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# A novel dual NO-donating oxime and c-Jun N-terminal kinase inhibitor protects against cerebral ischemia–reperfusion injury in mice

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## Abstract

The c-Jun N-terminal kinase (JNK) has been shown to be an important regulator of neuronal cell death. Previously, we synthesized the sodium salt of 11*H*-indeno[1,2-*b*]quinoxalin-11-one (IQ-1S) and demon-strated that it was a high-affinity inhibitor of the JNK family. In the present work, we found that IQ-1S could release nitric oxide (NO) during its enzymatic metabolism by liver microsomes. Moreover, serum nitrite/nitrate concentration in mice increased after intraperitoneal injection of IQ-1S. Because of these dual actions as JNK inhibitor and NO-donor, the therapeutic potential of IQ-1S was evaluated in an animal stroke model. We subjected wild-type C57BL6 mice to focal ischemia (30 min) with subsequent reperfu-sion (48 h). Mice were treated with IQ-1S (25 mg/kg) suspended in 10% solutol or with vehicle alone 30 min before and 24 h after middle cerebral artery (MCA) occlusion (MCAO). Using laser-Doppler flowmetry, we monitored cerebral blood flow (CBF) above the MCA during 30 min of MCAO provoked by a filament and during the first 30 min of subsequent reperfusion. In mice treated with IQ-1S, ischemic and reperfusion values of CBF were not different from vehicle-treated mice. However, IQ-1S treated mice demonstrated markedly reduced neurological deficit and infarct volumes as compared with vehicle-treated mice after 48 h of reperfusion. Our results indicate that the novel JNK inhibitor releases NO during its oxidoreductive bioconversion and improves stroke outcome in a mouse model of cerebral reperfusion. We conclude that IQ-1S is a promising dual functional agent for the treatment of cerebral ischemia and reperfusion injury.

## 1. Introduction

Reperfusion injury is a major clinical problem in several organs, including heart, liver, kidney, and brain. The important role of mitogen-activated protein kinases (MAPKs) has been demonstrated in the pathogenesis of reperfusion injury and stroke [23,39]. C-Jun N-terminal kinase (JNK) is a critical MAPK activated by various brain insults and is implicated in neuronal injury triggered by reperfusion-induced oxidative stress [30]. Three distinct JNKs,

designated as JNK1, JNK2, and JNK3, have been identified, and at least 10 different splicing isoforms exist in mammalian cells [17]. While JNK1 and JNK2 are ubiquitously expressed, JNK3 is found almost exclusively in the brain [43], but it is not dominant, as JNK3 knockout results only in a weak attenuation of the total JNK pool in brain tissue [5]. Increased JNK phosphorylation and JNK activity in the hippocampus have been reported after cerebral ischemia and reperfusion injury [19,29]. Sustained JNK activation has been shown to be associated with neuronal death and apoptosis following ischemic stroke, and in animal models of cerebral ischemia, acute inhibition of JNK reduces infarction and improves outcomes [13,31]. Because the inhibition of JNK isoforms has neuroprotective effects in animal models, it has been suggested that pan-JNK inhibitors may represent promising therapeutic agents for treatment of stroke.

Increasing evidence suggests that the JNK signaling pathway is tightly coupled with nitric oxide (NO) production during ischemia and reperfusion [44]. The effects of NO on the ischemic brain are thought to be dependent on the sources of its production and the stage of the ischemic process [10,20]. The low concentration of NO that is produced by endothelial NO synthase (eNOS) confers protective effects during cerebral ischemia, whereas the high concentrations of NO produced from neuronal NOS (nNOS) and inducible NOS (iNOS) are detrimental to the ischemic brain [10,28]. Exogenous NO had a neuroprotective role in reperfusion-induced brain injury [20,28]. Based on the coupling of NO and JNK pathways and the protective role of exogenous NO, we hypothesized that agents with dual functions as JNK inhibitors and NO donors could have protective effects against cerebral reperfusion injury. To date, some oxime derivatives have been demonstrated *in vivo* and *ex vivo* as NO donors [11,22]. Recently, we described novel oxime-derived, specific JNK inhibitors based on the 11*H*-indeno[1,2-*b*]quinoxalin-11-one scaffold [35,36]. One of these compounds, IQ-1S, was found to be active *in vivo* and had a higher affinity toward JNK3 over JNK1/JNK2 [35,36]. Because the neutral form of IQ-1S has an oxime group, we suggest that, similar to other aryl oxime derivatives [1,9,11], this compound could release NO during its oxidoreductive bioconversion and, together with JNK inhibition by the parent molecule, will improve stroke outcome in a model of cerebral reperfusion. Here, we show that indeed, the JNK inhibitor IQ-1S releases NO during its enzymatic metabolism with liver microsomes, resulting in increased serum NO concentration in mice after IQ-1S injection. Moreover, mice treated with IQ-1S demonstrated markedly reduced infarct volume and neurological deficit after 48 h of reperfusion.

