Nitrones as neuroprotective agents in cerebral ischemia, with particular reference to NXY-059

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Abstract

Stroke is a major clinical problem, and acute pharmacological intervention with neuroprotective agents has so far been unsuccessful. Recently, there has been considerable interest in the potential therapeutic benefit of nitrone-derived free radical trapping agents as neuroprotective agents. Nitrone compounds have been shown to be beneficial in animal models of various diseases, and the prototypic compound α-phenyl-N-tert-butylnitrone (PBN) has been extensively demonstrated to be neuroprotective in rat models of transient and permanent focal ischemia. The nitrone radical trapping agent disodium 2,4-disulfophenyl-N-tert-butylnitrone (NXY-059) has also been shown to be neuroprotective in these models. Furthermore, it has recently been shown to improve neurological function and reduce infarct volume in a primate model of permanent focal ischemia even when given 4 hr postocclusion. While radical trapping activity is demonstrable with NXY-059 and other nitrone compounds such as PBN, this activity is weak. Arguments for and against ascribing radical trapping as the therapeutic mechanism of action are discussed. This compound is well tolerated in human stroke patients and can be administered to produce plasma concentrations exceeding those effective in animal models; crucially, at the same time, it has also been shown to be effective in animal models. NXY-059 may thus be the first compound to be examined in stroke patients using drug exposure and time to treatment that have been shown to be effective in animal models of stroke.

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Keywords: Nitrones; PBN; NXY-059; Free radical trapping agents; Neuroprotection; Stroke

Abbreviations: 4-POBN, α-(4-pyridyl-1-oxide)-N-tert-butylnitrone; 5-HT, 5-hydroxytryptamine or serotonin; AR-R15896AR, S-(+)-α-phenyl-2-pyridine ethanamine dihydrochloride; ClCr, creatinine clearance; DMPO, 5,5-dimethyl-1-pyrroline N-oxide; ICH, intracerebral hemorrhage; MCA, middle cerebral artery; MPP+, 1-methyl-4-phenylpyridinium; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; MRI, magnetic resonance imaging; NIHSS, National Institutes of Health Stroke Scale; NMDA, N-methyl-D-aspartate; NXY-059, disodium 2,4-disulfophenyl-N-tert-butylnitrone; PBN, α-phenyl-N-tert-butylnitrone; SAE, serious adverse events; S-PBN, 2-sulfophenyl-N-tert-butylnitrone; STAIR, Stroke Therapy Academic Industry Roundtable; TBI, traumatic brain injury; TEMPO, 2,2,6,6-tetramethylpiperidine-N-oxyl; t-PA, tissue plasminogen activator.

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1. Introduction

1.1. Stroke and neuroprotective agents

Stroke is the third leading cause of death in major industrialized countries (Bonita, 1992), with an incidence of ~ 350 per 100,000 population aged 45–89 (Murray & Lopez, 1997). While the rate of stroke has fallen in the last 15 years, the absolute number has increased and will continue to rise due to the increasingly elderly population. Approximately 20% of stroke patients do not survive longer than 1 month, and a third of those who are alive after 6 months are dependent on others (Warlow, 1998). Stroke is therefore a major cause of long-lasting disability, and this has major repercussions not only for the survivor but also for the family and society as a whole.

We now have a reasonable understanding of the biochemical consequences of an acute ischemic stroke based on many years of detailed investigation in the brain of experimental animals subjected to an acute ischemic stroke and appropriate in vitro studies. The fact that the pathological consequences of stroke, in terms of both histology and functional outcome, appear to be similar in experimental animals and humans has led to the view that the biochemical mechanisms following the ischemic insult are likely to be similar in humans and animals. Consequently, substantial numbers of experimental drugs have been investigated over the last 20 years, which have been targeted at various parts of the “ischemic cascade” (i.e., the biochemical chain of events that is initiated by the cerebral ischemia) (Fig. 1). The hope has been that by attenuating or preventing some of the biochemical consequences of stroke, the neurological damage will be lessened; thus, the functional disabilities that occur will also be prevented or reduced. Compounds designed to interfere with the mechanisms of the neurodegenerative process have been named neuroprotective agents (or drugs). This identifies them as being in a separate category from the thrombolytic or “clot buster” drugs whose mechanism is that of restoring blood flow to the compromised region.

While it is probable that the cerebral tissue in the area immediately surrounding the infarct (the core) is probably damaged beyond recovery, the surrounding area (the penumbra), although with compromised perfusion, is probably capable of recovery, given the right conditions. Without appropriate treatment, however, this penumbral tissue will also become severely damaged (see, e.g., Snape et al., 1993) and thus presumably worsen the clinical outcome further.

To date, no neuroprotective approaches have been approved in the United States or Europe despite the success of a variety of compounds in animal models. Reasons for the failures to establish clinical efficacy have been the subject of a substantial number of reviews, including some questioning the value of animal models (De Keyser et al., 1999; Gladstone et al., 2002). There appears, however, to be opportunities to employ greater stringency in the use of animal models to aid the selection of new candidate neuroprotectants and guide clinical design. In many trials, particularly those investigating N-methyl-D-aspartate (NMDA) antagonists, adverse events prevented the administration of doses sufficient to produce plasma levels in patients that were neuroprotective in animal studies. A recent study has further demonstrated that some compounds that have been examined clinically even produced marked adverse events in rodents when
given at doses required to produce neuroprotection (Dawson et al., 2001).

Recent experimental studies, which we have conducted in rats, have demonstrated that the plasma levels that were adequate to protect in animal models of transient (or reperfusion) focal ischemia were still not sufficient to protect in a permanent focal ischemia model (Sydserff et al., 2002). Given the fact that many infarcts reperfuse slowly, if at all (Ringelstein et al., 1992), it is reasonable to assume that a drug must be given clinically at doses that are effective in rat permanent ischemia models if it is to be broadly effective in stroke.

Because of the many failures in clinical trials of putative neuroprotective agents, an academic industrial roundtable group (Stroke Therapy Academic Industry Roundtable, 1999) met and devised guidelines for drug development (the Stroke Therapy Academic Industry Roundtable [STAIR] criteria), which included selection criteria that should be met before a compound is progressed to clinical development (Stroke Therapy Academic Industry Roundtable, 1999). These criteria are outlined in Table 1. It can be seen that adequate dose-response data and use of permanent ischemic models to demonstrate efficacy are included. However, an additional criterion that is also listed is the use of time window studies and we would suggest that effective use of such data will be a major determinant for future clinical success. This therapeutic window of opportunity, specifically the time between the occurrence of the stroke and the time that treatment is initiated, has, until recently, often been assumed to differ in animals and humans. That is, there had been a view that damage develops more slowly in the cerebral tissue of humans and that a short time window in a rat model did not preclude giving the drug after a longer interval between stroke and drug administration in humans. A good example of this is the clinical investigation of NMDA antagonists. Despite substantial evidence for these compounds only providing protection when given shortly (60–90 min) after the ischemic insult (Fig. 2) (Massieu et al., 1993), they have nevertheless been administered to stroke patients up to 6 hr after the onset of the stroke (Davis et al., 2000). One may well ask why. The predominant reason is probably that of practicality because it is difficult to get patients to hospital and diagnosed within 90 min of stroke onset whereas 6 hr is a reasonable time frame for presentation and treatment. Indeed, the problems of carrying out a clinical trial with a short time window are substantial. However, the success of the tissue plasminogen activator (t-PA) trial with a 3-hr

Table 1
The STAIR criteria
Adequate dose-response plus serum concentrations measured, thereby defining minimally and maximally effective doses
Time window studies confirming efficacy
Physiological monitoring of animals undertaken
Randomized, blinded studies; reproducible effects (one independent)
Infarct volume measured and functional tests used, short-term and long-term assessment
Small rodent studied with permanent MCAO model; if only transient MCAO model used, then target reperfusion in clinic
Larger species used for novel, first-in-class compound
Studies published in peer-reviewed journal

Adapted from the criteria proposed in the paper of Stroke Therapy Academic Industry Roundtable (1999).
time window (NINDS t-PA Stroke Study Group, 1995) shows that such studies are possible. It is noteworthy that t-PA is also efficacious in animal stroke models in the same time frame (Brinker et al., 1999), which suggests that animals and humans may be similar in their time window of opportunity.

