction due to reperfusion (Kuroda and Siesjö, 1997), and PBN's protective capacity against brain ischemia was compared with new antioxidants, pterin-6-aldehyde (Mori *et al.*, 1998) and 3-methyl-1-phenyl-2pyrazolin-5-one (Nakashima *et al.*, 1999). These compounds showed similar or higher activity than PBN, but do not have apparent free radical-trapping functions.

Ischemia/reperfusion injury in other organs

The initial results on the effect of PBN on heart I/R injury were negative. PBN (50 mg/kg i.v.) was infused to dogs that had shown occlusion/reperfusion-induced arrhythmias. Neither PBN nor the xanthine oxidase inhibitor allopurinol was effective in improving arrhythmias (Parratt and Wainwright, 1987). Spin-trapping experiments using PBN as a spin trap to detect free radical formation in a dog coronary artery occlusion/reperfusion model were successful in observing myocardial release of free radicals immediately after reperfusion. The investigators also noticed that recovery of contractile function (measured as systolic wall thickening) after reperfusion was significantly greater in dogs given PBN than in controls (Bolli *et al.*, 1988). Later, this protection was studied in detail in the PBN dose range from 1.7 m*M* to 10 m*M* (Li *et al.*, 1993), and PBN was very effective in bringing various vascular parameters back to normal. The previous spin trapping results were confirmed later by using spin traps other than PBN (Culcasi *et al.*, 1989; Pietri *et al.*, 1989). PBN, but not DMPO, was reported to protect partly against I/R injury in perfused rat heart (Bradamante *et al.*, 1992, 1993; Li *et al.*, 1993; Vrbjar *et al.*, 1998). In contrast, PBN did not attenuate postischemic cell death in perfused porcine hearts (Klein *et al.*, 1993) or perfused rat hearts subjected to global ischemia (Baker *et al.*, 1994). In this latter experiment, an hydroxy radical adduct of PBN was detected in the perfusate.

In a kidney I/R injury event in intact rabbits, free radical formation was detected by spin trapping with PBN immediately after reperfusion (Pincemail *et al.*, 1990). However, PBN's protective effect on kidney I/R injury has not been tested.

Drug-induced neurodegeneration

Systemic administration of neurotoxins is known to cause neuronal cell loss or death. Preadministration of PBN in such drug-induced neurodegeneration models has been shown to protect neuronal cells. For example, PBN (150 mg/kg, i.p.) given 10 min before 3,4-methylenedioxy-methamphetamine (MDMA 10 mg/kg, i.p.) prevented the loss of 5-hydroxytryptamine and its metabolite 5-hydroxyindoleacetic acid in the cortex and hippocampus (Colado and Green, 1995; Colado *et al.*, 1997). PBN did not protect rats against fenfluramine-mediated neuronal damage, but it did in the case of *p*-chloroamphetamine-mediated damage (Murray *et al.*, 1996). The authors concluded that fenfluramine-mediated damage did not involve free radical reactions. In other studies, pretreatment with 2-SPBN significantly attenuated striatal excitotoxic lesions in rats produced by *N*-methyl-D-aspartate, kainic acid, and α-amino-3-hydroxy-5-methyl-isoxazole-4-propionic acid (Schulz *et al.*, 1995). In a similar manner, it has been shown that striatal lesions produced by 1-methyl-4-phenylpyridinium (MPP^+), malonate, and 3-acetylpyridine were significantly attenuated by either S-PBN or PBN treatment (Schulz *et al.*, 1995). In these events, production of hydroxy radicals was assessed by the conversion of salicylate to 2,3- and 2,5-dihydroxybenzoic acid (DHBA), resulting in the significant reduction of DHBA in 2-SPBN-treated animals. However, in similar studies with a plant micotoxin, 3-nitropropri-onic acid-induced striatal lesions, 2-SPBN, and PBN worsened injury, whereas DMPO was protective (Schulz *et al.*, 1996). Hydroxy radical formation evaluated with salicylate hydroxylation indicated reduction with all three nitrones. In other drug-induced neurodegeneration

