Neuronal Nitric Oxide Synthase Inhibition Prevents Cerebral Palsy following Hypoxia-Ischemia in Fetal Rabbits: Comparison between JI-8 and 7-Nitroindazole

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Abstract

Cerebral palsy and death are serious consequences of perinatal hypoxia-ischemia (HI). Important concepts can now be tested using an animal model of cerebral palsy. We have previously shown that reactive oxygen and nitrogen species are produced in antenatal HI. A novel class of neuronal nitric oxide synthase (nNOS) inhibitors have been designed, and they ameliorate postnatal motor deficits when administered prior to the hypoxic-ischemic insult. This study asks how the new class of inhibitors, using JI-8 (K_i for nNOS: 0.014 μM) as a representative, compare with the frequently used nNOS inhibitor 7-nitroindazole (7-NI; K_i: 0.09 $\pm$ 0.024 μM). A theoretical dose equivalent to 75 K_i of JI-8 or equimolar 7-NI was administered to pregnant rabbit dams 30 min prior to and immediately after 40 min of uterine ischemia at 22 days gestation (70% term). JI-8 treatment resulted in a significant decrease in NOS activity (39%) in fetal brain homogenates acutely after HI, without affecting maternal blood pressure and heart rate. JI-8 treatment resulted in 33 normal kits, 2 moderately and 13 severely affected kits and 5 stillbirths, compared with 8 normal, 3 moderately affected and 5 severely affected kits and 10 stillbirths in the 7-NI group. In terms of neurobehavioral outcome, 7-NI was not different from saline treatment, while JI-8 was superior to saline and 7-NI in its protective effect (p < 0.05). In the surviving kits, JI-8 significantly improved the locomotion score over both saline and 7-NI scores. JI-8 was also significantly superior to saline in preserving smell, muscle tone and righting reflex function, but 7-NI did not show significant improvement. Furthermore, a 100-fold increase in the dose (15.75 μmol/kg) of 7-NI significantly decreased systolic blood pressure in the dam, while JI-8 did not. The new class of inhibitors such as JI-8 shows promise in the prevention of cerebral palsy and is superior to the previously more commonly used nNOS inhibitor.

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Introduction

Cerebral palsy and death are severe consequences of perinatal hypoxia-ischemia (HI). Cerebral palsy has one of the highest indices of disease burden because of the life-long consequences for patients, caretakers and social

institutions. The costs to society are huge from the loss of potentially productive members of the society. The prevention of a single cerebral palsy patient would save almost a million dollars (in 2003 [CDC, 2004]). Yet there has been little progress in developing ways of preventing cerebral palsy. We have developed a rabbit model in which a cerebral palsy phenotype develops after acute placental insufficiency at preterm gestation, based on the clinical paradigm of abruptio placentae [Derrick et al., 2004, 2007; Tan et al., 2005]. We have previously shown that reactive oxygen species and reactive nitrogen species are generated in antenatal HI [Tan et al., 1998, 1999].

The free radical nitric oxide ($\cdot$NO) appears to play a critical role in perinatal HI brain injury. $\cdot$NO is produced by nitric oxide synthase (NOS) and there are at least three isoforms of NOS. While endothelial NOS (eNOS) inhibition is deleterious, neuronal NOS (nNOS) inhibition is considered neuroprotective in perinatal HI and in stroke [Bolaños and Almeida, 1999; Moro et al., 2004]. After HI and reperfusion, there is increased $\cdot$NO generated by nNOS [Wei et al., 1999], which can react with superoxide produced by either xanthine oxidase or the mitochondrial respiratory chain to form peroxynitrite and other reactive nitrogen species. The reactive nitrogen species then can cause profound oxidative damage and functional changes in part due to protein nitration and 3-nitrotyrosine formation [Thomas et al., 2008].