If we look at the simplified model of the neurodegenerative cascade (Fig. 1), it can be seen that most compounds investigated to date act on the early events (glutamate antagonists, ion channel compounds, clomethiazole, etc.). Consequently, one can speculate that these drugs must be given rapidly after the ischemic insult if they are to be of any value. If, as we believe, the time window of neurodegenerative events is similar in experimental animals and humans, then we have to use 1 of 2 approaches. (1) Administer the drug very soon after the stroke; an approach that is practically very difficult. (2) Develop a compound acting on a later part of the ischemic cascade that can be given in experimental animals at a time after the ischemic insult, which can be also achieved practically in clinical practice. Data from animal stroke models suggest that nitrone radical trapping compounds allow us to employ the second approach, as these compounds have a large therapeutic window of opportunity in experimental animals.

1.2. The nitrone radical trapping agents

The nitrone-derived free radical trapping agents were originally developed as tools for studying free radical chemistry and named spin traps. They allowed the indirect detection of short-lived free radical species, which, because of their reactivity, never accumulate in sufficient concentration to allow direct measurement (Janzen & Blackburn, 1968). The nitrone compound reacts with the free radical to form a compound called a spin adduct (Fig. 3). Once the adduct is formed, it is relatively stable and the radical thus becomes inactivated and unable to damage cellular tissues or biochemical processes.

Free radicals have been implicated in the pathology of a substantial number of disease processes and even normal aging. Any compound that interferes with free radical formation may therefore have wide utility. A variety of compounds have been developed (Fig. 4) and examined for their biological activity. Because this review focuses on cerebral ischemia, discussion of the studies of nitrone radical traps and other applications is out of place. However, the list shown in Table 2, which is adapted from that published by Hensley et al. (1997), gives a feel for the many pharmacological actions of nitrone-based...
Table 2 (continued)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Effects</th>
</tr>
</thead>
</table>
| S-PBN    | 1. Inhibited ROS generation and apoptosis in neurones cultured from fetal Down’s syndrome brain (Busciglio & Yankner, 1995)  
2. Mitigated MPTP-induced striatal dopamine depletion and neurotoxicity in Swiss-Webster mice (Schulz et al., 1995b)  
3. Attenuated malonate-induced neurotoxicity (Schulz et al., 1995a)  
4. Neuroprotective against ischemia/reperfusion peripheral nerve injury (Gray et al., 2003) |  
TMPO (MPO)  
1. Suppressed methylprednisolone-induced apoptosis of thymocytes (Slater et al., 1995a, 1995b) |

radical trapping compounds and their possible therapeutic applications.

2. α-Phenyl-N-tert-butylnitrone, 2-sulphophenyl-N-tert-butylnitrone, and neuroprotection

2.1. Effect of α-phenyl-N-tert-butylnitrone in global and focal ischemia models in rats

The first reports on the neuroprotective action of α-phenyl-N-tert-butylnitrone (PBN) in experimental models of stroke came in 1990 with publications from 2 unrelated laboratories. Oliver et al. (1990) reported on the protective efficacy of PBN in a gerbil reperfusion model, although damage was not measured by conventional histological techniques but rather by measurement of protein carbonyl derivatives and glutamine synthase activity. PBN (300 mg/kg) injected prior to ischemia attenuated the ischemia-induced rise in carbonyl derivatives and loss of glutamine synthase activity. In addition, Floyd (1990) reported that PBN administration decreased the rate of mortality following prolonged global ischemia in gerbils. Phillips and Clough-Helfman (1990) observed that PBN (100 mg/kg) both prevented the ischemia-induced rise in locomotor activity and significantly reduced damage to hippocampal pyramidal cell layers produced by global ischemia in Mongolian gerbils. Histological evidence for protection in this model was subsequently also reported by Yue et al. (1992).

In focal reperfusion models, PBN has also been shown to be effective, reducing the size of the infarct produced by occlusion of the middle cerebral artery (MCA) followed by reperfusion (Zhao et al., 1994; Schulz et al., 1997; Mori et al., 1998; Kuroda et al., 1999; Pazos et al., 1999; Li et al., 2001). Schulz et al. (1997) observed that PBN protected the striatum in addition to the cortex. Pazos et al. (1999) also demonstrated that the neuroprotective efficacy of PBN could be separated from the hypothermic effect that this drug can have in rodents.

There appears to have been only one study on the neuroprotective actions of PBN in permanent focal ischemia model using occlusion of the MCA. Cao and Phillips (1994) examined its activity in rats when administered at various
times following the start of a permanent MCA occlusion (MCAO) electrocoagulation. The investigation demonstrated the substantial neuroprotective effect of the compound even when given 12 hr after the start of the ischemic episode. A marked reduction in edema was also observed (Table 3).

### 2.2. Effect of α-phenyl-N-tert-butylnitrone and 2-sulfophenyl-N-tert-butylnitroxine in embolic stroke

Most studies on focal ischemia use models that occlude the MCA by use of either an intraluminal thread or electrocoagulation. However, recently, Yang et al. (2000) reported on the use of a model that involves injection of an autologous thrombus into the MCA, a model that is said to closely mimic the clinical situation in stroke.

This model was used to examine the effect of both PBN and 2-sulfophenyl-N-tert-butylnitroxine (S-PBN) in attenuating ischemic damage. Both compounds were given as a 100-mg/kg i.p. injection once daily for 3 days starting 2 hr after the introduction of the clot, and both produced a significant reduction in infarct size (Table 4).

A rather different approach to the utility of radical trapping compounds was employed by Ashai et al. (2000). This group examined the effect of PBN administration on the incidence of intracerebral hemorrhage (ICH) following t-PA. At present, thrombolysis therapy using t-PA is complicated by the risk of secondary ICH following thrombolysis (Wardlaw et al., 1997; Jean et al., 1998). The reperfusion that occurs may also be associated with both further damage and free radical production (Pahlmark & Siesjö, 1996; Facchinetti et al., 1998; Nakashima et al., 1999). Therefore, studies on the effect of t-PA combined with a radical trapping agent might appear to be a logical approach.

Ashai et al. (2000) introduced clot emboli to occlude the MCA in spontaneously hypertensive rats and observed high rates of cerebral hemorrhage 24 hr later when t-PA had been administered 6 hr postischemia. Infarction and neurological deficits were also worsened by t-PA. When PBN was combined with t-PA, the t-PA-induced hemorrhage volumes were reduced by 40%, with a reduction in infarction and neurological deficits also occurring. Parallel studies with Wistar-Kyoto rats failed to demonstrate similar negative effects of t-PA administration, which suggests that blood pressure is an important correlate of t-PA-induced hemorrhage. Crucially, the study suggests that PBN and other radical trapping agents might reduce the severity of t-PA-induced hemorrhage and brain injury following cerebral ischemia.