models, necrosis of subtantia nigra and other brain regions in fluorothiyl-induced status epilepticus in rats was shown to be ameliorated by preadministered PBN (He *et al.*, 1997). MPP^+-induced deficits in motor activity were restored by preadministered PBN (Fredriksson *et al.*, 1997). In the same model, substantia nigra cell loss produced by MPP^+ administration into rat striatum were significantly reduced by 2-SPBN (Fallon *et al.*, 1997), and azulenyl nitrone spin traps protect against MPTP (1-methyl-4-phenyl-1,2,3,6-tetramethylpyridine) neurotoxicity (Klivnyi *et al.*, 1998). PBN decreased methamphethamine-induced depletion of striatal dopamine in rats without altering hyperthermia (Cappon *et al.*, 1996).

In studies involving glutamate microdialysis, PBN attenuated excitotoxicity in rat striatum (Lancelot *et al.*, 1997) and striatum injury (Ferger *et al.*, 1998). Other studies have shown that PBN protected against MDMA-induced depletion of serotonin in the CNS (Yeh, 1999), and a cyclic nitrone inhibited iron-dependent CNS damage (Thomas *et al.*, 1997). In a rat model of spinal cord injury, PBN given 30 min before (30 mg/kg, i.v.) and 2 hr after (10 mg/kg, i.v.) trauma improved energy metabolism in the spinal cord (Farooque *et al.*, 1997). However, PBN did not protect from compression injury of rat spinal cord (Li *et al.*, 1997). Vagotomy-induced noncholinergic bronchoconstriction *in vivo* in guinea pigs was significantly ameliorated by PBN preadministration (Zhang *et al.*, 1996). A majority of these studies have interpreted protective effects of PBN and its analogs as evidence that free radicals were involved in the drug-induced neurodegenerative events. Most recently, PBN, given 30 min before seizure induction with kainic acid administration reduces the decrease in ATP concentration and adenylate energy charge, without significantly reducing the amount of lactate accumulated, or the decrease in intracellular pH (Folbergrova *et al.*, 1999). The authors suggest that PBN preserves the structural and functional integrity of substantia nigra, pars reticulata neurons by protecting the mitochondria against oxidative damage. Most recently, pretreatment of mice with the PBN for 7 days prior to and during 3 days of KCN markedly reduced cyanide-induced cortical DNA fragmentation (Mills *et al.*, 1999).

Lifespan and aging

Another form of neuroprotective effect, PBN's anti-aging effect has attracted considerable attention. The study on the reversal of age-related parameters in gerbils by chronic PBN administration (Carney *et al.*, 1991) appeared to trigger later and anti-aging studies. Chronic administration of PBN (30 mg/kg daily, i.p.) to a senescence-accelerated mouse (SAM) model has been shown to extend 50% mean survival from 42 weeks to 56 weeks (Edamatsu *et al.*, 1995). Recently, wild-type C57BL/6J male mice (24.5 months old) were chronically treated with 0.25 mg/ml PBN (approximately 75 mg/kg/per day) in drinking water, and they showed an extended lifespan by 4% that was statistically significant (Saito *et al.*, 1998). In a mechanistic study on PBN's anti-aging effect in the SAM model, the effect of chronic PBN treatment (14 days, 30 mg/kg, i.p. daily) was evaluated on the physical state of cortical synaptosomal membrane proteins using EPR spin labeling. The protein from SAM-P8 (senescence-prone) mice returned EPR parameters toward normal values, but SAM-R1 (senescence-resistant) mice did not change (Butterfield *et al.*, 1997). In other anti-aging studies, old rats (24 months) chronically (9.5 months) treated with PBN showed improvement in cognitive performance and survival (Socci *et al.*, 1995; Sack *et al.*, 1996). But, another study showed that PBN did not expand lifespan of the house fly (Dubey *et al.*, 1995).