Tremendous efforts have been put into the development of new NOS inhibitors to find better compounds that can protect the immature brain from HI. Some examples of those inhibitors include L-nitro-arginine methyl ester, an inhibitor of eNOS and nNOS (but not inducible NOS, iNOS), and 1400W, an inhibitor of iNOS. 7-Nitroindazole (7-NI) is a highly efficient NOS inhibitor in vitro; however, it shows a remarkable specificity for nNOS in vivo without altering blood pressure. The protective effects of these inhibitors in stroke models are not conclusive [Willmot et al., 2005]. One of the reasons could be that inhibition of eNOS decreases cerebral blood flow [Willmot et al., 2005], causing hypoxia. For example, the in vitro potency (IC_{50}) of 7-NI, the specific noncompetitive nNOS inhibitor, is in the range of 6.9–9.7 μM for iNOS, 0.09–8.3 μM for nNOS and 2.1–14.8 μM for eNOS inhibition [Mayer et al., 1994; Willmot et al., 2005]. The overlap between the values for different isoforms points to a low specificity for nNOS.

To test the role of nNOS activity in the etiology of cerebral palsy, it was felt that a more specific inhibitor was urgently needed which would specifically target nNOS while not affecting other isoforms. We have developed a series of nNOS inhibitors based on the structure of the nNOS active site and shown very promising results derived from our rabbit cerebral palsy model [Ji et al., 2009b]. We selected one of the compounds, JI-8 (compound 5 in the previous publication [Ji et al., 2009b]), with IC_{50} of 28, 0.014 and 4.1 μM for iNOS, nNOS and eNOS, respectively, and compared its protective effect to that of 7-NI. We found that JI-8 was superior to 7-NI in terms of survival and neurobehavior.

Materials and Methods

Our study was approved by the animal review committee of the NorthShore University HealthSystem Research Institute. All animals received humane care in compliance with the Principles of Laboratory Care formulated by the National Society for Medical Research and with the National Institute of Health Guide for the Care and Use of Laboratory Animals prepared by the National Academy of Sciences.

Animal Model and NOS Inhibitor Delivery
In vivo, global HI of fetuses was induced by uterine ischemia at 70% gestation (embryonic day 22, E22) in pregnant New Zealand white rabbits (Myrtle's Rabbitry, Thompson's Station, Tenn., USA) as previously described [Tan et al., 2005; Derrick et al., 2007]. E22 corresponds to approximately 22–27 weeks gestation in humans, a value derived from previous work on oligodendroglial maturation [Buser et al., 2010]. Based on the inhibitory concentration of nNOS in vitro (K_i), a dose of JI-8 was calculated for administration to the dam that was equivalent to $75 \times K_i$ of nNOS based on the dam's weight and the assumptions of homogeneous distribution in the circulation and entire blood volume of the dam as the targeted volume of distribution. This dose of 0.1575 μmol/kg was meant to theoretically achieve a concentration of JI-8 in the dam's blood that would be $75 \times K_i$ for nNOS. The dose was administered into the descending aorta of the dam 30 min prior to 40 min of uterine ischemia. The same dose was repeated immediately after uterine ischemia. These dams were compared with another group of dams administered an equimolar dose of 7-NI. The same volume of saline was injected as a vehicle control. For toxicity analysis, the experiment was repeated with a 100-fold increase in the doses of both compounds to 15.75 μmol/kg, administered in the same volume (n = 4; dams not previously exposed to low dose). Blood pressure and heart rate were measured every minute in the left leg with a Vet/BP 600 device (Sensor Devices Inc., Waukesha, Wisc., USA).

nNOS Activity Measurement
In a subset of animals, fetal brains were removed either immediately or 24 h after HI (n = 3 for each group and time point). nNOS activity was measured as previously described [Porter et al., 2005; Vásquez-Vivar et al., 2009].