A somewhat related study was performed by Lapchak et al. (2001) using a rabbit model of focal embolic stroke. This group also observed a marked increase in hemorrhage rate if t-PA was administered 60 min after injection of blood clots into the MCA. They also observed that pretreatment with either PBN or another nitrone, 2,2,6,6-tetramethylpiperidine-N-oxyl (TEMPO), 5 min after the blood clot injection decreased the rate of hemorrhage. However, this group also reported that administration of PBN alone increased hemorrhage rate, an effect not seen with TEMPO. These data cannot unfortunately be fully compared with the rat study because Ashai et al. (2000) did not study the effect of PBN alone on the hemorrhage rate.

### 2.3. Effect of α-phenyl-N-tert-butylnitroxine in a model of hemorrhagic stroke

While the majority of strokes are thromboembolic, 10–15% of strokes in Western populations (Anderson et al., 1994; Gillum, 1995) and up to 30% in Oriental populations (Burchfiel et al., 1994; Lo et al., 1994) are due to ICH. Hemorrhagic transformation can also occur in a significant number of patients presenting with ischemic stroke (Lyden & Zivin, 1993).

It is known that iron compounds can markedly accelerate free radical processes by catalyzing the formation of hydroxyl radical compounds (Halliwell, 1992), and iron compounds such as hemoglobin and its degradation products are present in high concentration during ICH (Sadrzadeh et al., 1987). Iron compounds could therefore worsen the situation during ICH because free radicals are already being formed as a consequence of the ischemia.

Peeling et al. (1998) examined 2 free radical inhibitors, dimethylthiourea and the nitrone PBN, in a collagenease-induced model of ICH. Effects of drug treatment were examined by use of behavioral models, magnetic resonance imaging (MRI) and histopathology. Administration 2 hr after the ICH of either of the compounds resulted in an improved neurological deficit score. However, treatment did not reduce edema, resolution of the

---

**Table 3**

Cerebral infarct volume and brain edema following PBN administration

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>Infarct volume (mm³)</th>
<th>Brain edema (% change)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ischemic control</td>
<td>15</td>
<td>194.9 ± 9.2</td>
<td>6.8 ± 0.8</td>
</tr>
<tr>
<td>Ischemia + PBN (0.5 hr prior)</td>
<td>9</td>
<td>80.2 ± 17.9</td>
<td>3.0 ± 0.7²</td>
</tr>
<tr>
<td>Ischemia + PBN (0.5 hr post)</td>
<td>15</td>
<td>98.9 ± 18.1</td>
<td>3.3 ± 0.9²</td>
</tr>
<tr>
<td>Ischemia + PBN (5 hr post)</td>
<td>12</td>
<td>97.9 ± 29.9</td>
<td>2.2 ± 1.6²</td>
</tr>
<tr>
<td>Ischemia + PBN (12 hr post)</td>
<td>13</td>
<td>130.9 ± 22.8</td>
<td>2.7 ± 0.8²</td>
</tr>
</tbody>
</table>

Data taken from Cao and Phillis (1994).

² Different from ischemic control: P < 0.01.

² Different from ischemic control: P < 0.05.

---

**Table 4**

Effect of focal ischemia in rats on the volume of infarct and the effect of PBN and S-PBN

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Infarct volume (%)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>32.8 ± 9.4</td>
<td>–</td>
</tr>
<tr>
<td>PBN</td>
<td>21.2 ± 10.9</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>S-PBN</td>
<td>21.2 ± 13.1</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

Data taken from Yang et al. (2000).
hematoma, or neuronal damage in the tissue adjacent to the hemorrhage.

2.4. Effect of α-phenyl-N-tert-butyl-nitrone and 2-sulfophenyl-N-tert-butyl-nitrone on traumatic brain injury

Li et al. (1997) examined the effect of PBN on compression injury to rat spinal cord (the compound being given both before and subsequent to the injury) but found no protective effect in either functional tests or histological outcome measures. However, relatively low doses of the drug were given in comparison with those required in cerebral ischemia models and this could have influenced the outcome.

In contrast, in a model of traumatic brain injury (TBI), both PBN and S-PBN have been demonstrated to reduce the loss of ipsilateral hemispheric tissue when the nitrones were administered 30 min after a fluid percussion injury (Marklund et al., 2001a). Interestingly, both compounds attenuated increased free radical production although S-PBN, in contrast to PBN, could not be detected in cerebral tissue. The authors concluded that a major site of free radical production was in TBI at the blood-endothelial interface (Marklund et al., 2001b).

2.5. Effect of α-phenyl-N-tert-butyl-nitrone and 2-sulfophenyl-N-tert-butyl-nitrone on neurotoxin-induced damage to cerebral tissue

A variety of neurotoxins are considered to induce damage through free radical-mediated events, and it is not surprising therefore that nitrones have been extensively investigated for their effects in neurotoxin-induced damage. A full review of this area is outside the brief of this article. However, given the association between cerebral ischemia and excitotoxicity, the effects of PBN and S-PBN in some studies on neurotoxin-induced cerebral damage will be mentioned.

Two studies by Schulz et al. (1995a, 1995b) have demonstrated the efficacy of S-PBN against neurotoxin-induced damage in mice. In one study, a comparison was made of the protective properties of dizocilpine, lamotrigine, and S-PBN against damage produced by the mitochondrial toxin malonate (Schulz et al., 1995a). While dizocilpine and lamotrigine were only effective when given up to 1 hr following the malonate injection, S-PBN was effective up to 6 hr later, supporting the view that nitrones have a large window of opportunity. In the second study, the same group showed that PBN and S-PBN administration attenuated malonate, 1-methyl-4-phenylpyridinium (MPP+), and NMDA-induced damage and also lessened the malonate-induced rise in free radical production in the rat brain (Schulz et al., 1995b). Available evidence from other models points to S-PBN having poor brain penetration (Marklund et al., 2001b; Dehouck et al., 2002), which raises questions as to why it appears to be acting as a radical trapping agent within the brain. One explanation may be that the neurotoxins were injected via a probe, thereby damaging the blood-brain barrier. The mechanism of action of nitrone compounds is discussed further in Section 5.

The same laboratory has also studied the effect of S-PBN on the dopamine neurotoxin MPP+ in the rat and again found a protective effect of the nitrone compound (Fallon et al., 1997). Because MPP+ is injected via intracerebral injection, this also might be expected to damage the blood-brain barrier.

Guidetti and Schwarz (1999) examined whether PBN would protect against striatal damage produced by 1,3-dihydroxykynurenic acid, which is a metabolite of tryptophan and is known to be a generator of free radicals. S-PBN was found to be an effective neuroprotectant when this neurotoxin was injected directly into the cerebral tissue.

One neurotoxic compound that has been shown to produce free radicals in cerebral tissue when injected systemically is the recreationally used amphetamine derivative 3,4-methylenedioxyxymethamphetamine (MDMA or ecstasy). This compound has been found to produce free radicals in the striatum and also produce a subsequent degeneration of hydroxytryptamine or serotonin (5-HT) nerve endings (Colado et al., 1997). PBN administration attenuated the free radical production (Colado et al., 1997) and markedly decreased the degree of damage to 5-HT nerve endings as measured by both binding of [3H]-paroxetine to 5-HT nerve endings and loss of tissue 5-HT content (Colado & Green, 1995; Colado et al., 1997; Yeh, 1999).