PBN's anti-aging effects were evaluated by assessing parameters other than the lifespan: Nonadrenergic receptor function has been shown to return to normal in PBN-treated (2 weeks) aged rats (Gould and Bickford, 1994); PBN has been shown to ameliorate age-related deficits in striatal muscarnic receptor sensitivity (Joseph *et al.*, 1995) and in the phorbol ester stimulation of synapsin phosphorylation (Eckles *et al.*, 1997). Age-related reductions in oxometric enhancement of K^+-evoked dopamine release from superfused striatal slices were restored by PBN treatment (Joseph *et al.*, 1996a, b). PBN preadministration has been shown to

prevent hyperoxia-induced oxidation of cortical synaptosomal membrane proteins in young gerbils (Howard *et al.*, 1996).

Carcinogenesis

Dietary choline deficiency has been known to cause hepatocellular carcinoma in rats, and preventive effects of PBN were tested in this model (Nakae *et al.*, 1998). Rats were given drinking water that contained 0.013–0.13 wt% PBN (average dose 50–100 mg/kg/per day) along with a choline deficient, L-amino acid defined (CDAA) diet for 12 weeks. The results showed dose-dependent inhibition by PBN of the changes that are normally induced in the livers of rats by the CDAA diet feeding, *i.e.*, development of putative preneoplastic lesions, proliferation of connective tissue, reduction of glutathione *S*-tranferase activity, formation of 8-hydroxy deoxyguanosine (8-OHdG) in DNA, and an increase in inducible cyclooxygenase (COX2) activity (Nakae *et al.*, 1998). These results indicate that PBN at least inhibited the early phase of carcinogenesis caused by the CDAA diet. Whether PBN is able to inhibit the development of hepatocellular carcinoma is yet to be tested. In other studies, the protective effects of PBN, resveratrol, melatonin, and vitamin E against the kidney carcinogen $KBrO_3$ were tested in rats (Cadenas and Barja, 1999). The increase in 8-OHdG in the kidney DNA after $KBrO_3$ challenge was partially prevented by pretreatment with PBN, melatonin, and vitamin E and was completely abolished by resveratorol treatment. However, free radical involvement of this renal injury is not known.

PBN as an antidote

PBN has shown activities as an antidote in other animal models than drug-induced neurodegeneration. Preadministration of PBN has been shown to inhibit thalidomide-induced teratogenicity in rabbits (Wells *et al.*, 1997; Parman *et al.*, 1999). PBN (40 mg/kg, i.v.) was administered to pregnant rabbits with gestational days (GD) 8 to GD 12, 15 min prior to a teratogenic dose of thalidomide (400 mg/kg, i.v.) and the birth defect at GD 29 was evaluated. The results showed that several indices of birth defect were lowered to close to control levels. 8-OHdG levels in multiple organs in fetus from PBN-pretreated animals were dramatically lower than those treated by thalidomide alone. The authors suggested that decreased ROS levels may have caused this protection. Almocalant-induced teratogenicity in rats was prevented by PBN preadministration [0.85 mmol (150 mg)/kg, s.c.] (Wellfelt *et al.*, 1999).

PBN preadministration was shown to reduce significantly the size of liver edema in rats that was formed with carbontetrachloride (CCl_4) administration (Janzen *et al.*, 1990). In a later study, a DMPO-type nitrone, 2-phenyl DMPO (2-PhDMPO) has shown similar protective effects (Towner *et al.*, 1993). In those studies, the edema size was evaluated using magnetic resonance imaging (MRI) technique. Preadministration of PBN (100 mg/kg, i.p., 1 hr before) protected guinea pigs from carbon monoxide-mediated impairment of high-frequency auditory sensitivity (Fechter *et al.*, 1997). The authors showed that allopurinol preadministration also provided similar protection. In other reports, PBN protected from adriamycin-mediated cardiotoxicity in intact rats (Paracchini *et al.*, 1993).