Neurobehavioral Evaluation
Following HI, the dams were allowed to spontaneously deliver at term gestation (31.5 days). Evaluations of postural deficits, hy-
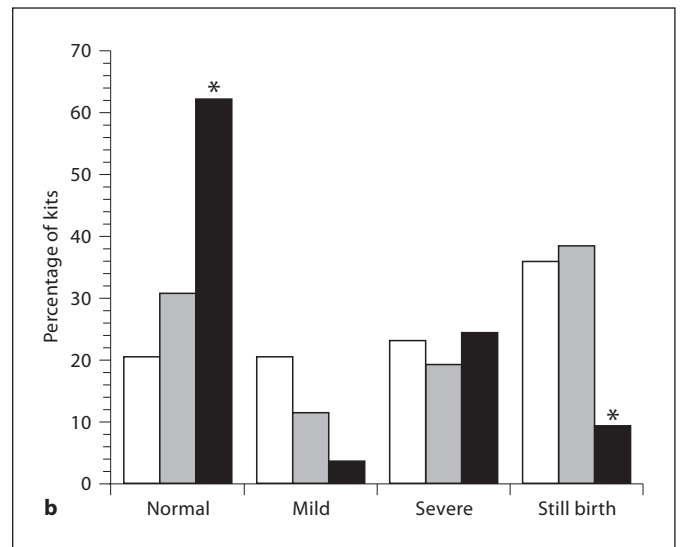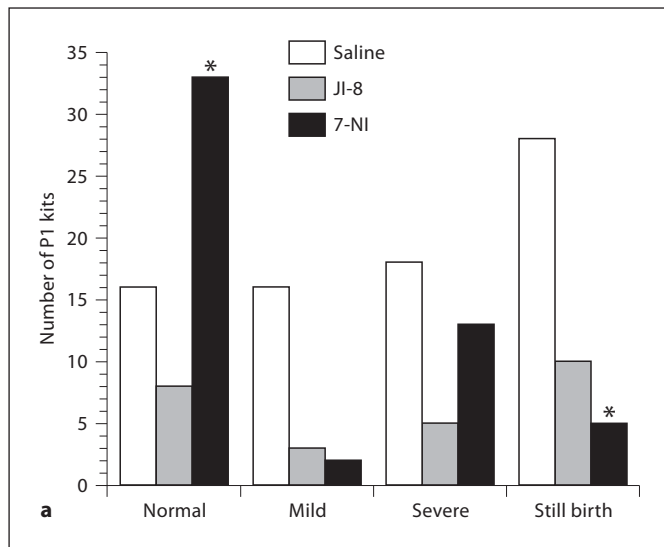
Fig. 1. E22 pregnant rabbits received global HI for 40 min followed by reperfusion for 40 min. Doses of 0.1575 µmol/kg of JI-8 or 7-NI were administered before and again after HI, and compared with the same volume of saline. Newborn kits at P1 were categorized into normal, mild and severe based on neurobehavior. The saline groups only had 20%, the 7-NI group had 31% and the JI-8 group 62% normal P1 kits. Numbers of dams in each group: saline = 10; JI-8 = 8; 7-NI = 4. * The JI-8 group had a significantly better outcome than the 7-NI group (Kendall's tau-b 95% confidence interval: –0.107 to –0.514). **a** Absolute number. **b** Percentage distribution of each neurobehavioral category after treatment.

pertonia and other neurobehavioral abnormalities were performed on postnatal day 1 (P1; E32) and their results were published before [Derrick et al., 2004]. The evaluations included tests for smell, righting reflex, muscle tone and locomotion, which were videotaped and scored by blinded observers on an ordinal scale [Derrick et al., 2007]. The P1 rabbits were then categorized into normal, mild (absence of hypertonia but with other abnormalities), severe (postural deficits and/or hypertonia) and dead groups.

Total Radical-Trapping Antioxidant Parameter Assay

The total radical-trapping antioxidant parameter (TRAP) assay was performed as previously described [Tan et al., 1996], with minor modifications. Measurement of antioxidant activity is based on the reduction by antioxidants of the radical cation of 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonate) (ABTS$^{\bullet+}$). This radical is produced from the reaction of ABTS (7 mM) with potassium persulfate (molar ratio 1:0.35) and by allowing the mixture to stand for 12–16 h in the dark and at room temperature. The ABTS$^{\bullet+}$ concentration was calculated from its absorbance measured on a spectrophotometer the next day at 734 nm ($\varepsilon 734 = 0.015$ M^{-1}cm^{-1}; Lambda 14; Perkin-Elmer, Norwalk, Conn., USA) every 7 s for 4 min at 25°C. The first 4 readings were used to establish the baseline before adding the sample or standards. The readings of samples were compared with a standard curve generated from similar absorbance readings obtained from known concentrations of Trolox in isotonic 5 mM PBS at pH 7.4 and 25°C [Re et al., 1999].

Gender Assessment

Estimate of gender was made in the rabbit kits by visual inspection of abdominal organs [Nielsen and Torday, 1983], which was shown to be 100% accurate by PCR in our laboratory. In the saline and JI-8 groups, a subpopulation of kits was tested for gender.

