Functional and Histological Evidence for the Protective Effect of NXY-059 in a Primate Model of Stroke When Given 4 Hours After Occlusion

Jonathan W.B. Marshall, PhD; Rosalyn M. Cummings; Laura J. Bowes, BA; Rosalind M. Ridley, ScD; A. Richard Green, DSc

Background and Purpose—NXY-059 has substantial protective effects when administered immediately after the onset of ischemia in a primate model of stroke. This study examined the efficacy of this drug when administered 4 hours after onset, a more clinically relevant time point.

Methods—Before surgery, marmosets were trained and tested on a number of neurological tests, which assessed general neurological function, motor ability, and spatial awareness. Four hours after permanent middle cerebral artery occlusion (pMCAO), marmosets received a bolus of saline (n = 13) or NXY-059 (n = 13), and osmotic minipumps were implanted, providing 48-hour saline or drug (85 μmol/kg per hour) infusion. The monkeys were retested 3 and 10 weeks after surgery. Finally, infarct size was evaluated with histological analysis.

Results—The unbound plasma NXY-059 concentration was 200±9 μmol/L after 24-hour infusion, a concentration well tolerated in stroke patients. Drug treatment ameliorated the long-term motor impairment produced by pMCAO; the marmosets were better at using their contralesional, stroke-affected arm than controls at both 3 and 10 weeks. Saline-treated animals had a debilitating spatial neglect at 3 weeks with residual signs evident at 10 weeks. NXY-059 treatment substantially attenuated neglect at 3 weeks, with no deficit being seen at 10 weeks. NXY-059 reduced the overall infarct size by 28% (saline, 324±46 mm³; NXY-059, 234±30 mm³) with protection to the cortex, white matter, and subcortical structures.

Conclusions—NXY-059 is an effective neuroprotective agent when administered 4 hours after pMCAO in a primate species, attenuating both motor and spatial neglect. The compound also substantially lessened the volume of cerebral damage. (Stroke. 2003;34:2228-2233.)

Key Words: behavior, animal | free radicals | neuroprotection | nitrogen oxides | monkeys

Despite clear efficacy of many compounds in animal models of stroke, all subsequently failed in the clinic. In an attempt to improve selection, the Stroke Therapy Academic Industry Roundtable (STAIR) published guidelines to be met before a compound progresses to clinical development.1 Recommendations included a greater use of functional tests in animal models and long-term outcome measures and use of primate models incorporating neurological assessment. These suggestions are encompassed in our marmoset model of stroke, which is particularly relevant since clinically important measures of functional deficits (motor function and spatial neglect) are quantified.2,3 Although marmosets are not gyrencephalic, they are closer in the phylogenetic tree to humans and have a larger brain-weight to body-weight ratio than rodents.

Lees et al4 suggested that the radical trapping agent NXY-059 (disodium 4-[(tert-butylimino)-methyl]benzene-1,3-disulfonate N-oxide) appeared to meet the STAIR criteria. However, the time window studies of neuroprotective efficacy were conducted in rats, and evaluation was based on histological analysis. While NXY-059 has been found to be protective in the marmoset model,2 drug administration began only 5 minutes after permanent middle cerebral artery occlusion (pMCAO), and the plasma level achieved (76 μmol/L) was significantly lower than that required to produce maximal neuroprotection in a rat pMCAO model.5 In the present study the dose was increased to produce a plasma concentration similar to that required for the maximum protective effect in pMCAO in rats but still below the level that is safely tolerated in stroke patients (unbound drug plasma level 260 μmol/L),6 and the therapeutic time window was extended to 4 hours after pMCAO, which is relevant to clinical treatment of acute ischemic stroke.
Materials and Methods

Animals
Twenty-eight laboratory-bred, adult common marmosets (Callithrix jacchus), aged approximately 18 months (weight, approximately 400 g at the start of the experiment), were used. They were kept within a large colony and had good visual and auditory interaction with other monkeys. All procedures were performed in accordance with UK Home Office regulations, which ensure humane and proper care of research animals.

Preliminary Pharmacokinetic Analysis
A preliminary pharmacokinetic study was performed on 2 marmosets with the use of an intravenous bolus of NXY-059 (50 mmol/kg) and a mean constant-rate subcutaneous infusion of 70 μmol/kg per hour. Data were fitted to a 1-compartment model with intravenous bolus and maintenance infusion, which showed a good fit. These data were applied to dose calculations for the main study. The unbound plasma drug values in the 2 animals were 66%, 61%, and 65% in a spiked plasma sample. A value of 65% was therefore assumed for the main study. The drug was assayed by coupled column liquid chromatography and UV detection at 299 nm. Plasma samples were treated with caprylic acid in buffer (pH 7), followed by ultrafiltration and centrifugation before injection of the supernatant into the liquid chromatographic system. The limit of quantification was 0.6 to 0.8 μmol/L with accuracy 101% to 102%. Unbound NXY-059 was assayed after adjustment of the plasma pH to 7.4 and ultrafiltration at 37°C.