Other animal models

Early indications of PBN's influence on the animal energy state have been shown in the study that the pretreatment of mice with PBN, 4-POBN, or DMPO increased swimming endurance in mice (Novelli *et al.*, 1990). Long-Evans Cinnamon (LEC) rats have been known to develop acute hepatitis spontaneously as a result of abnormal copper accumulation. In this model, the development of copper-mediated spontaneous hepatitis with severe jaundice was inhibited by PBN, which was administered s.c. every 2 days at the dose of 128 mg/kg, beginning with 13-week-old rats and continuing for 17 weeks (Yamashita *et al.*, 1996). PBN prevented the loss of body weight, reduced the death rate, and improved liver activity indice. Ocular inspection also confirmed the suppressive effects of PBN on jaundice. PBN's protection from copper-mediated oxidative stress was speculated to be the mechanism of this effect.

In an animal model of diabetes, systemic injection of streptozotocin (STZ) has been shown

to cause type I (juvenile) diabetes in rodents, and PBN showed protection against this diabetogenesis (Tabatabaie *et al.*, 1997). Mice were co-administered PBN (150 mg/kg, i.p.) and STZ (30 mg/kg, i.p.) for 5 consecutive days. Animals given PBN plus STZ maintained their blood glucose level (180 mg/dl) close to those treated with saline or PBN alone (140 mg/dl) for up to 25 weeks, whereas animals treated with STZ alone showed a high blood glucose level (430 mg/dl). The rise of glycated hemoglobin levels and the weight loss in this disease were also suppressed by PBN treatment. It is suggested that the immune response brought about by STZ was suppressed by PBN (Tabatabaie *et al.*, 1997). The same study showed that STZ-induced NO formation in the pancreas was inhibited by PBN. In an AIDS dementia complex (ADC) animal model, the envelope protein of human immunodeficiency virus-1 (HIV-1), gp120 has been shown to cause behavioral anomalies in rat neonates. Animals co-injected with PBN (50 mg/kg) and gp120 (2.5–3.5 ng/animal) did not show the cognitive deficit or the motor and behavioral dysfunctions that were seen in animals treated with gp120 alone (Tabatabaie *et al.*, 1996). In other models, PBN preadministration (200 mg/kg, i.p.) 30 min before restraint–cold stress reduced the gastric ulcer index 4.5-fold in 2 hr after stress as compared with animals that received stress alone (Das *et al.*, 1997). In the same study, a similar reduction of the index was obtained by the pretreatment with dimethylsulfoxide (100 mg/kg, i.p.) (Das *et al.*, 1997). PBN preadministration protected double-stranded DNA breakage in brain cells in rats exposed to radiofrequency electromagnetic radiation (Lai and Singh, 1997a, b). All disease models described in this section have been rarely mentioned as diseases where any free radicals are involved in the initiation or developmental stages.

PHARMACOLOGIC EFFECTS OF PBN *IN VITRO*

Numerous reports indicate that PBN has inhibitory effects against oxidative events *in vitro* or in cellular systems. The earliest study on PBN's pharmacologic activity was conducted *in vitro* on the protective effect of phenylhydrazine-induced hemolysis in human erythrocytes (Hill and Thornalley, 1983). Later, PBN, but not 4-POBN was shown to maintain the integrity of isolated primary rat hepatocytes (Albano *et al.*, 1986). In a series of studies, metal-induced oxidative damage by endothelial cells to low-density lipoprotein (LDL) was inhibited by the presence of PBN, and PBN-treated LDL was not degraded by macrophages as readily as those incubated in the absence of PBN (Kalyanaraman *et al.*, 1991). A lipid-derived radical formed during oxidation of LDL was detected by spin trapping with PBN. It is suggested that PBN inhibits the oxidative and biological modification of LDL by scavenging the LDL-lipid-derived radicals (Kalyanaraman *et al.*, 1993). Lipophilicity of the nitrone appears to be an important factor for determining the efficacy of inhibition, because 4-POBN was not effective; however, novel lipophilic cyclic nitrones were more effective than PBN (Thomas *et al.*, 1994b). Some cyclic nitrones were effective in inhibiting lipid peroxidation (Fevig *et al.*, 1996; Thomas *et al.*, 1996a, b). In these studies, nitrones with higher lipophilicity showed higher activities. But the effective doses of PBN and PBN analogs *in vitro, i.e.*, in the micromolar range, are considered to be suprapharmacologic. PBN was shown to affect the function of isolated rat diaphragm with a dose less than 1 m*M* (Andersen *et al.*, 1996).