Statistical Analysis

As the scoring was on an ordinal scale, the neurobehavioral tests of smell, righting reflex, muscle tone and locomotion were analyzed first by the nonparametric Kruskal-Wallis test, comparing the three groups; type I error was set at <0.05. Post hoc comparisons were done by the Wilcoxon two-sample test to compare two groups with a type I error of <0.0167, in view of the multiple comparisons. For heart rate and blood pressure, comparison between groups was done by ANOVA, and post hoc comparison of means using the Student-Newman-Keuls multiple range test. Comparison of JI-8 and 7-NI was also done by the two-sample t test with a type I error of <0.0167. The comparison of normal, mild, severe and dead outcomes was analyzed using Kendall's tau-b and was considered significant if 95% confidence intervals were not encompassing 0. All statistical analyses were done with SAS version 9.1 (SAS Institute Inc., Cary, N.C., USA).

Results

JI-8 Was Superior to an Equimolar Dose of 7-NI in Ameliorating Neurobehavioral Deficits in Kits

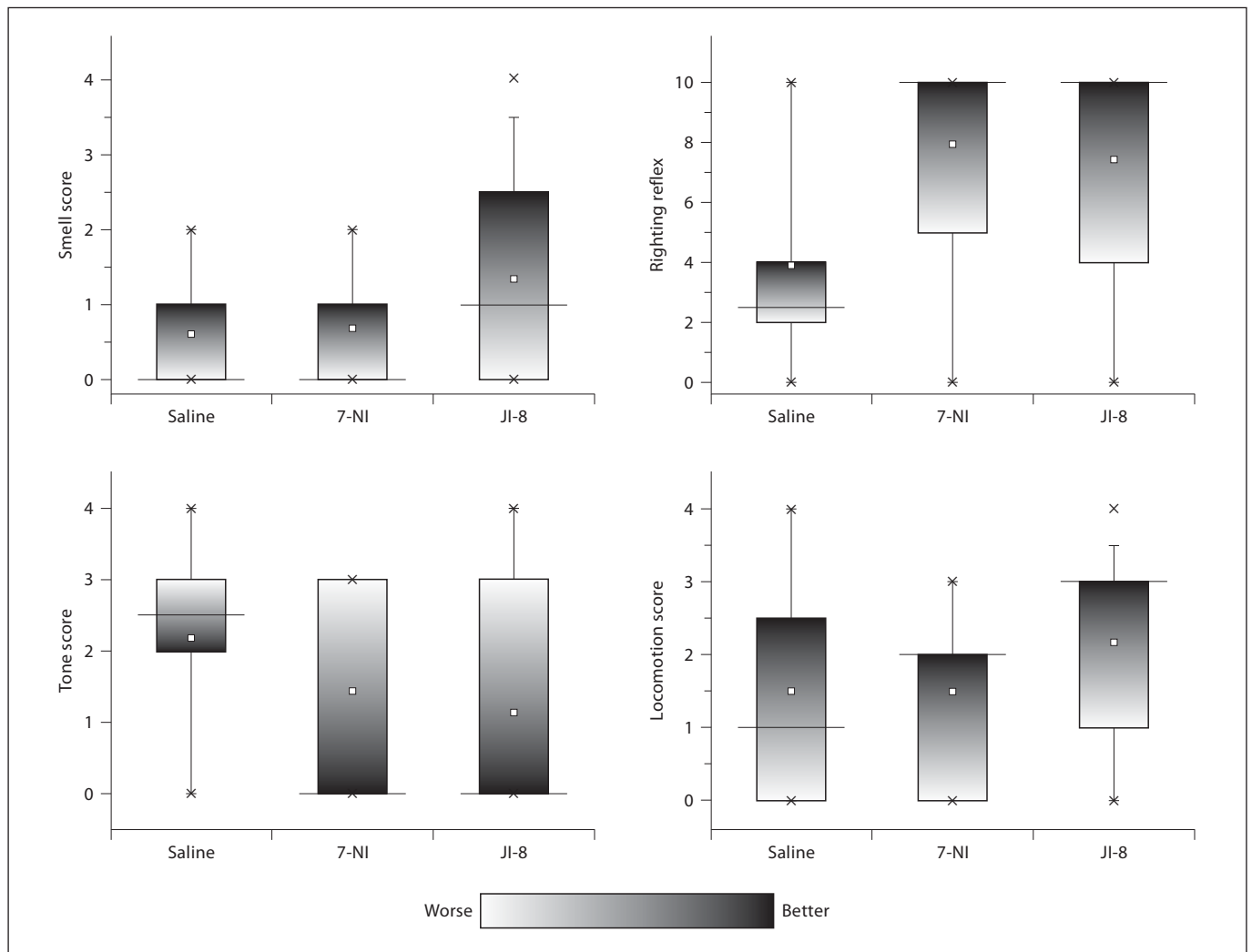The 7-NI group included 26 kits and was compared with 53 kits treated with JI-8. As controls, we used saline-