## 2. Materials and methods

### 2.1. Compounds

Sodium salt of 11*H*-indeno[1,2-*b*]quinoxalin-11-one (IQ-1S) was synthesized, as described [34], and the structure of the compound was confirmed by NMR and mass spectroscopy [36]. For *in vitro* experiments, IQ-1S was dissolved in dimethyl sulfoxide (DMSO) (EMD Chemicals, Darmstadt, Germany); for animal treatment, IQ-1S was suspended in 10% solutol HS15 (Sigma-Aldrich, St. Louis, MO).

### 2.2. Determination of NO in microsomal suspension and serum

Pooled liver microsomes from male CD-1 mice were obtained from Sigma-Aldrich. IQ-1S was incubated for the indicated times with the microsomal suspension (2 mg/ml) and NADPH (2 mM) in potassium phosphate buffer (100 mM, pH 7.4; 37 °C). Control samples contained DMSO (up to 2%). The incubation was ended by heating the reaction for 5 min at 100 °C, and the mixture was centrifuged (10 min × 10,000 × *g*). Aliquots from the reaction were directly mixed with Griess reagent (equal volumes of 1% sulfanilamide in 0.4 N HCl and 0.1% *N*-(1-naphtyl) ethylenediamine in 0.4 N HCl) to determine $NO_2^-$ [9].

After single intraperitoneal (*i.p.*) injection of IQ-1S (50 mg/kg; the dose was selected based on the total amount received during treatment in the ischemia model, see below), C57BL6/J mice were bled, and sera were separated. NO production in serum was determined by measuring levels of nitrite plus nitrate by the Griess reaction after reduction of nitrate to nitrite by nitrate reductase using a Nitrite/Nitrate Colorimetric Assay Kit (Cayman Chemical, Ann Arbor, MI). Before the reaction, serum samples were ultrafiltered using 30 kDa molecular weight cut-off filters (Amicon).

### 2.3. Cerebral reperfusion model

The transient middle cerebral artery (MCA) occlusion (MCAO) model was performed on 10–12 week old male C57BL6/J mice under anesthesia (1.5% isoflurane in 30% $O_2$ and 70% $N_2O$). A fiberoptic probe was affixed to the skull over the area supplied by the MCA for relative CBF measurements by laser Doppler flowmetry (LDF) before, during, and 30 min after MCAO. Body temperature was monitored continuously, and temperature was maintained at 36.5–37.5 °C with a heating plate (FHC). MCAO was caused by inserting a nylon filament covered by silicon (Doccol) into the internal carotid artery and advancing it to the origin of the MCA for 30 min of ischemia with subsequent reperfusion for 48 h [3]. All procedures were performed in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and approved by the Massachusetts General Hospital Subcommittee on Research and Animal Care.

### 2.4. Evaluation of neurological deficit

Mice were examined after 48 h of reperfusion using a 4-point scale, as described [3]. Normal motor function was scored as 0, flexion of the contralateral torso and forearm on lifting the animal by the tail as 1, circling to the contralateral side as 2, leaning to the contralateral side at rest as 3, and no spontaneous motor activity as 4.

### 2.5. Measurement of infarct volume

After measurements of neurological deficit at 48 h of reperfusion, brains were cut into 2-mm-thick coronal sections and stained with 2% 2,3,5-triphenyltetrazolium chloride (TTC) for 1 h at 37 °C in the dark [2]. The area of infarction in each section was expressed as a fraction of the non-ischemic part of ipsilateral hemisphere (indirect volume of infarct) [2].

### 2.6. IQ-1S treatment

Mice were treated intraperitoneally with IQ-1S (25 mg/kg) suspended in 10% solutol (100 μl) or with vehicle (10% solutol, 100 μl) 30 min before and 24 h after MCAO. This dosing of IQ-1S was selected based on previously published studies that used this compound to target JNK in mouse models *in vivo* [35,36]. This dose regimen represents a combination of prophylactic (IQ-1S administration before ischemia-reperfusion) and therapeutic (after reperfusion) treatments. Because an acute (first minutes after stroke) and delayed treatment (5–7 days after stroke) with JNK inhibitors could have different outcomes after cerebral ischemia [38], 24 h after stroke was used for the second administration of IQ-1S.