Surgery
Marmosets were anesthetized with Saffan (alphaxolone [9 mg/mL]/alphadolone acetate [3 mg/mL], 0.15 mL/100 g; Glaxo Vet Ltd) administered intramuscularly. The middle cerebral artery (MCA) was permanently occluded as previously described. After surgery, the monkeys were placed in incubators to maintain body temperature and administered the long-acting analgesic flunixin meglumine (1 mg/kg SC; Finadyne, Schering-Plough) once daily for 2 days. Animals were monitored with a Tiger pulse oximeter (Thames Medical), and perfused transcardially with 200 to 300 mL saline followed by 250 to 300 mL of 10% formal saline. Brains were removed and analyzed after histological preparation and analysis, as described elsewhere.

NYX-059 Treatment
Four hours after pMCAO, monkeys received a 1-mL intravenous infusion of either saline (n = 13) or NXY-059 (77 μmol/kg) (n = 13) and a 0.2-mL subcutaneous injection of saline or NXY-059 (154 μmol/kg), and primed osmotic minipumps (Alzet model 2001D) were implanted subcutaneously to provide 24-hour drug (85 μmol/kg per hour) or saline infusion. The minipumps were replaced after 24 hours with additional minipumps, under Saffan anesthesia, thereby providing continuous drug administration for a total of 48 hours, at which time the pumps were removed.

Physiological Variables
During surgery, blood pressure was monitored with an Ultrasonic Doppler Flow Detector (Perimed UK Ltd). Heart rate and P O 2 levels were measured with a Tiger pulse oximeter (Thames Medical), and body temperature was measured with a rectal thermometer. Rectal temperature was monitored at 4, 28, and 52 hours after pMCAO.

Postoperative Disability
Postoperative records were kept detailing food, water, drug administration, well-being, and behavior. Postoperative disability (maximum score, 17) was graded at 4, 28, and 52 hours after surgery with a scale derived from the National Institutes of Health Stroke Scale (see Lees et al).

Neurological Testing
All neurological tasks were performed in a modified home cage with an internal acrylic plastic sheet (Plexiglas) enclosure, as described previously. Before surgery, all monkeys had been familiarized with and tested on the tasks. Rotation and head bias were performed in the same cage but without the internal Plexiglas enclosure and perch. Monkeys were retested 3 and 10 weeks after surgery to conform to previous studies. Surgery, neurological testing, and histological analysis were all done with the investigators blind to treatment condition.

Rotation and Head Bias
The number and direction of spontaneous 360° rotations of monkeys were recorded for a 15-minute period. In addition, head position was scored (normal/no bias=0; slight/occasional bias=1; moderate/frequent bias=2; severe/permanent bias=3) over a 1-minute period on 3 consecutive days. Head bias was not scored before pMCAO because it is induced by occlusion, and baseline scores are 0 or near 0.

Two-Tube Choice Test
Two black plastic tubes, with a food reward in each, were presented to the monkey, as described previously. After right-sided pMCAO, monkeys reach almost exclusively to the right of the 2 rewarded tubes, even when both tubes are presented on the monkey’s ipsilesional or right side. This is a test of “extinction,” ie, the tendency for items in ipsilesional hemisphere to overshadow attention to items in contralesional hemisphere.

Six-Tube Search Task
The monkeys were required to search for 1 marshmallow piece hidden in 1 of 6 black plastic tubes, as described previously. Because there was only 1 reward available for each trial, this task is a test of spatial neglect.

Hill and Valley Staircase Tasks
In these tasks the monkeys have to reach through vertical slots in the Plexiglas screen to retrieve food rewards from the steps of 2 staircases outside the cage. In the Hill task, there were 2 laterally positioned slots, and the staircases rose toward the center of the apparatus. The monkeys therefore used their right hand to reach to the right staircase and their left hand to reach to the left staircase. In the Valley task, there was 1 centrally positioned slot, and the staircases rose toward the outside of the apparatus. The right arm was therefore used to reach to the left staircase and vice versa. Examination of the use of each arm to reach into either hemisphere allows examination of the effects of a unilateral motor impairment, confined to 1 arm in either hemisphere, to be dissociated from a unilateral perceptual spatial impairment, confined to 1 hemisphere with either arm.