In the presence of PBN in cell culture medium, the maximum cell division number of human diploid fibloblast was shown to increase by 50% as compared to cells cultured in the absence of PBN, implicating an action similar to PBN's anti-aging effect (Chen *et al.*, 1995). In human neutrophils *in vitro*, self-inflicted cell death induced by a phorbol ester-mediated oxidative burst was inhibited in the presence of a relatively high concentration of PBN (10 m*M*) (Seawright *et al.*, 1995). In a similar category of study, hydrogen peroxide (H_2O_2) toxicity to PC12 cells was counteracted by PBN (Joseph *et al.*, 1997). A new type of PBN effect in cells was demonstrated: A low concentration of PBN (*e.g.*, 5.6 μM) doubled the developmental rate of rat embryo to the two-cell stage, and this effect was attributed to PBN's free radical detoxification capability (Yamashita *et al.*, 1997). Other reports showed that PBN and 4-POBN had no effect on the survival of embryonic or

adult dopamine neurons (Karlsson *et al.*, 1998). Cooperative neuroprotection by brain-derived neurotrophic factor and 2-SPBN against axotomy-induced retinal ganglion cell death was reported (Klocker *et al.*, 1998).

Mitochondria are speculated to be the site of free radical formation in the event of oxidative stress. An effect of PBN was demonstrated on rat brain mitochondrial functions *in vitro*. In this system, PBN potently inhibited complex I-mediated H_2O_2 synthesis with an IC_{50} of approximately 100 μM (Hensley *et al.*, 1998). Although this inhibition is a mechanism that may explain many known pharmacologic activities of PBN, this effect has not been demonstrated *in vivo*. In other systems, PBN was shown to regulate the content of intracellular thiol in murine hematopoietic progenitor cells *in vitro* (Kashiwakura *et al.*, 1997). In primary rat glial cell culture, PBN has been shown to decrease basal protein phosphorylation and increase phosphatase activity in a concentration-dependent manner (Robinson *et al.*, 1999a). For example, 1 mM PBN decreased protein phosphorylation by 30%. In the same cell culture, PBN or NAC pretreatment significantly suppressed interleukin-1β (IL-1β), H_2O_2, and sorbitol-mediated activation of p38-mitogen activated protein kinase (p38-MAPK), and H_2O_2 biosynthesis (Robinson *et al.*, 1999b). This kinase is considered to play a key role in inflammatory response to oxidative stress.

In the field of radiation biology, 8-OHdG in gamma ray-irradiated DNA solution was suppressed by the presence of PBN *in vitro*, indicating that PBN is potentially radioprotective (Young *et al.*, 1996).

MECHANISTIC STUDIES

Interaction with cytokine production

The presence of PBN's protective effect in animal disease models has been frequently used as a tool to suggest the free radical involvement in the disease initiation and development, and this assumption is naturally based on the PBN's free radical scavenging action. There have been several reports that claimed that EPR detection of PBN spin adducts during or after the protective episode was the self-explanatory evidence that a trapping reaction was responsible for the protection. However, because of the lack of quantitative evaluation as well as the possibility that these spin adducts could be the products of side reactions, such observations have not been accepted as hard convincing evidence as yet.