### 2.7. Molecular modeling

To evaluate the ability of IQ-1S to permeate the blood-brain barrier (BBB), we calculated parameters important for this permeation [37] using the structure of the neutral form of IQ-1S, which is the most abundant form at physiological pH [35]. The octanol-water partition coefficient aLogP was calculated according to the Ghose-Crippen additive scheme [14] with the use of HyperChem 7.0 software. Polar surface area (tPSA) was obtained by a summation of atomic increments [12]. Number of rotatable bonds ($N_{rot}$) was counted directly in the structural formula of the molecule. Based on calculated parameters (aLogP, tPSA, and $N_{rot}$) the prediction of BBB permeation for IQ-1S was obtained using previously reported classification trees [37].

### 2.8. Statistical analysis

All results are expressed as mean ± S.D. Statistical analysis was performed with t-test or Kruskal-Wallis 1-way analysis of variance on ranks (neurological deficit). Differences of $p < 0.05$ were considered significant.

## 3. Results

### 3.1. IQ-1S releases NO

Previous publications reported that aryl oximes could be metabolized by cytochrome P450 and NO synthase to release NO [1,9,40]. Because of the biological effects of NO during reperfusion, we studied whether the aryl oxime IQ-1S could act as a NO precursor after an *in situ* oxidation catalyzed by endogenous enzymes under physiological conditions (liver microsomes with NADPH and $O_2$). The results indicate that the NADPH-dependent metabolism of IQ-1S by liver microsomes resulted in accumulation of $NO_2^-$, which was formed via oxidation of NO radical (Fig. 1A and B). $NO_2^-$ production increased linearly with increasing concentrations of IQ-1S but not reaching saturation at 100 μM of the compound. In contrast, incubation of the control DMSO with microsomes did not result in accumulation of $NO_2^-$. Production of $NO_2^-$ was also not observed in the absence of NADPH (Fig. 1B) or with heat-inactivated microsomes (5 min at 100 °C; data not shown), indicating that the cytochrome P450 mixed-function oxidase system was involved in the oxidation IQ-1S, in accordance with the general mechanism of other aryl oximes [1,9,40]. Likewise, single administration of IQ-1S (50 mg/kg, i.p.) significantly increased serum nitrite/nitrate concentrations in vivo at 30–60 min after treatment (Fig. 1C).