Histology
Eleven weeks after surgery, the monkeys were deeply anesthetized and perfused transcardially with 200 to 300 mL saline followed by 250 to 300 mL of 10% formal saline. Brains were removed and immersed in 10% formal saline and examined to ensure that the MCA had been occluded at the site adjacent to the olfactory tract before histological preparation and analysis, as described elsewhere.

Data Analysis
Data were analyzed with multifactorial ANOVA with post hoc Newman-Keuls t tests. ANOVA was performed with the software package GB-Stat version 6.5 PPC (Dynamic Microsystems, Inc). All results are shown as mean and SEM.

Results
Two animals (1 from each group) died within 48 hours of surgery, and 1 saline-treated animal was killed at 2 weeks because of poor recovery. One NXY-059–treated animal had
a spontaneous cerebral hemorrhage at 5 weeks after surgery and died, but because it had appeared healthy until the hemorrhage, results from the 3-week analysis were included in the evaluation. Twenty-two monkeys were used in the final analysis in 2 groups: NXY-059 (6 male and 6 female) and saline (6 male and 4 female). There was no difference in sex response to either drug or stroke.

NXY-059 Plasma Level
The plasma total drug concentration of NXY-059 was 308±13 μmol/L after 24-hour infusion, which was computed to a plasma unbound drug concentration of 200±9 μmol/L.

Physiological Variables
The body temperature (both during and after surgery), heart rate, blood pressure, and Po2 levels in saline-treated (control) and NXY-059–treated monkeys did not reveal any significant differences (data not shown).

Postoperative Disability
Just before drug administration, the control group had a lower neurological deficit score (10.9±0.8) than the NXY-059–treated group (13.3±0.5; P<0.03). Both 24 and 48 hours later, there was no discernible difference in the scores between the groups (saline, 9.7±0.8 and 9.5±0.8, respectively; NXY-059, 9.5±0.6 and 9.1±0.6, respectively), and there were no other side effects to identify drug-treated monkeys.

Rotation and Head Bias
ANOVA with group (control versus NXY-059) and time (preoperative versus 3 weeks postoperative versus 10 weeks postoperative) as factors was used to analyze the number of spontaneous rotations. The group effect was significant (F1,21 = 4.61, P = 0.044). Post hoc analysis revealed a significant decrease in the abnormal rotation of the NXY-059–treated group at 3 weeks (P<0.01), with the rotation in this group not differing from zero. The abnormal rotation was markedly decreased in the control group at 10 weeks compared with 3 weeks, and the lack of rotation in the NXY-059–treated group was maintained (Figure 1a).

The stroke produced marked head bias at 3 weeks. ANOVA with group and time as factors was used to analyze results. The group effect was significant (F1,21 = 4.698, P = 0.042). NXY-059 decreased the abnormal head bias at both 3 weeks (P<0.01) and 10 weeks (P<0.05) (Figure 1b).

Two-Tube Choice Task
Two-factorial ANOVA, with group and time as factors, compared the tendency of monkeys to reach to the left of the 2 tubes when the tubes were presented on the monkeys’ ipsilesional side. The overall group effect was F1,21 = 3.75, P = 0.068. Post hoc tests showed that at 3 weeks the NXY-059–treated group had less of a deficit (P<0.05). At 10 weeks there was little deficit in the control group and no beneficial effect of NXY-059.

Six-Tube Search Task
ANOVA with group, tube (1 to 6), and time as factors was used to analyze data. The overall group effect was significant (F1,21 = 3.535, P = 0.074). Post hoc tests showed that at 3 weeks, NXY-059–treated monkeys were better at finding rewards hidden in the 4 leftmost tubes than controls (tubes 3 to 6: P<0.01). Ten weeks after surgery, the NXY-059–treated group was still significantly better at finding a reward hidden in tube 5 (P<0.01) (Figure 2).

Hill and Valley Staircase Tasks
Three-way ANOVA with group, hemispace (ipsilesional versus contralesional), and time as factors was used to analyze the data. There was a significant difference between the groups on the Valley task (F1,21 = 5.02, P = 0.037), and analysis approached significance on the Hill task (F1,21 = 3.68, P = 0.069). Post hoc tests showed that at 3 and 10 weeks, the NXY-059–treated monkeys were significantly better at reaching with their affected, left arm into neglected left hemispace than were controls in the Hill test (3 weeks: P<0.05; 10 weeks: P<0.01). In the Valley test, there was no significant post hoc difference at reaching with the affected left arm into right unneglected hemispace at 3 weeks, but there was a clear effect at 10 weeks (P<0.05). At 3 weeks, the NXY-059–treated monkeys were significantly better than controls at reaching with their unaffected, right arm into neglected hemispace on the Valley task (P<0.01). At 10 weeks, both groups could reach into contralesional neglected space with their unaffected right arm without any significant deficit. Results are shown in Figure 3a and 3b.