As early as 1991, in the mouse endotoxin shock model, PBN's protective action against a lethal dose of endotoxin was shown to be associated with significant down-regulation of some cytokine genes and proteins (Pogrebniak *et al.*, 1991). In this study, northern blot and protein analyses of tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), IL-6, and c-Fos were conducted in liver tissues obtained from mice treated with PBN (300 mg/kg) 15 min before LPS (30 mg/kg) injection. TNF-α protein levels were significantly lower in the PBN-treated animals at 1–6 hr, whereas IFN-γ protein levels were depressed at 8 hr. PBN down-regulated TNF-α mRNA at 30 min, with maximum lowering of all cytokine mRNA at 3 hr. PBN depressed c-*fos* transcription within 15 min after LPS injection. This activity of PBN appeared to have no relationship to its free radical trapping action; in fact, the authors suggested the presence of two concurrent mechanisms, *i.e.*, free radical trapping and cytokine down-regulation. Later, in the PBN-treated rat endotoxin shock model, gene expression of 11 cytokines were simultaneously determined, using a ribonuclease protection assay (RPA) (Sang *et al.*, 1999). The results not only reproduced the previous results of PBN's inhibition for TNF-α, IFN-γ, and IL-6 in the mouse model, but other cytokines such as IL-1α, IL-1β, TNF-β, IL-3, IL-4, and IL-5 were also inhibited (Fig. 4). It is noteworthy that in 30 min after LPS administration, PBN pretreatment enhanced the expression of the anti-inflammatory cytokine IL-10 as compared to animals treated by LPS alone. IL-10 protein in plasma was also confirmed to be amplified by PBN treatment (Kotake *et al.*, 1999b). The enhancement of IL-10 was reported in the mouse endotoxin shock model where animals were pretreated with the NF-κB inhibitor pyrrolidine dithiocarbamate (PDTC) (Nemeth *et al.*, 1998). Moreover, the preadministration of recombinant IL-10 has been shown to protect mice from lethal endotoxin shock (Berg *et al.*, 1995). These

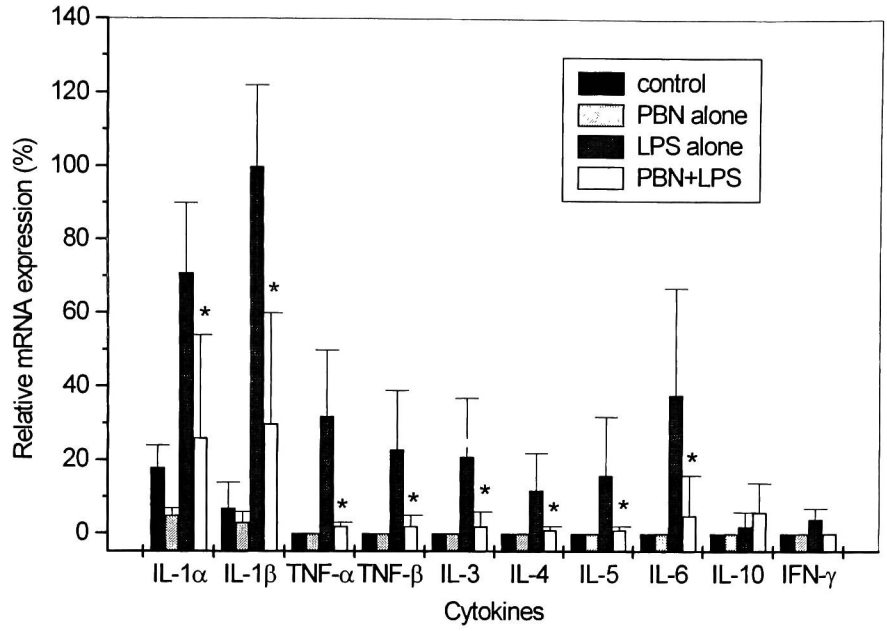


FIG. 4. Inhibition of cytokine mRNA expression in rats treated with PBN and LPS. This graph is made based on the previously published data (Sang *et al.*, 1999). Rats were administered with physiologic saline (n = 8) or PBN (150 mg/kg, i.p.) (n = 8). Also 5 of 8 saline-administered rats received i.p. injection of LPS (3 mg/kg) and the same number of PBN-administered rats received i.p. injection of LPS (3 mg/kg). Three hours later, liver mRNA was isolated and subjected to ribonuclease protection assay and the result was analyzed by densitometry. The differences between the columns marked with * (PBN+LPS) and those of LPS alone are statistically significant.

facts justify the assumption that the IL-10 overexpression promoted by PBN treatment is a functional mechanism for protection.