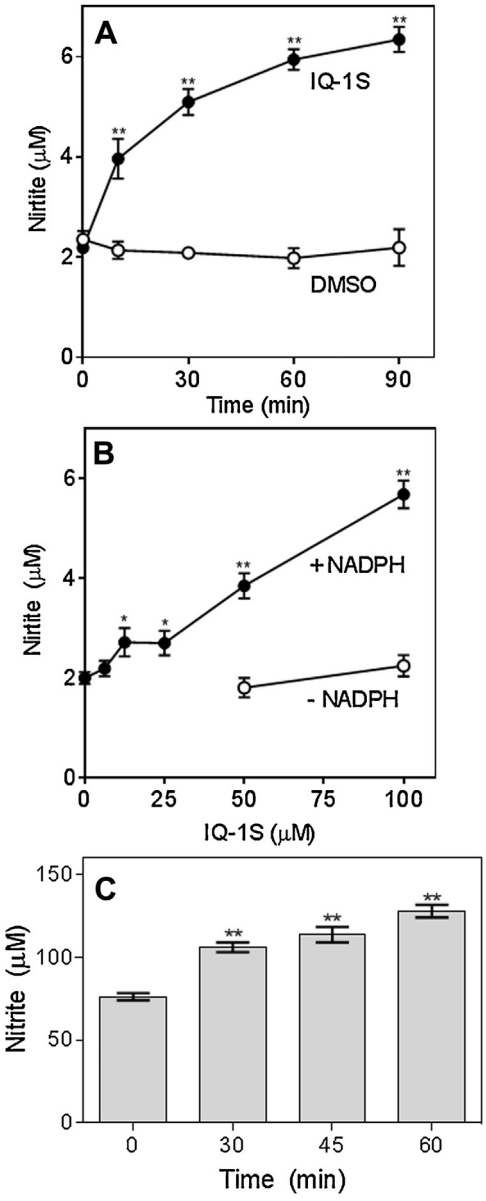


**Fig. 1.** NO production by mouse liver microsomes and in serum.
Panel A. NO production during NADPH-dependent metabolism of IQ-1S by mouse liver microsomes. IQ-1S (100 μM) was incubated with microsomes and 2 mM NADPH for the indicated times, and nitrite production was measured. Panel B. Microsomes were incubated with the indicated concentrations of IQ-1S for 60 min without or with 2 mM NADPH, and nitrite production was measured. Panel C. NO production in serum after IQ-1S injection. IQ-1S was administrated *i.p.* (single dose 50 mg/kg), mice (n = 3) were sacrificed after indicated time, and serum was collected. Serum nitrite levels were evaluated as described. For Panels A and B, values are the mean ± S.D. of triplicate samples from one experiment, which is representative of two independent experiments. Statistically significance differences (*$p < 0.05$; **$p < 0.01$) between IQ-1S- and DMSO-incubated microsomes are indicated. For Panel C, the data are presented as means ± S.D. of triplicate samples from different mice and are representative of two independent experiments. Statistically significance differences (**$p < 0.01$) between animals treated with IQ-1S and vehicle are indicated.

### 3.2. IQ-1S decreased reperfusion injury

To ensure that comparable ischemic insults were reproducibly induced, we used laser Doppler to monitor the decrease in cortical perfusion and confirmed no differences between mouse groups in CBF during MCAO and the first 30 min of reperfusion. Compared to the control untreated and vehicle-treated mice, two injections of IQ-1S administered 30 min before and 24 h after MCAO significantly reduced infarct volumes measured at 48 h after reperfusion (Fig. 2A). Moreover, mice treated with IQ-1S demonstrated less severe neurological deficit after 48 h of reperfusion, as compared to control untreated and vehicle-treated mice (Fig. 2B). In the course of these studies, 3 animals died in each of the control and vehicle-treated groups of mice, whereas 2 animals died in the IQ-1S-treated group. All deaths occurred during the first night after stroke. These animals were excluded from statistical evaluation of infarct volume and neurological deficit.

### 3.3. Computation of blood-brain barrier (BBB) permeability of IQ-1S

Although NO has a good permeability via the BBB, intact IQ-1S is necessary for JNK inhibition in brain tissue. We used a modern machine learning algorithm to predict the BBB permeability-surface area (PS) product (logPS) [33] from physical-chemical descriptors [37]. The values of a LogP and tPSA were found to be

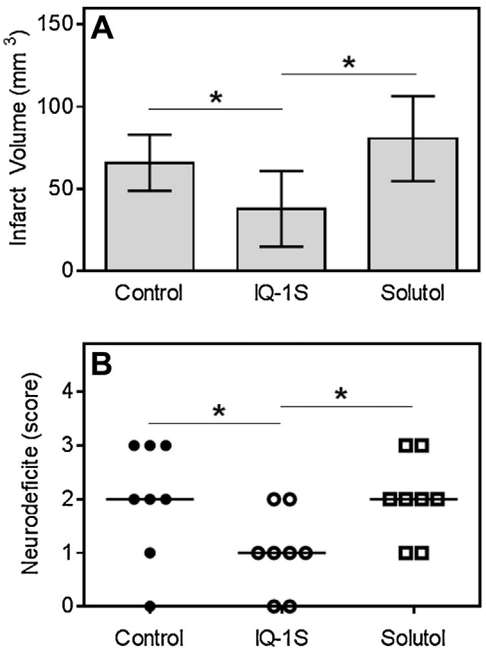


**Fig. 2.** IQ-1S treatment improved stroke outcome. Mice were treated with IQ-1S (*i.p.*, 25 mg/kg) or with vehicle control (10% solutol), 30 min before and 24 h after MCAO or without any treatment (control).
Panel A. IQ-1S decreased infarct volume. After 48 h of reperfusion, the animals were euthanized, and the area of infarction in stained brain sections was measured. n = 8 mice per group. *P < 0.05 *vs.* control and vehicle. Panel B. IQ-1S treatment decreases neurological deficit. Mice were examined for neurological deficits after 48 h of reperfusion using a 4-point scale. n = 8 mice per group.

2.91 and 57.31, respectively. Using these descriptors, together with number of rotatable bonds ($N_{rot}$ = 1) for this molecule and implementation of classification and regression trees, we predict that IQ-1S has strong (CNSp + ) BBB permeation.