Measurement of the defined reaches with the left arm revealed a higher score by the NXY-059–treated monkeys in...
both tests, although this did not reach statistical significance ($F_{1,21} = 3.905$, $P = 0.062$) (Figure 3c). However, post hoc tests demonstrated a significantly greater use of the left arm by NXY-059–treated animals at both 3 and 10 weeks on both tasks ($P < 0.01$ in all cases).

**Quantitative Histological Analysis**

The control animals had a large infarct in their right hemispheres extending to subcortical structures, with almost total loss of caudate and putamen. ANOVA with group and stereotaxic level (A14.5 to A2.5) as factors showed a significant group × stereotaxic level interaction ($F_{2.50}, P < 0.01$). Post hoc analysis showed significant reductions in the size of infarct between stereotaxic levels A10.5 and A7.5 (Figure 4a). The total infarct volume in the NXY-059–treated group was decreased by 28.9% compared with the control group (NXY-059: 234 ± 46 mm$^3$; saline: 324 ± 46 mm$^3$). Damage to the cortex was decreased by 55 ± 30 mm$^3$, white matter by 13 ± 3 mm$^3$, caudate by 11 ± 4 mm$^3$, and putamen by 5 ± 2 mm$^3$ (Figure 4b). However, although this reduction in damage approached significance for the caudate ($z = 1.76$, $P = 0.078$) and white matter ($z = 1.69$, $P = 0.091$), it was significant only for the putamen ($z = 2.01$, $P = 0.045$).

**Discussion**

This study has shown unequivocal evidence for the protective efficacy of NXY-059 when administered 4 hours after the start of pMCAO in marmosets, with efficacy evident with the use of both functional neurological measures and histological analysis.

The mean plasma free drug concentration was 200 μmol/L, which is only 75% of a well-tolerated level achieved in a recent clinical study (260 μmol/L). Since protection by NXY-059 in the rat pMCAO model is related linearly to plasma level, a greater protective effect might have been achieved in marmosets had the plasma concentration been increased further.

Monkeys were allocated in a manner blind to treatment groups before pMCAO. Just before drug administration, the NXY-059 group had a higher disability score and thus apparent severity of neurological impairment, suggesting that the random distribution, if anything, biased the study against drug intervention.

While rotation and head bias are not features normally seen in stroke patients, these behaviors can be a sensitive marker of unilateral brain dysfunction in animal models of disease.
NXY-059 treatment essentially abolished the marked rotation at both 3 and 10 weeks and produced a similar amelioration of the abnormal head bias.

Without drug treatment, marmosets have a marked neglect of contralesional space after pMCAO. In humans, spatial neglect is incapacitating and can markedly hinder the patient’s rehabilitation from a stroke. Patients tend to recover from the more severe symptoms, as do marmosets, but some symptoms can persist and pose challenges for rehabilitation of concomitant motor disability. Furthermore, neglect in marmosets may be a surrogate for a wider category of higher cortical dysfunctions, including aphasia and apraxia in humans, from which recovery is less rapid and which may cause permanent problems. In the present study the control group had marked neglect, as observed in the 6-tube test at 3 weeks and a small significant deficit at 10 weeks. NXY-059 substantially lessened spatial neglect at 3 weeks, and the modest deficit remaining at 10 weeks was also lessened. Similar beneficial effects were also witnessed in the 2-tube task. In the Hill staircase test, all monkeys, whether treated with saline or NXY-059, had normal use of the right ipsilesional arm in ipsilesional space, successfully retrieving most, if not all, rewards. However, control animals failed almost entirely to retrieve any rewards with their contralesional left arm in contralesional neglected space at both 3 and 10 weeks. This result is effectively identical to our previous study. In contrast, the NXY-059–treated group displayed some ability to use this arm at both time points. While the deficit in using the contralesional left arm on this task reflects both a spatial neglect and motor problem, the fact that other tests had shown that spatial neglect is almost absent by 10 weeks allows for a strong argument that the left arm use in the NXY-059–treated animals at 10 weeks reflects improvement in motor function. This interpretation is given further credence by results obtained in the Valley staircase test because use of the left arm in right hemispace is a reflection solely of motor function. Again, after the stroke, control animals usually failed to retrieve any rewards with this arm, while the NXY-059–treated group showed some success.