3. MDL 101,002, 2,2,6,6-tetramethylpiperidine-N-oxyl, and neuroprotection

While there have been a variety of nitrone-derived spin traps synthesized (Fig. 4), published data on the biological activity of these compounds are scarce with the exception of PBN. Of the other compounds examined, probably the most investigated is the cyclic nitrone MDL 101,002, which is one of a series of cyclic nitrone spin traps that have been synthesized (Thomas et al., 1996). All the cyclic compounds examined in this study were more potent as in vitro inhibitors of lipid peroxidation than PBN, with the unsubstituted variant MDL 101,002 being ~ 8-fold more potent than PBN. In general, the potency correlated with lipophilicity (Craig et al., 1997; Thomas et al., 1997).

MDL 101,002 has been found to be neuroprotective in both transient and focal ischemia models in rats. In a reperfusion model, administration of the compound at the start of the reperfusion period following a 3-hr occlusion provided a dose-dependent neuroprotection against ischemic damage of up to 70% (Johnson et al., 1998), and protection of up to 90% was observed in a mixed permanent/transient distal MCAO model. A reduction in damage of 40% was also observed in a permanent MCAO model when the drug
was given 30 min after the start of the occlusion (Johnson et al., 1998).

MDL 101,002 is also efficacious in a rat embolic stroke model when administered 5 min after embolization, both decreasing the volume of ICH and improving the behavioral deficit score related to vehicle-treated animals (Hu et al., 1999).

Finally, administration of MDL 101,002 has been found to produce dose-dependent protection against malonate-induced striatal lesions and also significant protection against the depletion of dopamine and its metabolite induced by the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Mathews et al., 1999).

Some other compounds have been studied in models of cardiac ischemia and therefore will not be discussed here. However, TEMPO was studied in a rabbit model of embolic stroke and is discussed in Section 2.2.

4. Disodium 2,4-disulfophenyl-N-tert-butyl nitrone and animal models of stroke

4.1. Effect of disodium 2,4-disulfophenyl-N-tert-butyl nitroine in transient focal ischemia models in rats

The first report of the neuroprotective effect of disodium 2,4-disulfophenyl-N-tert-butyl nitroine (NXY-059) in a rat model of transient focal ischemia was that of Kuroda et al. (1999) who compared its effect with PBN. The model used the insertion of an intraluminal filament to occlude the MCA for 2 hr. The first experiments established the dose-response relationship of NXY-059 in this stroke model. NXY-059 was administered 1 hr following a 2-hr MCAO. Rats were injected with a loading dose of NXY-059 (0.3, 3.0, or 30 mg/kg, respectively) followed immediately by a 24-hr i.v. infusion (0.3, 3.0, or 30 mg/kg/hr). NXY-059 produced dose-dependent neuroprotection (Fig. 5) when damage was assessed histologically following 48-hr reperfusion. NXY-059 (3.0 mg/kg/hr) was markedly more effective than PBN given at an equimolar infusion dose (bolus: 1.4 mg/kg and 1.4 mg/kg/hr) because this dose of PBN was not neuroprotective (Fig. 5). NXY-059-treated rats also displayed fewer neurological deficits than the control or PBN-treated rats both 24 and 48 hr after reperfusion.

The main findings of the Kuroda et al. (1999) study were supported by the observations of Sydserff et al. (2002). This group administered doses of NXY-059 of 3, 10, or 30 mg/kg/hr by i.v. infusion starting 5 min after the end of the 2-hr occlusion. No loading dose was given. The dose-dependent degree of neuroprotection reported by Sydserff et al. was similar to that observed by Kuroda et al. (Fig. 6). The increased efficacy seen by Kuroda et al. at low doses of NXY-059 may have been due to the initial loading dose used in that study and which was not used by Sydserff et al. The data on plasma concentrations presented in Fig. 6 have all been extrapolated from dose-plasma concentration values obtained in the Sydserff et al. study and may thus be slightly in error in the Kuroda et al. projections.

The Kuroda et al. (1999) study also included investigation into the therapeutic time window in the transient MCAO model. NXY-059 provided effective neuroprotection when administered 5 hr after MCAO (3 hr after the start of reperfusion) and the protective effect was almost significant 8 hr after occlusion. Finally, this group also made a brief study of the duration of protection afforded by NXY-059 following the ischemic insult. Similar protection was observed when outcome was measured either 2 or 7 days after the ischemic episode, indicating that damage was probably being prevented rather than the drug slowing the development of damage.

4.2. Effect of disodium 2,4-disulfophenyl-N-tert-butyl nitroine in permanent focal ischemia models in rats

A major study on the effect of NXY-059 in the permanent MCAO model has recently been reported (Syd-
Doses of 30, 50, and 70 mg/kg/hr for 24 hr were administered by s.c. osmotic minipumps circumventing the problem of keeping an indwelling i.v. line patent for 24 hr, although it increased the problem of delivering the drug reliably from the moment of implantation. As before, an appropriate loading dose (30, 50, or 70 mg/kg, respectively) was given at the start of the treatment period. NXY-059 produced a dose-dependent neuroprotection in permanent MCAO. Protection was demonstrated not only when measured as a total volume of damage but also when measured in terms of cortical and subcortical damage (Fig. 7).

The plasma concentration at 24 hr appeared to be linearly related to dose, and results suggested a linear relationship between dose and neuroprotection (Fig. 7). Extrapolation of these values (given the linear relationship between dose and plasma concentration) suggested that around 80% neuroprotection could be achieved when the plasma “free” concentration was around 150 μmol/L at 24 hr of infusion (Fig. 8) (Sydserff et al., 2002).

The final experiment in the study examined the therapeutic window of opportunity in rats given NXY-059 (50 mg/kg/hr for 24 hr). Treatment was initiated up to 4 hr after the start of the permanent MCAO and histological measurement of damage performed at the end of the infusion period. Substantial neuroprotection was observed up to 4 hr postocclusion (Fig. 9). In a further experiment, measurement was made 6 hr postocclusion when a 22% decrease in infarct size was observed, although this failed to reach statistical significance ($P = 0.23$).

Zhao et al. (2001), using a rather different model of permanent focal ischemia, also demonstrated that NXY-059 produced dose-dependent neuroprotection. The method used involved placement of an aneurysm clip on the MCA of spontaneously hypertensive rats. Five minutes postocclusion, rats were infused with 30 or 60 mg/kg/hr of NXY-059 for 24 hr. Infarct volume was measured by microtubule-associated protein-2 and hematoxylin and eosin staining (which gave comparable results). A modest decrease in

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**Fig. 7.** The relationship between the plasma free concentration of NXY-059 and the degree of neuroprotection in a rat model of permanent focal ischemia. Data taken and recalculated from Sydserff et al. (2002).

**Fig. 8.** The relationship between the plasma free concentration of NXY-059 and the degree of neuroprotection in a rat model of permanent focal ischemia. Data taken and recalculated from Sydserff et al. (2002).
cortical damage was seen with 30 mg/kg/hr and a significant protection at a dose of 60 mg/kg/hr. Administration of NXY-059 in this study produced no significant changes in the physiological variables measured (mean arterial blood pressure, pCO₂, pO₂, pH, glucose, and hematocrit) compared with values in saline-injected animals, confirming the lack of significant action of NXY-059 on major physiological parameters previously reported by Kuroda et al. (1999) and detailed in Section 4.1.