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Vásquez-Vivar/Tan

Fig. 2. Scoring of neurobehavioral tests in P1 kits. All surviving kits were evaluated for smell, muscle tone, righting reflex and locomotion. The worst scores are depicted as box-and-whisker plots. JI-8 (n = 54) significantly improved all tested functions compared with the saline group (n = 78) and also showed a trend for improved locomotion compared with 7-NI (p = 0.0356; n = 26). 7-NI only improved righting reflex compared with saline. Box: interquartile range. Line inside box: median. Empty dot: mean. Vertical line: range of 1–99% tiles. Small crosses: minimum and maximum values.

treated kits in experiments performed at the same time (78 kits). JI-8 treatment significantly increased the number of normal-appearing kits and decreased the number of severely affected and dead kits following HI compared with the saline-treated group (fig. 1). 7-NI decreased the number of deaths, but JI-8 treatment had a significantly better outcome than 7-NI treatment if all kits were considered (Kendall's tau-b 95% confidence interval: –0.107 to –0.514).

For surviving kits, the worst scores for smell, righting reflex, muscle tone and locomotion functions of each kit in each category were compared between groups. There were significant differences among the three groups in all parameters. JI-8 therapy significantly improved smell (p = 0.0081), muscle tone (p = 0.0041) and righting reflex (p = 0.0002), and showed a trend for locomotion (p = 0.0354, higher than type I error set a priori) compared with the saline group, while 7-NI therapy only significantly improved righting reflex (p = 0.0012) compared with the saline group. More interestingly, JI-8 also showed a trend for improved locomotion (p = 0.0356) compared with 7-NI (fig. 2). There were no differences between male and female rabbit kits in any of the three treatment groups

(online suppl. fig. 1; for all online suppl. material, see www.karger.com/doi/10.1159/000327244).

7-NI Had Significant Cardiovascular Effects in Dams

We have previously demonstrated that JI-8 and its structure-related compounds did not affect the blood pressure of dams during the HI and reperfusion procedure [Ji et al., 2009b]. When 7-NI was delivered the same way as JI-8, there was a significant decrease in the heart rate of dams during and after the HI period (fig. 3). Furthermore, to test the acute toxicity of both compounds, we increased the previous doses 100-fold, and the administration of both compounds was performed in the same way as before. There were no significant changes in blood pressure or heart rate in JI-8-treated dams, but the systolic blood pressure in 7-NI dams was significantly decreased (88 ± 1.6%, n = 3, compared with 96.6 ± 2.4% in JI-8 group; control group set to 100%; p < 0.01) after 30 min of administration (fig. 4). The heart rate after the 100-fold increased dose of 7-NI (n = 3) was significantly slower than that in the JI-8 group (n = 4) at 2 time points, i.e. 10 min before (p < 0.01) and 10 min after the second dose (p < 0.05), when normalized to baseline.

Fetal Brain nNOS Activity in the 7-NI Group Did Not Change

Because we were using an equimolar strategy to compare equivalent doses, the dose of 7-NI in our experiment was much lower than in previously published studies [Willmot et al., 2005]. We tested the activity of nNOS in fetal brains immediately after the period of HI. There was no significant change in nNOS activity in the 7-NI group (117.7 ± 40.7 pmol/min/mg protein; n = 6) compared with the control group (102.6 ± 13.5 pmol/min/mg protein; n = 10). JI-8 significantly decreased nNOS activity (62.2 ± 10.5 pmol/min/mg protein; n = 11; p < 0.05) compared with the control group. Since these two inhibitors were delivered at the same molar amount, this result suggests that therapy with JI-8 was a more successful inhibitor strategy than that with 7-NI. There was no significant difference in nNOS activity among the 24-hour samples.