Because performance in the Hill and Valley tests involves both major motor movement (reaching into the apparatus) and fine motor control (picking up the reward), we also scored each defined attempt to reach the food, even if it was not retrieved. This evaluation showed that NXY-059–treated monkeys made considerably more reaches with their left contralesional arm than controls in both tests at 3 and 10 weeks.

The histological data support the functional evidence for NXY-059 being protective. Infarct volume was 28% less in the NXY-059–treated group. NXY-059 treatment reduced damage to the cortex by 27%, to the white matter by 30%, to the caudate by 36%, and to the putamen by 20%, although only the protection to the putamen was statistically significant because of wide variability in response within the groups. This suggests that NXY-059 protects both gray and white matter as well as cortical and subcortical structures, an interpretation that is supported by other data.

The importance of matching the therapeutic window of opportunity in animal studies with those to be used clinically cannot be overestimated, and it has been proposed that time windows for clinical and experimental models should be matched. The present study and results obtained in rats demonstrate that this is an achievable goal for NXY-059.

It is possible to make limited comparisons regarding the relative efficacy of clomethiazole, the N-methyl-D-aspartate antagonist AR-R15896AR, and NXY-059 in the marmoset model because all have been given at doses producing clinically relevant plasma concentrations. Neither clomethiazole given 60 minutes after occlusion nor AR-R15896AR given 5 minutes after occlusion produced any restoration of motor function in the paretic arm, while NXY-059 given 4 hours after occlusion (this study) had a clear beneficial effect. This emphasizes both the long therapeutic time window and the efficacy of NXY-059.

NXY-059 is a nitrones free radical trapping agent with limited brain penetration across the blood-brain barrier after transient MCAO. This might be relatively unimportant in the ability of the compound to protect against reperfusion injury since radicals are produced at the blood-endothelial interface. However, NXY-059 is also efficacious in protecting...
against damage in models of pMCAO in rodents\textsuperscript{5,11} and primates,\textsuperscript{2} which raises questions regarding its mechanism of action. In vitro data suggest that prolonged ischemic conditions increase brain penetration of NXY-059,\textsuperscript{5,9} and hemorrhagic stroke\textsuperscript{15} and embolic stroke in rabbits,\textsuperscript{16} has now been shown to substantially lessen both motor and cognitive disability and to reduce infarct volume in marmosets when administered 4 hours after the onset of ischemia. Clinical trials can now be conducted with NXY-059 with the same drug exposure and time window shown to be effective in animal models.

In conclusion, NXY-059, a compound with demonstrable neuroprotective efficacy in transient MCAO,\textsuperscript{5,9} pMCAO,\textsuperscript{5,11} and hemorrhagic stroke\textsuperscript{15} models in rats, pMCAO in marmosets,\textsuperscript{2,3} and embolic stroke in rabbits,\textsuperscript{16} maintains Akt activation and inhibits release of cytochrome c after focal ischemia,\textsuperscript{14} it may interact directly with neurons in the damaged area or interact at the level of the endothelial cell to alter signal transduction pathways and thereby affect mitochondrial function. Further studies are required to ascertain fully both the site and mechanism of action of NXY-059.

Acknowledgments

This study was supported by AstraZeneca and conducted within a component of the Medical Research Council Centre for Behavioral and Clinical Neuroscience in Cambridge, UK. AstraZeneca is developing NXY-059 under a license agreement with Renovis Inc (California). We thank Jan Lundström for pharmacokinetic modeling and Kerstin Lanbeck-Vallén for analysis of plasma NXY-059 concentrations. Clinical trials can proceed with NXY-059 under a license agreement with Renovis Inc (California). We thank Jan Lundström for pharmacokinetic modeling and Kerstin Lanbeck-Vallén for analysis of plasma NXY-059 concentrations. We also thank Catherine Maclean, Debbie Mills, and Amy Milton for their assistance.

References

Functional and Histological Evidence for the Protective Effect of NXY-059 in a Primate Model of Stroke When Given 4 Hours After Occlusion
Jonathan W.B. Marshall, Rosalyn M. Cummings, Laura J. Bowes, Rosalind M. Ridley and A. Richard Green

Stroke. 2003;34:2228-2233; originally published online August 14, 2003;
doi: 10.1161/01.STR.0000087790.79851.A8

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://stroke.ahajournals.org/content/34/9/2228

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Stroke can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Stroke is online at:
http://stroke.ahajournals.org/subscriptions/