4.3. Effect of disodium 2,4-disulfophenyl-N-tert-butylnitrone in permanent focal ischemia in marmosets

Over the last few years, a primate model of acute ischemic stroke has been developed in Cambridge University, which allows not only histological assessment of damage but also measurement of functional impairment (Marshall & Ridley, 1996; Marshall et al., 1999). The value of the model lies in the fact that it reproduces many of the clinical features of human stroke. The problems of motor neglect, perceptual spatial neglect, and extinction deficits are separately quantified during a 10-week period following the permanent focal ischemia. Finally, the volume of cerebral ischemic damage can be assessed histologically (Marshall et al., 1999, 2000a, 2000b). Two studies have now been made on the efficacy of NXY-059 in this model.

The first study was designed to determine whether NXY-059 was an effective neuroprotective agent (Marshall et al., 2001). Accordingly, the drug was given only 5 min after the onset of ischemia. The dose given was designed to produce a plasma level that at that time been shown to be well tolerated in stroke patients in a phase II study (Lees et al., 2001a), and the drug was given by loading dose followed by osmotic minipump implantation starting 5 min after the start of the ischemic episode and treatment continued for 48 hr. This produced a plasma free drug concentration of 76 ± 6 µmol/L in monkeys.

NXY-059 administration produced no overt behavioral effects in the animals nor were there significant change in physiological parameters such as blood pressure, pO₂, and pCO₂.

Three weeks after the MCAO, the stroked animals were unable to reach with the contralesional arm into the contralesional space. No improvement was seen in the use of the arm 10 weeks later. This major motor deficit was substantially lessened by NXY-059 administration, the drug-treated animals being able to use this arm at around 50% of normal function at both 3 and 10 weeks. This demonstrates clearly that the improvement seen shortly after the infarct in the drug-treated animals is due to neuroprotection and not merely a slowing in the appearance of damage.

With regard to spatial hemineglect, this deficit, which can be a major clinical problem in stroke patients (Robertson et al., 1993), was marked in the stroked animals at 3 weeks. Like its human clinical equivalent, the deficit was much less apparent at 10 weeks. However, in the NXY-059-treated marmosets, there was considerably less neglect at 3 weeks than in the control animals, and at 10 weeks, no residual deficit was observed.

When brain damage was examined histologically (at 10 weeks following the final functional tests), a substantial volume of damage was observed in the stroked animals, which was reduced by more than 50% in the NXY-059-treated animals (Fig. 10).

In a second study (Marshall et al., 2003a), an investigation was made into the time window of opportunity for NXY-059 in marmosets. The time chosen was 4 hr post-occlusion, a clinically relevant time point, and the dose chosen was higher because a recent clinical study had demonstrated that plasma free drug concentrations of 260 µmol/L were well tolerated by stroke patients (Lees et al., 2003; see later). The study was conducted with almost the same protocol as the first study reviewed above but the changes in time of administration and dose as detailed
above. A plasma level of 200 ± 9 μmol/L was achieved in this second study, and this was well tolerated by the monkeys. Spatial neglect was again a major problem in the control group at 3 weeks and this problem was markedly attenuated by NXY-059 administration at 3 weeks and absent in the drug-treated animals at 10 weeks.

The degree of motor impairment in the control animals was almost total at both 3 and 10 weeks, replicating the data seen in control animals in the first study. NXY-059 administration at 4 hr resulted in a significant use of the paretic arm at both 3 and 10 weeks as measured by the tests used in the previous study and also when assessed by the use of a new measure that evaluated attempts by the animals to obtain the food reward (Fig. 11).

Finally, histological studies found that the NXY-059-treated animals had a 28% smaller infarct than the control saline-treated animals with protection seen in cortical areas, subcortical areas, and white matter (Fig. 12).

What is particularly striking in these studies is the time window. Not only does this primate study confirm that NXY-059 has a therapeutic window of opportunity of at least 4 hr as had been indicated in earlier studies in rats (Kuroda et al., 1999; Sydserff et al., 2002) but it also emphasizes the difference in this window when compared with other neuroprotective agents. Both clomethiazole (Marshall et al., 2000a) and the low-affinity NMDA antagonist S-(+)-α-phenyl-2-pyridine ethanamine dihydrochloride (AR-R15896AR) (Marshall et al., 2000b) have been examined in the same model using doses designed to produce plasma levels that were relevant for clinical studies. Neither clomethiazole, given 1 hr after the start of the ischemia, nor AR-R15896AR, given only 5 min after the start of ischemia, improved the motor deficit induced by the insult. In contrast, NXY-059 produced a clear improvement when given 4 hr later (Marshall et al., 2003b).

4.4. Disodium 2,4-disulfophenyl-N-tert-butylnitrone and models of embolic stroke

Two studies have examined the effect of NXY-059 in rabbit embolic stroke models. One involved the injection of small clots (Lapchak et al., 2002a) and the other involved injecting large clots (Lapchak et al., 2002b). In both studies, NXY-059 was synthesized locally in the university of the investigating scientists, and purity values were not reported.
In the small clot model, rabbits were injected with small clots intraarterially, and neurological function was examined 2 and 24 hr later. Treatment consisted of i.v. administration over 30 min of NXY-059 (100 mg/kg). NXY-059 administration resulted in a significant improvement in the embolism-induced neurological deficit (measured by an increase in the number of emboli required to produce the same deficit) when administered 5 min after the insult and a near statistical improvement when given 3 hr later (Table 5). Administration of t-PA also produced an improvement in the neurological deficit score when given 60 min but not 3 hr after the insult (Table 5). When NXY-059 was administered in combination with t-PA, the efficacy was greater than either compound alone (Table 5).

It is difficult to extrapolate these data to clinical conditions because t-PA is normally likely to be administered prior to NXY-059, and the NXY-059 will be given as a prolonged infusion (see later). Nevertheless, the data do demonstrate the efficacy of NXY-059 as a neuroprotective agent in an embolic stroke model.

The second study with NXY-059 examined the effect of the compound in a large clot model. This model has been used by the investigating group in several studies to examine the effect of combining putative neuroprotective agents with t-PA on the incidence of production of ICH. The group had previously reported that PBN significantly increased the hemorrhage rate (Lapchak et al., 2001) but, in combination with t-PA, paradoxically decreased the hemorrhage rate seen with t-PA alone (see Section 2.2). The study with NXY-059 reported a numerical, but not statistically significant, increase in hemorrhage rate following administration of this nitrone compound. A statistically significant lowering of hemorrhage rate was reported when NXY-059 was given in combination with t-PA compared with the hemorrhage rate produced by t-PA administration alone.

4.5. Effect of disodium 2,4-disulfophenyl-N-tert-butylnitrone in a rat model of hemorrhagic stroke

An ICH can be produced in the rat brain by intracerebral infusion of collagenase and its progression can be monitored by MRI. Subsequent neurological deficits in the animal can also be quantified.

PBN administration provided long-term functional improvement in this model even when treatment was initiated 2 hr after the hemorrhage (Section 2.3) (Peeling et al., 1998). In a subsequent study, the same group examined the consequences of administering NXY-059 on the outcome following ICH in rats (Peeling et al., 2001). NXY-059 was administered as a s.c. loading dose followed by implantation of osmotic minipumps, the dosing schedule being designed to achieve a steady-state plasma concentration of around 150 μmol/L for 72 hr. Treatment was initiated 30 min after the ICH. MRI was performed 24 hr after the ICH and again 7 and 42 days later. The initial imaging confirmed that the initial hemorrhage was similar in size in both control and NXY-059-treated groups. The later MRI results showed that the hematoma resolved similarly in both groups.