The obligatory outcome of endotoxin shock in the liver is considered to be multiple cytokine expression and the later apoptotic liver cell death. An RPA determination of multiple apoptosis-associated genes was conducted in the livers of rats treated with PBN and LPS, and the result indicated that PBN preadministration showed broad spectrum inhibition to these genes, both 30 min and 3 hr after LPS administration (Stewart *et al.*, 1999). Major reductions were observed for the expression of genes such as *YAMA* (caspase 3) and *fas-A*, which are located downstream of the NF-κB pathway. This inhibition is again consistent with the hypothesis that PBN is an NF-κB inhibitor.

Interaction with NO pathways

In a mouse endotoxin shock model, PBN pretreatment (300 mg/kg, i.p.) 30 min before LPS injection (50 mg/kg, i.p.) was shown to inhibit NO formation in the liver that was monitored 6 hr after LPS challenge (Miyajima and Kotake, 1995). A NO trapping method with iron complexes of dithiocarbamate combined with EPR spectroscopy was used to detect NO directly in the liver tissue. In the same and later studies, the reduction of NO was shown to be caused by the inhibition of the expression of inducible NO synthase (iNOS) gene and protein (Miyajima and Kotake, 1995, 1997). This reduction of NO formation was confirmed by other NO trapping methods in mice and rats (Reinke *et al.*, 1996; Fujii *et al.*, 1997; Kotake *et al.*, 1999a). The effective time window for PBN treatment to reduce NO formation in mice was estimated to be from 30 min before to 30 min after LPS treatment (Miyajima and Kotake, 1997).

Interaction with NF-κB

Because nuclear factor κB (NF-κB) is considered to be a major transcription factor for iNOS gene transcription (Xie *et al.*, 1994), PBN was speculated to be an inhibitor for NF-κB activation. This notion was first tested in cells (Kotake *et al.*, 1998). Mouse peritoneal macrophages were stimulated with LPS and IFN-γ for 30 min in the presence or absence of PBN (3–10 m*M*), and nuclear extracts were subjected to electrophoretic mobility shift assay (EMSA), to

quantify DNA binding activity of NF-κB. PBN inhibited NF-κB activity with an IC_{50} about 7 m*M*. In addition, PBN was found to be an inhibitor of COX2 induction and COX catalytic activity. But PBN was not an inhibitor of iNOS catalytic activity. Later, PBN was shown to inhibit NF-κB and activator protein-1 (AP-1) *in vivo* in a rat endotoxin shock model (Sang *et al.*, 1999). The DNA binding activity of NF-κB was quantified by EMSA in the livers of rats 30 min after LPS administration (4 mg/kg), and the liver nuclear extract of PBN-treated (150 mg/kg, i.p., 30 min before) animals showed a significant reduction of NF-κB. AP-1 was not expressed until 3 hr after LPS administration, but this transcription factor was also inhibited by PBN pretreatment. PDTC is a widely used NF-κB inhibitor (Schreck *et al.*, 1992b) and its preadministration has shown antisepsis activity *in vivo* (Lauzurica *et al.*, 1999). These facts are consistent with the hypothesis that PBN is an inhibitor of NF-κB. PDTC inhibits NF-κB more potently than PBN (approximately 100-fold), but the protective dose against rat sepsis is similar (*e.g.*, 100 mg/kg), suggesting that the pharmacologic mechanisms for these drugs are not the same.