JI-8 Slowly Reduces ABTS•⁺ in the TRAP Assay

We tested whether any of the compounds had any total antioxidant activity. 7-NI did not show any detectable activity in this assay, similar to that of the saline controls. JI-8 slowly reacted with ABTS•⁺ over time (fig. 5). This reaction is different from that of soluble antioxidants such as ascorbate that rapidly reduce ABTS•⁺ precipitously, similar to that of Trolox.
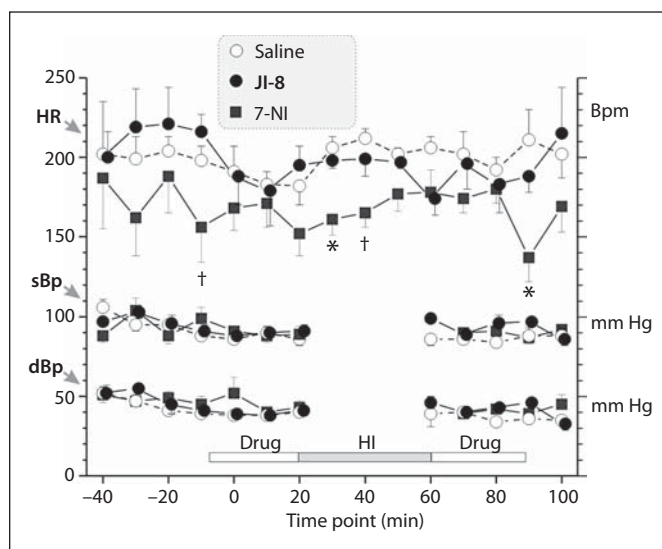

Fig. 3. JI-8 did not affect the cardiovascular function of the dams. Doses of 0.1575 μmol/kg of JI-8 (to n = 9 dams) or 7-NI (n = 5) or equivolume saline (n = 8) were given before and after HI. 7-NI significantly decreased the heart rate (HR) at multiple time points, while JI-8 had no effect. Symbols for JI-8 have been offset a little for clarity. sBp = Systolic blood pressure; dBp = diastolic blood pressure. † p < 0.05 by ANOVA and post hoc Student-Newman-Keuls test; * in addition, p < 0.0167 by t test comparison of JI-8 and 7-NI groups.

Discussion

Although there has been much evidence concerning the probable role of nNOS inhibitors in perinatal asphyxia [Bolaños and Almeida, 1999], there has been insufficient evidence to move forward to a clinical trial, especially as the benefit in ameliorating motor deficits in animals has not been shown until recently [Derrick et al., 2004]. Since the effects of nNOS inhibition are opposite to those of eNOS inhibition [Bolaños and Almeida, 1999], the most desirable property of a potential neuroprotectant is to have a wide margin between inhibition of nNOS and eNOS in the effective dose range. 7-NI is one of the most popular nNOS inhibitors, purportedly for its specificity [Moore et al., 1993]. However, the margin for inhibition between nNOS and eNOS is at best 4-fold. It has been speculated that 7-NI may act through an unknown mechanism other than nNOS inhibition [Matsumura et al., 2008].