Behavioral testing was performed 1, 3, 7, 14, and 21 days after the ICH. This showed that the total neurological deficit score comprising assessment of beam walking, circling, and posture reflex tests was consistently lower at all times examined in the animals treated with NXY-059 compared with controls. More complex behaviors (food pellets retrieved by the contralateral paw and rotameter test) were not improved by NXY-059 administration.

Neutrophil infiltration into the region of the hematoma was significantly less 2 days after the ICH in the NXY-059-treated rats.

Table 5

<table>
<thead>
<tr>
<th>Treatment</th>
<th>ES_{50} (mg)</th>
<th>% Increase</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.04 ± 0.31</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NXY-059 (5 min)</td>
<td>2.54 ± 0.22</td>
<td>144</td>
<td>0.0313</td>
</tr>
<tr>
<td>NXY-059 (180 min)</td>
<td>2.01 ± 0.35</td>
<td>93</td>
<td>0.0586</td>
</tr>
<tr>
<td>t-PA (60 min)</td>
<td>2.64 ± 0.15</td>
<td>125</td>
<td>0.0221</td>
</tr>
<tr>
<td>t-PA (180 min)</td>
<td>0.63 ± 0.35</td>
<td>4172</td>
<td></td>
</tr>
<tr>
<td>NXY-059 (5 min) + t-PA (60 min)</td>
<td>3.15 ± 0.50</td>
<td>202</td>
<td>0.0015</td>
</tr>
<tr>
<td>NXY-059 (5 min) + t-PA (180 min)</td>
<td>2.66 ± 0.82</td>
<td>155</td>
<td>0.0375</td>
</tr>
</tbody>
</table>

Results calculated as the amount (mg) of clots required to produce neurological impairment in 50% of rabbits (ES_{50}). Data taken from Lapchak et al. (2002a).
5. Neuroprotective mechanism of action of nitrone compounds

Nitrones are generally assumed to produce their neuroprotective effects by trapping free radicals that are known to be involved in the production of cell death after cerebral ischemia (Chan, 2001). PBN was shown to be a free radical trapping reagent and antioxidant several years ago (Janzen & Blackburn, 1968; Janzen et al., 1994). It is plausible that many of the pharmacological effects of PBN (Table 2) are secondary to its radical trapping properties, although other mechanisms of action are possible. However, no specific target such as a receptor, ion channel, or enzyme has yet been identified. It has been suggested that PBN may modulate various intracellular systems sensitive to oxidative stress rather than simply trap free radicals before they directly damage proteins, lipids, and DNA (Hensley et al., 1997; Chan, 2001). A variety of biochemical changes have been observed to occur following nitrone administration in vivo or addition of the compound in vitro (Table 6). However, how many of these are, in some way, associated with a radical trapping action is unknown and some changes may relate to a direct action of the nitrone on the mechanism under study.

Recent work has compared the radical trapping ability of PBN with S-PBN and NXY-059 (Maples et al., 2001). All 3 compounds trapped carbon and oxygen centered radicals and could prevent the oxidation of salicylate in vitro. However, PBN was not particularly potent as an antioxidant and could prevent the oxidation of salicylate in vitro. NXY-059 was the least effective.

A number of studies have shown an increased production of free radicals during 1–6 hr of reperfusion following transient focal ischemia (Matsuo et al., 1995; Morimoto et al., 1996; Solenski et al., 1997) but only one study has tested the effect of nitrones on this. Gido et al. (2000) measured hydroxyl radical production by oxidation of 4-hydroxybenzoic acid to 3,4-dihydroxybenzoic acid (3,4-DHBA) using brain microdialysis following transient (2 hr) MCAO in the rat. There was a 3-fold increase in 3,4-DHBA during reperfusion but no effect of PBN when it was given either prior to ischemia or immediately before reperfusion. In contrast, Marklund et al. (2001b) have shown that PBN reduced radical production in a model of TBI. There are little published data showing an increased production of free radicals during permanent focal ischemia. Peters et al. (1998) followed radical production in the infarct border during 3 hr of ischemia and observed a modest 1.6-fold increase in contrast to the 4.9-fold increase seen during reperfusion following 1 hr of ischemia.

PBN readily crosses the blood-brain barrier and cell membranes (Chen et al., 1990; Cheng et al., 1993; Dehouck et al., 2002) so it could exert its effects intracellularly and extracellularly in the brain or at the level of the blood-endothelial interface. In contrast, both NXY-059 and S-PBN have negatively charged sulfo groups and blood-brain barrier permeability, and cerebral endothelial uptake has been shown to be low under normoxic conditions in an in vitro model of the blood-brain barrier (Dehouck et al., 2002). Permeability was increased 3.5-fold after 3 hr of ischemia, suggesting that some brain penetration might be predicted to occur in vivo under these conditions. This would be consistent with observations of a breakdown of the blood-brain barrier starting 3–4 hr after onset of 2-hr MCAO (Belayev et al., 1996). However, brain uptake of NXY-059 in animals subjected to transient (2 hr) MCAO was low 1 and 4 hr after the start of reperfusion (Kuroda et al., 1999) and S-PBN did not penetrate the brain in a model of TBI (Marklund et al., 2001b). In addition, the Dehouck et al. (2002) study demonstrated that, in contrast to PBN, the cell penetration of both NXY-059 and S-PBN was negligible.

Table 6

<table>
<thead>
<tr>
<th>Neuroprotective effects</th>
<th>In vivo or ex vivo</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inhibition of iNOS induction in HIV-1 envelope</td>
<td>(Tabatobaie et al., 1996)</td>
</tr>
<tr>
<td>Protection of primary cerebellar neurones from glutamate toxicity</td>
<td>(Yue et al., 1992)</td>
</tr>
<tr>
<td>Suppression of caspase-3 activation following global ischemia</td>
<td>(Li et al., 2001)</td>
</tr>
<tr>
<td>Inhibition of endotoxin-induced induction of nitric oxide synthase</td>
<td>(Miyajima &amp; Kotake, 1995)</td>
</tr>
<tr>
<td>Improved rate of metabolic recovery, acidosis rebound, and ATP renewal in rat brain following transient focal ischemic injury</td>
<td>(Folbergrova et al., 1995)</td>
</tr>
<tr>
<td>Suppressed c-fos expression in postischemic gerbil brain</td>
<td>(Carney et al., 1994)</td>
</tr>
<tr>
<td>Attenuated secondary mitochondrial dysfunction after transient focal ischemia</td>
<td>(Kuroda et al., 1996)</td>
</tr>
<tr>
<td>Reduced number of positive τ-oligodendrocytes after focal ischemia</td>
<td>(Irving et al., 1997)</td>
</tr>
<tr>
<td>Prevention of cytotoxic ischemia following malonate</td>
<td>(Asanuma et al., 2002)</td>
</tr>
<tr>
<td>NXY-059</td>
<td></td>
</tr>
<tr>
<td>In vitro</td>
<td></td>
</tr>
<tr>
<td>Ex vivo</td>
<td></td>
</tr>
<tr>
<td>1. Inhibition of Akt activation after focal ischemia</td>
<td>(Yoshimoto et al., 2002)</td>
</tr>
<tr>
<td>2. Inhibited release of cytochrome c after focal ischemia</td>
<td>(Yoshimoto et al., 2002b)</td>
</tr>
</tbody>
</table>
Concentrations of NXY-059 in the blood are relatively high following neuroproprotective doses in both rats and primates (30–200 μmol/L), and Kuroda et al. (1999) have suggested that its neuroprotective effects in transient focal ischemia are due to trapping of extracellular free radicals produced either directly from endothelial cells and inflammatory cells such as circulating polymorphonuclear leukocytes and macrophages or following interactions between them (Betz, 1996; Hallenbeck, 1996). The free radicals would normally cause local damage to the endothelium and this could lead to blood-brain barrier breakdown (Tasdemiroglu et al., 1994) that could be prevented by nitrones. Indeed, high concentrations of PBN have been shown to reduce the enhancement in paraendothelial permeability and endothelial cell damage produced by H2O2-induced oxidative stress (Blasing et al., 2002) although no data are available with NXY-059. The free radicals would also be expected to cross the blood-brain barrier, producing damage to brain tissue (Matsu et al., 1995), so a trapping of the radicals in the blood would also prevent their damaging effects on neurones. Drugs that interfere with the interaction between neutrophils and the endothelium are neuroprotective in animal models of transient ischemia but not in models of permanent ischemia (Clark et al., 1991; Zhang et al., 1995; Jiang et al., 1998; Prestigiacomo et al., 1999). This may be because the inflammatory response occurs later and plays only a minor role relative to the effect of a permanent reduction in blood flow (Prieto et al., 1988; Zhang et al., 1994). However, NXY-059 also has powerful neuroprotective effects in rat and primate models of permanent ischemia, suggesting that another neuroprotective mechanism or site of action may be more important in permanent ischemia.