NF-κB is called a redox-sensitive transcription factor because it was activated by pro-oxidant such as H_2O_2, and inhibited by antioxidants such as PDTC and NAC (Schreck *et al.*, 1991; Schreck *et al.*, 1992a). Initially NF-κB activation was speculated to occur through direct destruction of its endogenous inhibitor I-κB. However, later I-κB was shown to be phosphorylated and ubiquitinated before its proteolytic destruction (DiDonato *et al.*, 1997). A 900-kD kinase complex that can phosphorylate I-κB has been identified and named I-κB kinase (IKK) (Zandi *et al.*, 1997). In the rapidly developing studies on I-κB destruction pathways, the assumption that free radicals are directly involved in I-κB destruction seems to have lost its ground. Recently, one functional isoform of IKK, IKKβ has been shown to be inhibited by salicylate and aspirin, suggesting that this kinase is redox-sensitive (Yin *et al.*, 1998). This assumption is yet to be tested. It is speculated that kinases existing more upstream of IKK are redox-sensitive (Engelhardt, 1999). We speculate that free radicals are acting as signaling molecules to activate such kinases, and PBN deactivates them by trapping free radicals.

PATENTS

So far, 13 U.S. patents have been issued on the potential use of PBN and its analogs to treat various diseases in humans (Carney 1995; Carney and Floyd 1995a, b, 1997a, b; Carr *et al.*, 1995; Floyd and Carney, 1991; Janzen and Wilcox, 1995; Janzen *et al.*, 1996; Janzen and Zhang, 1997; Proctor, 1998; Ribier *et al.*, 1997). There has been no report on clinical trials of PBN.

SUMMARY

PBN is a drug whose chemical properties were initially characterized and then, on the basis of that knowledge, pharmacologic effects were discovered. Perhaps this explains why the presence of PBN's pharmacologic effects have been thought to be a proof that free radicals are involved in the initiation or development of the disease. Unfortunately, this may be the reason why very few mechanistic studies have been performed. Recent mechanistic studies seem to indicate that PBN's protective action is not solely based on PBN's trapping and detoxifying activity against damaging free radicals. Rather, PBN's anti-inflammatory action through inhibition of the induction of inflammatory factors and enzymes is more likely a major mechanism. The necessity of early-stage treatment appears to support this notion.

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of Education, Science, Sports, and Culture, Japan.

ABBREVIATIONS

ADC	AIDS dementia complex
AP-1	activator protein-1
CDAA	choline deficient, amino acid defined
CNS	central nervous system
COX2	cyclooxygenase 2
DHBA	dihydroxybenzoic acid
DMPO	5,5-dimethylpyrroline *N*-oxide
EMSA	electrophoretic mobility shift assay
EPR	electron paramagnetic resonance
GC/MS	gas chromatography/mass spectrometry
GD	gestational days
HIV-1	human immunodeficiency virus-1
HPLC	high-performance liquid chromatography
I/R	ischemia/reperfusion
i.p.	intraperitoneally
i.v.	intravenously
IFN-γ	interferon-γ
IKK I-κB	kinase
IL-1β	interleukin-1β
iNOS	inducible NO synthase
LDL	low density lipoprotein
LEC	Long-Evans Cinnamon
LPS	lipopolysaccharide
MCAO	middle cerebral artery occlusion
MDMA	3,4-methylenedioxy-methamphetamine
MPP^+	1-methyl-4-phenylpyridinium
MPTP	1-methyl-4-phenyl-1,2,3,6-tetramethylpyridine
MRI	magnetic resonance imaging
NF-κ	B nuclear factor κB
NO	nitric oxide
NOS	nitric oxide synthase
8OHdG	8-hydroxy deoxyguanosine
4OHPBN	4-hydroxyphenyl *N-tert*-butylnitrone
p38-MAPK	p38-mitogen activated protein kinase
PBN	phenyl *N-tert*-butylnitrone
PDTC	pyrrolidine dithiocarbamate
2-PhDMPO	2-phenyl-5,5-dimethylpyrroline *N*-oxide
4-POBN	4-pyridyl-*N*-oxide *N-tert*-butylnitrone
ROS	reactive oxygen species
RPA	ribonuclease protection assay
s.c.	subcutaneously
SAM	senescence-accelerated mouse
SOD	superoxide dismutase
2-SPBN	2-sulfoxyphenyl *N-tert*-butylnitrone
STZ	streptozotocin
2-SPBN	2-sulfoxyphenyl *N-tert*-butylnitrone
TNF-α	tumor necrosis factor-α

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