A new series of nNOS inhibitors was designed using fragment hopping [Ji et al., 2008, 2009b; Silverman et al., 2009], a new fragment-based approach, for de novo in-
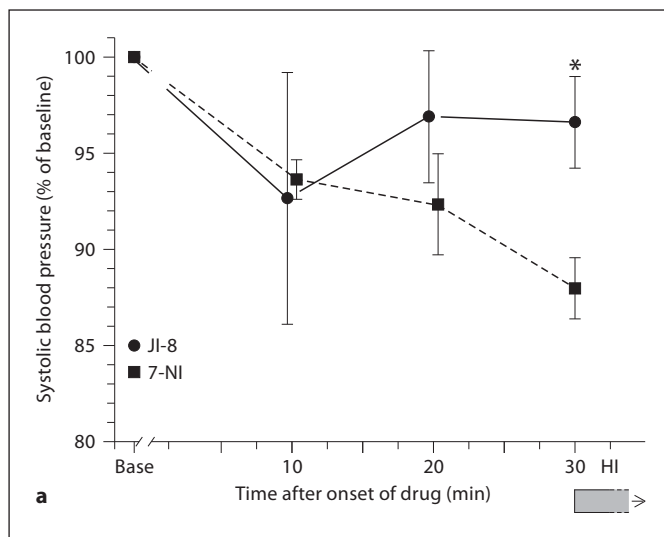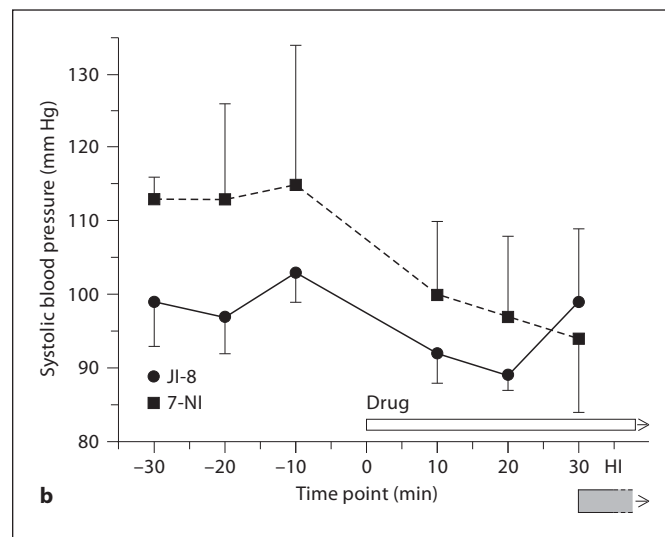
Fig. 4. a Blood pressure recorded every minute after onset of drug and averaged over 10-min blocks before HI. The means and SEM of the averages are depicted as percentage changes from baseline. A dose of 15.75 μmol/kg of JI-8 (n = 4) did not affect the blood pressure of the dam, but the same dose (K_i) of 7-NI caused blood

pressure changes (n = 3). For the middle 2 time points, the symbols have been offset a little for clarity. * $p < 0.01$ compared with JI-8 for change from baseline (Base). **b** Means and SEM of spot systolic blood pressure recordings at the time points.
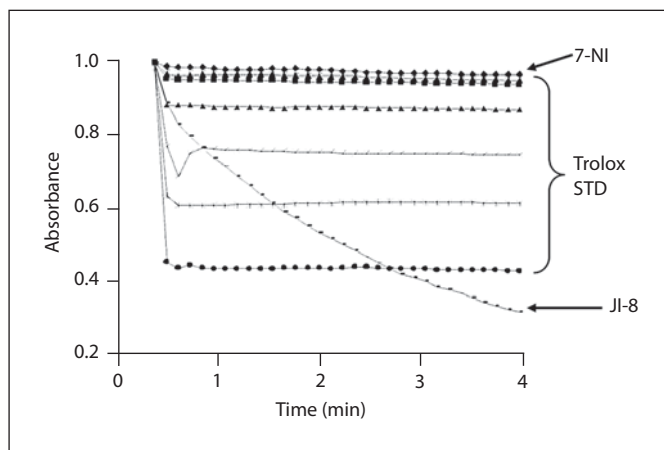

Fig. 5. TRAP assay showing that JI-8 can slowly decolorize ABST$^{•+}$. Trolox standards (STD) were prepared at the concentrations of 0, 0.1, 0.25, 0.5, 0.75 and 1 mM. The first 4 readings were used to set the baseline level. 7-NI had no effect.

hibitor design focusing on ligand diversity and isozyme selectivity. This led to a series of compounds that has a much higher in vitro specificity than all nNOS inhibitors currently available [Ji et al., 2008, 2009a]. We selected one of the most effective of these compounds, JI-8 (compound 5 in Ji et al. [2009b]), and compared its neuronal

protection with the effect of 7-NI in our rabbit cerebral palsy model.

The dose of JI-8 was chosen based on previous experience [Ji et al., 2009b], and 7-NI was given to the animals at the same molar concentration to compare equivalent doses. In the neurobehavioral tests, we found that the JI-8 group had significant improvement over the control group and was significantly better than the 7-NI group. Interestingly, the percentage of severe outcomes was not different between the JI-8 group and the 7-NI group. One possible explanation would be that JI-8 rescues those kits that would otherwise have been stillbirths, thus shifting the distribution to survival and to the left on the abscissa of figure 1. The other possibility would be that the optimal dose of JI-8 was not employed. Although JI-8 only showed transient inhibition of nNOS activity [Ji et al., 2009b], the long-term effect of JI-8 still needs further investigation. Our data suggest that JI-8 is more potent than 7-NI, but the optimal dosing strategy still needs to be worked out to test for increased survival and decreased incidence of severely affected kits.