Kastrup et al. (1999) have shown that the blood-brain barrier does break down during permanent ischemia but this does not begin until around 4 hr after onset. There are no data available on the brain uptake of NXY-059 under these conditions. If NXY-059 does enter the brain during the first few hours of permanent or transient ischemia, the question arises as to whether sufficient concentrations are reached to trap free radicals or if it acts on a target requiring low (nM) concentrations of the drug for activation. No effects were found when NXY-059 was tested on a wide range of different receptor binding or modulatory sites, neurotransmitter reuptake sites, and various second messenger assays (AstraZeneca data on file). Nevertheless, the recent papers of Yoshimoto et al. (2002a, 2000b) are intriguing because they report that when NXY-059 was administered to rats subjected to a focal ischemic insult, it attenuated some ischemia-induced changes at the level of the mitochondrion such as cytochrome c release. It also maintained Akt activation. However, it remains unclear as to whether such changes occur as the result of free radical trapping or because of some more direct action at a cellular level.

6. Clinical studies with disodium 2,4-disulfophenyl-N-tert-butylnitronate

6.1. Studies conducted with disodium 2,4-disulfophenyl-N-tert-butylnitronate in acute stroke patients

Two randomized double-blind placebo-controlled studies have been performed with the primary objective to evaluate safety and tolerability of NXY-059 in subjects with acute stroke. The 2 doses chosen for the first study (Lees et al., 2001a) aimed to achieve exposures shown to be neuroprotective in transient MCAO models in the rat. The results of this study, coded SA-NXY-0003, did not indicate any dose-limiting adverse events and concurrent information suggested (as shown in Fig. 8) that higher exposure levels provided greater neuroprotective effects in the permanent MCAO model in the rat than those attained in SA-NXY-0003. A second study (Lees et al., 2003) was therefore designed to examine the safety and tolerability of NXY-059 at higher doses. This study was coded SA-NXY-0004.

The study drug was given in both studies as a 1-hr loading dose (at 3 times the maintenance dose) followed by 71 hr at a maintenance dose, which was adjusted for patients with impaired renal function as estimated by creatinine clearance (CLCR) because the clearance of NXY-059 is dependent on renal function (Edenius et al., 2002; Strid et al., 2002). Plasma clearance of NXY-059 was in fact shown by Strid et al. (2002) to be directly proportional to glomerular filtration rate without any apparent contribution by nonrenal clearance. The half-life of NXY-059 was accordingly increased from ~2–4 to 10–12 hr in subjects with moderate and severe renal impairment (Strid et al., 2002). The fraction unbound of NXY-059 in these studies with pharmacokinetic objectives in healthy young and elderly as well as renally impaired subjects was ~0.6. For both studies, in stroke subjects, the study drug had to be initiated within 24 hr of the onset of symptoms of acute stroke and the follow-up period was 30 days, and the mean time to onset of symptoms ranged from 14 to 15 hr.

The SA-NXY-0003 study was performed in 15 centers in UK and Sweden enrolling 150 subjects (Lees et al., 2001a). Fifty-one subjects were randomized to placebo and 49 and 50 subjects to 170 or 85 mg/hr, respectively, maintenance doses of NXY-059. Ten percent of the subjects had a baseline diagnosis of hemorrhagic stroke and the rest were diagnosed as ischemic stroke. The groups were fairly well matched with respect to age and severity of stroke symptoms, but a disproportionate number (8 of 15) of the subjects with intracerebral hemorrhagic stroke were randomized to the 85-mg/hr group. The mean ± SD stroke severity as measured by National Institutes of Health Stroke Scale (NIHSS) score was 8.6 ± 6.4, 7.3 ± 5.4, and 7.8 ± 6.6 in the 170 mg/hr, 85 mg/hr, and the placebo group, respectively. Premature treatment termination occurred in 14% of the subjects in the placebo group and 17% and 16% in the 85 and 170 mg/hr, respectively, dose groups of NXY-
A total of 6 serious adverse events (SAE) were seen by the investigators as possibly causally related to the study drug, and the number of such reports was similar in the 3 treatment groups. Five of the 7 deaths occurred in the 85-mg/hr dose group, which reflected the disproportionate number of subjects in that group with an intracerebral hemorrhagic stroke, a condition with a well-known poor prognosis (Härdemark et al., 1999). Neither the incidence nor the causes of death suggested any association with the NXY-059 treatment.

The SA-NXY-0004 study was performed in 11 centers in UK, Sweden, and Germany and enrolled 135 subjects with symptoms of acute stroke and a CT scan result compatible with a diagnosis of acute ischemic stroke (Lees et al., 2003). Subjects were randomized 2:1 to NXY-059 versus placebo, 48 subjects were randomized to placebo and 39 and 48 subjects to 844 and 420 mg/hr, respectively, maintenance doses of NXY-059. An independent safety review committee assessed the outcome of the first 60 subjects randomized to NXY-059 (420 mg/hr) or placebo prior to proceeding to randomization to NXY-059 (844 mg/hr) or placebo.

The mean ± SD NIHSS scores were 6.9 ± 5.7, 9.9 ± 7.0, and 8.4 ± 6.8 in the 844 mg/hr, 420 mg/hr, and the placebo group, respectively. The difference in severity between the treatment groups was caused by a lower average severity in the subjects at the German sites, which just participated in the later part of the study. The analyses of safety and tolerability and stroke recovery were performed both on all subjects and separately within the 2 randomization strata (based on stroke severity on the NIHSS and age) to reduce the potential bias of the different baseline stroke severities in the treatment groups.

Premature treatment termination occurred in 6% of the subjects in the placebo group and 0% and 2% in the 844 and 420 mg/hr, respectively, dose groups of NXY-059. The subjects in the 844-mg/hr NXY-059 dose group experienced fewer adverse events, SAE, and deaths as compared with the NXY-059 low dose and the placebo group. Seven deaths occurred in the study, 4 in the 420-mg/hr dose group of NXY-059 and 3 in the placebo group.