## 4. Discussion

JNKs are involved in many neuropathological signaling events and play key roles in regulation of brain tissue survival [26]. For example, increased activity of JNK signaling pathways was observed after global and focal ischemia (for review [21]), including transient ischemia in rat and mouse brain [18]. A variety of small-molecule JNK inhibitors have been reported [24], and some have been shown to exhibit neuroprotective effects in animal models of stroke [8,13], suggesting that JNK inhibition could be a relevant strategy in the therapy of ischemic insult. For example, the most commonly used JNK inhibitor SP600125 demonstrated neuroprotective potential in various models, including cerebral ischemia–reperfusion injury after an acute stroke [13,16,31]. This JNK inhibitor decreased neuronal apoptosis and stroke injury [13,16,31]. However, SP600125 is relatively nonspecific, and 13 of 28 tested kinases were inhibited with similar or greater potency as the JNKs [4]. In contrast, IQ-1S is one of most specific small-molecule, non-peptide JNK inhibitors known and does not inhibit other kinases [35] that play roles in ischemic brain pathological events, including glycogen synthase kinase-3 (GSK-3) [25], phosphatidylinositol 3-kinase (PI3 K) [45], and cyclin-dependent kinase (Cdk) [32].

Previously, we found that intraperitoneal administration of IQ-1S led to a rapid rise in the serum concentration of this compound, with an $AUC_{0-12\,h}$ value of 7.4 $\mu M \times h^{-1}$ [36]. Here, we calculated that IQ-1S likely has strong BBB permeation and showed that IQ-1S could be enzymatically converted to NO by liver microsomes (*in vitro*). Additionally, treatment with IQ-1S led to enhanced NO production *in vivo*. Thus, we suggest that the beneficial effects of IQ-1S on stroke outcome could be a combined result of JNK inhibition by the parent compound in brain tissue, as well as NO generation during its bioconversion.

JNK exacerbates stroke injury by provoking pro-inflammatory cellular signaling and ischemic cell death [29]. For example, high expression of pro-inflammatory cytokines was found in a model of focal cerebral ischemia [15]. Because IQ-1S decreases levels of these cytokines in cultures of monocytes and lymphoid cells and inhibits transcriptional activity of NF-κB/AP-1 [35,36], this compound could protect against brain reperfusion injury via inhibition of pro-inflammatory pathways. Moreover, we recently found that IQ-1S can inhibit expression of matrix metalloproteinase (MMP)-1 and -3 in cultured fibroblast-like synoviocytes [35], and MMPs are known to cause neurovascular damage during early times after stroke [7]. Indeed, significant increases in MMPs levels were observed during the acute stage of reperfusion following transient cerebral ischemia in mice [27]. Clearly, further work is important to evaluate additional JNK-dependent mechanisms involved in therapeutic effects of IQ-1S after focal ischemia.

While IQ-1S is a specific JNK inhibitor, we found here that its enzymatic bioconversion led to NO generation. There is a growing body of evidence that synthetic NO donors have efficacy in animal models of cerebral ischemia. For example, intraperitoneal injection of NO donor Rut-bpy prior to ischemia/reperfusion reduced brain infarct and improved viability of hippocampal neurons [6]. Although the exact mechanisms of NO-induced protection against brain ischemic injuries are not yet clear, NO is known to inhibit the NF-κB pathway in endothelial cells [41]. Similarly, exogenous NO donors attenuated the S-nitrosylation of mixed lineage kinase 3 (MLK3) induced by reperfusion and inhibited activation of the JNK pathway [20]. It should be noted that NO donors could modulate BBB permeability [42], and many attempts have been made to capitalize on this NO mechanism to enhance drug delivery across the BBB [38]. Thus, we suggest that the dual functionality of IQ-1S could thereby potentially enhance neuroprotective activity of this compound during early times after ischemia and reperfusion.

In conclusion, we show here that IQ-1S protects against damage in a mouse model of cerebral reperfusion injury when administered before and during the reperfusion period. We also show that this compound exhibits NO donor activity, which together with previously reported JNK inhibitory activity may contribute to its protective effects. In addition, the known inhibitory effects of IQ-1S on inflammatory cytokine and MMP expression may also contribute to the beneficial therapeutic effects of this compound. Thus, future studies on the relative contributions of vascular and inflammatory mechanisms to the protection by this compound are warranted.

## Conflict of interest

There are no actual conflicts of interest for the authors.

## Acknowledgements

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