JI-8 significantly decreased nNOS activity in fetal brains. This suggests that JI-8 could pass both the placental and blood-brain barriers. 7-NI only showed limited protection, although it has to be said that its dose was much lower (1/100 to 1/50) than those used by other re-

Comparison between JI-8 and 7-NI

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searchers [Moore et al., 1993; Alderton et al., 2001; Willmot et al., 2005]. We also did not observe any inhibition of fetal brain nNOS activity by 7-NI. This implies that 7-NI is a much less potent nNOS inhibitor than JI-8, but it might be beneficial due to a mechanism other than inhibition of nNOS. The difference between 7-NI and JI-8 confirms findings in the existing literature that decreasing NO during HI is an important mechanism of neuroprotection. The most likely mechanism affected is that of oxidative stress from NO and NO-derived radicals. The developing fetal brain is most vulnerable to oxidative stress because of immature antioxidant enzyme systems [Mishra and Delivoria-Papadopoulos, 1988; Khan and Black, 2003], early development of prooxidant systems such as xanthine oxidase [Vettenranta and Raivio, 1990] and NOS [Keilhoff et al., 1996], and having a relative deficiency in tetrahydrobiopterin that predisposes to uncoupling of nNOS and the production of superoxide from nNOS [Vásquez-Vivar et al., 1999, 2009]. NO reacts with available superoxide to form peroxynitrite [Beckman, 1991] and reactive nitrogen species [Bolaños et al., 2009], which are powerful biological oxidants [Knapp et al., 2001]. In rat neuronal cultures, peroxynitrite is believed to cause neuronal damage following hypoxia-reoxygenation [Cazevieille et al., 1993] and it leads to necrosis and apoptosis in motor neurons [Estevez et al., 1998]. Low levels of peroxynitrite may result in apoptosis in cortical neurons, while high levels induce necrotic cell death [Bonfoco et al., 1995]. Further studies are needed to establish the link between reactive nitrogen species and motor deficits.

During the experiment, JI-8 did not show any measurable effects on the blood pressure and heart rate of dams as both remained unchanged for 40 min after receiving the drug. However, even the low dose of 7-NI had some undesirable effects. It significantly decreased the dam's heart rate during the HI procedure following drug delivery (fig. 3). This decrease may not be clinically significant but does not help in recovery. A decreased heart rate, if not compensated by increased cardiac output, has the potential to further compromise uterine blood flow. Furthermore, when we administered the 100-fold higher dose, JI-8 again did not show any detectable effects on blood pressure and heart rate. In contrast, when a 100-fold higher dose of 7-NI was delivered (in the concentration range used in other studies), we observed a significant drop in systolic blood pressure in the dams (fig. 4). The later onset of cardiovascular effects on the rabbit dams suggests that 7-NI may be either inhibiting eNOS or acting through other, additional mechanisms.

We used the TRAP assay to discover whether there was any antioxidant effect of JI-8 or 7-NI. The procedure of our TRAP assay is also called the Trolox-equivalent antioxidant capacity assay [Huang et al., 2005]. Usually, the results are read at the end of the reaction [Huang et al., 2005], but we calculated the capacity of the solution based on the areas under the curve. Because the reaction rates are different, our method was more suitable to represent the true antioxidant capacity over time. JI-8 did not behave like a classic antioxidant as the reaction curve for JI-8 is quite different from that for Trolox (fig. 5). There was a slow reaction during the TRAP assay. This can be explained by the aminopyridine group in its structure [Ji et al., 2009b], which can react with $ABTS^{\bullet+}$. Since the reaction with the artificial oxidant $ABTS^{\bullet+}$ is slow, whether JI-8 could function as a true antioxidant or scavenger of reactive nitrogen species in vivo needs further study.

In summary, we found that JI-8 showed superior neuroprotection to 7-NI in our rabbit cerebral palsy model. JI-8 had not only higher potency and specificity, but also had fewer side effects on the cardiovascular system. It is possible that JI-8 has some antioxidant capacity, but it does not behave like known antioxidants. With the development of newer nNOS inhibitors, further studies may reveal even more potent and specific drugs that may be suitable for ultimate translation to clinical studies.

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