### 6.2. Integrated safety analysis, exposure relative to neuro-protective concentrations in stroke models, and stroke outcome

NXY-059 appeared to be well tolerated in both studies with no increases in total adverse events, any specific type of adverse event, or treatment withdrawals in the subjects treated with NXY-059. Subsequent integrated analyses were performed across the 2 studies, and no effects of the NXY-059 treatment were seen on laboratory parameters. Overall, the most frequent adverse events in the combined NXY-059 treatment groups were headache, fever, and hyperglycemia. For the combined placebo groups, the most frequent adverse events were headache, fever, and hypertension. The incidences of common types of adverse events were similar between the combined NXY-059 treatment and the placebo groups (Table 7). The events noted in both treatment groups are commonly encountered in patients with acute stroke, as a consequence of either the stroke itself or the concomitant diseases (e.g., diabetes mellitus or pneumonia).

The unbound NXY-059 concentration achieved in the highest dose group of NXY-059 (844 mg/hr) was 237 ± 46 μmol/L at 1 hr after start of treatment and the concentration at steady state was 260 ± 79 μmol/L. The attained exposures in stroke patients were hence ~100- and 5-fold greater, respectively, than the exposure required for neuro-protection in the transient and permanent MCAO model in the rat (Figs. 7 and 8). Importantly, using the more rapid loading dose regimen, the concentrations were reached already at 1 hr after onset of treatment. Further, the exposures in these stroke patients were consistently also well above those required for maximal levels of neuro-protection in both models as well as for the plasma concentrations in the 2 studies performed in marmosets.

The studies performed to date in stroke patients were not designed to determine efficacy. The late average time to

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**Table 7**

Summary of Incidence of Adverse Events during the infusion period from Studies SA-NXY-0003 and SA-NXY-0004 of 5% or greater in either the total NXY-059 or placebo groups, ordered by Most Frequent in the Total group

<table>
<thead>
<tr>
<th>Preferred term</th>
<th>85 mg/hr (n = 48)</th>
<th>170 mg/hr (n = 49)</th>
<th>420 mg/hr (n = 48)</th>
<th>884 mg/hr (n = 39)</th>
<th>Total NXY-059 (n = 184)</th>
<th>Placebo (n = 97)</th>
<th>Total (n = 281)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Headache</td>
<td>4 (8.3%)</td>
<td>10 (20.4%)</td>
<td>13 (27.1%)</td>
<td>6 (15.4%)</td>
<td>33 (17.9%)</td>
<td>19 (19.6%)</td>
<td>52 (18.5%)</td>
</tr>
<tr>
<td>Fever</td>
<td>6 (12.5%)</td>
<td>11 (22.4%)</td>
<td>10 (20.8%)</td>
<td>0</td>
<td>27 (14.7%)</td>
<td>14 (14.4%)</td>
<td>41 (14.6%)</td>
</tr>
<tr>
<td>Hyperglycemia</td>
<td>4 (8.3%)</td>
<td>4 (8.3%)</td>
<td>2 (4.2%)</td>
<td>0</td>
<td>18 (9.9%)</td>
<td>11 (11.3%)</td>
<td>29 (10.3%)</td>
</tr>
<tr>
<td>Hypertension</td>
<td>4 (8.3%)</td>
<td>2 (4.1%)</td>
<td>5 (10.4%)</td>
<td>2 (5.1%)</td>
<td>13 (7.1%)</td>
<td>13 (13.4%)</td>
<td>26 (9.3%)</td>
</tr>
<tr>
<td>Nausea</td>
<td>5 (10.4%)</td>
<td>3 (6.1%)</td>
<td>6 (12.5%)</td>
<td>3 (7.7%)</td>
<td>17 (9.2%)</td>
<td>9 (9.3%)</td>
<td>26 (9.3%)</td>
</tr>
<tr>
<td>Vomiting</td>
<td>3 (6.3%)</td>
<td>3 (6.1%)</td>
<td>3 (6.3%)</td>
<td>1 (2.6%)</td>
<td>10 (5.4%)</td>
<td>8 (8.2%)</td>
<td>18 (6.4%)</td>
</tr>
<tr>
<td>Pain</td>
<td>4 (8.3%)</td>
<td>1 (2.0%)</td>
<td>3 (6.3%)</td>
<td>4 (10.3%)</td>
<td>12 (6.5%)</td>
<td>4 (4.1%)</td>
<td>16 (5.7%)</td>
</tr>
<tr>
<td>APTT increased</td>
<td>4 (8.3%)</td>
<td>5 (10.2%)</td>
<td>2 (4.2%)</td>
<td>0</td>
<td>11 (6.0%)</td>
<td>4 (4.1%)</td>
<td>15 (5.3%)</td>
</tr>
<tr>
<td>Renal function abnormal</td>
<td>4 (8.3%)</td>
<td>6 (12.2%)</td>
<td>1 (2.1%)</td>
<td>0</td>
<td>11 (6.0%)</td>
<td>4 (4.1%)</td>
<td>15 (5.3%)</td>
</tr>
<tr>
<td>Bradycardia</td>
<td>2 (4.2%)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2 (1.1%)</td>
<td>5 (5.2%)</td>
<td>7 (2.5%)</td>
</tr>
<tr>
<td>Diarrhea</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1 (2.6%)</td>
<td>1 (0.5%)</td>
<td>5 (5.2%)</td>
<td>6 (2.1%)</td>
</tr>
</tbody>
</table>
initiation of treatment would not be appropriate in studies with the purpose of evaluating efficacy. Further, there was a sizable proportion of subjects with low stroke severity in both studies. The median stroke severity at baseline in the Lees et al. (2003) study was 6 on the NIHSS. As shown in Fig. 13, subjects with a lower NIHSS are likely to spontaneously have a good and very good outcome—prospectively defined in the study as a score of 2 or 3 and 0 or 1, respectively, on the modified Rankin scale. Subjects with a more significant stroke severity of NIHSS of 6 or greater are at greater risk of a poor outcome; numerically, there were more subjects with a good or very good outcome in the NXY-059-treated groups (Fig. 13). There were however no statistically significant differences between the total NXY-059 and the placebo groups on either functional or neurological recovery (Lees et al., 2003).

7. General conclusions

Nitrone-derived radical trapping agents have been shown to have a multiplicity of pharmacological effects both in vitro and in vivo. Several of these effects have potential clinical benefit. However, to date, there has been little development of compounds of this type into pharmaceutical compounds. The notable exception is NXY-059, which is being developed as part of a discovery program based on the robust findings of the neuroprotective action of PBN.

NXY-059 has been shown to be an effective neuroprotective agent in several animal models of stroke including transient and permanent focal ischemia and thromboembolic focal ischemia. It also has modest positive effects in a hemorrhagic stroke model. Efficacy has been shown in both rat and primate models.

What is notable is that NXY-059 has a large therapeutic window of opportunity and provides statistically significant neuroprotection (measured in terms of both functional neurological improvement and histological measurement of infarct size) when given 4 hr after MCAO in both rat and primate models of acute ischemic stroke. It can therefore be readily administered to stroke patients not only at a dose that produces plasma levels that are higher than those shown in rodents and primates to produce effective neuroprotection but also within a time frame after the stroke that is relevant to the time windows shown in animal models. It is therefore the first neuroprotective compound to be examined clinically, meeting both these key factors. NXY-059 has also been noted (Lees et al., 2001b) to meet all the STAIR criteria (see Table 1). It has been suggested that these are major features of the preclinical evaluation that should be adhered to before any compound progresses to clinical trial.

What remains an enigma with both NXY-059 and other nitrone compounds is whether its neuroprotective efficacy can be fully ascribed to its activity as radical trapping agents or whether other mechanisms, as yet unidentified, are also involved. Nevertheless, this lack of information should not detract from the impressive neuroprotective profile of this compound and its potential as a therapy for acute ischemic stroke.

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