Effective Plasma Concentration of a Novel Na\(^+\)/Ca\(^2+\) Channel Blocker NS-7 for Its Cerebroprotective Actions in Rats with a Transient Middle Cerebral Artery Occlusion

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ABSTRACT

The effect of a novel Na\(^+\)/Ca\(^2+\) channel blocker NS-7 [4-(4-fluorophenyl)-2-methyl-6-(5-piperidinopentyloxy) pyrimidine hydrochloride] on the cerebral infarction, edema, and mortality was examined in rats with a transient middle cerebral artery occlusion (MCAO), and the effective plasma concentration of this compound for producing the cerebroprotective action was subsequently determined. MCAO was occluded by inserting a thread through internal carotid artery for 2 h, and then recirculated for 6 h. NS-7 (0.125–1 mg/kg), when injected i.v. immediately after recirculation, significantly reduced the infarct volume as well as the cerebral edema. Delayed treatment with NS-7 at 1 h after recirculation produced an equivalent inhibition of the infarction, and was still effective, although to a lesser extent, when injected at 2 h but not 3 h after recirculation. Glycerol (4 g/kg) suppressed the cerebral edema but did not reduce the size of cerebral infarction in the cerebral cortex or striatum. Therefore, it is likely that the suppression of brain edema does not always lead to the reduction of the infarct size. NS-7 treated in combination with glycerol further decreased the water content in the occluded brain. Moreover, NS-7 significantly lowered the mortality observed up to 10 days after a transient MCAO. From these data, it is suggested that the presence of NS-7 in plasma during 1 to 3 h after recirculation is important for producing the neuroprotective action. To determine the pharmacologically effective plasma concentration of NS-7, the effect of continuous infusion of this compound on the cerebral infarction was examined. Infusion of NS-7 at 0.3 mg/kg over 2 h, starting immediately after recirculation, significantly reduced the infarct size. Its plasma concentration during 1 to 3 h was 14.5 to 28.5 ng/ml (36.9–72.3 nM). From these findings it is suggested that NS-7 has a potent anti-infarct action in addition to antiedema action in the rat transient MCAO model. Moreover, its effective plasma concentration was assumed to be 36.9 to 72.3 nM.

The cellular mechanisms underlying the ischemic brain damage have not yet been clarified. Excitotoxic role of glutamate and intracellular Ca\(^2+\) overload have still been considered as the major cause of neuronal degeneration after cerebral ischemia (Orrenius et al., 1989; Choi, 1990, 1992; Kristián and Siesjö, 1998). Several lines of evidence have shown that the activation of voltage-gated Na\(^+\) as well as Ca\(^2+\) channels are involved in the excessive glutamate release and subsequent elevation of the intracellular Ca\(^2+\) concentration under cerebral ischemia (Gembá et al., 1993; Graham et al., 1993; Barone et al., 1995; Taylor and Meldrum, 1995). Indeed, several Na\(^+\) channel blockers (Lysko et al., 1994; Ratand et al., 1994) and Ca\(^2+\) channel blockers, particularly N-type and P/Q-type Ca\(^2+\) channel blockers (Buchan et al., 1994; Asakura et al., 1997), have been reported to cause neuroprotection in animal models of focal as well as global cerebral ischemia.

NS-7 [4-(4-fluorophenyl)-2-methyl-6-(5-piperidinopentyloxy) pyrimidine hydrochloride] has been developed in our laboratories as a neuroprotective agent. This compound blocks both voltage-gated Na\(^+\) and Ca\(^2+\) channels: it inhibits Na\(^+\) and Ca\(^2+\) currents through L-type and N-type Ca\(^2+\) channels in NG108-15 cells (Suma et al., 1997), displaces the binding of \(^[^{3}H] \)batrachotoxin, a specific ligand for the neurotoxin receptor site2 of Na\(^+\) channel, in rat brain (Shimidzu et al., 1997), and blocks KCl-induced activation of nitric-oxide synthase through blockade of both L-type and P/Q-type Ca\(^2+\) channels in primary cultured neurons (Oka et al., 1999). We have previously shown that the exposure of the rat cerebrocortical slices to hypoxia and glucose deprivation causes an enhancement of nitric-oxide synthase activity and induces the leakage of lactate dehydrogenase after reoxygenation, both of which are attenuated by NS-7 (Oka et al., 2000). Moreover, in rats with permanent middle cerebral artery occlusion (MCAO), a single bolus injection of NS-7 immediately after occlusion inhibits the activation of a Ca\(^2+\)-activated neutral protease calpain and reduces the size of cerebral infarction (Takagaki et al., 1997). However, the in vivo...
neuroprotective action of NS-7 has been shown only in the rat permanent MCAO model and there have been hitherto no published data on the evaluation of this compound for the neuroprotective efficacy in other models of cerebral ischemia.

In the present study, the rat MCA was transiently occluded by inserting a nylon filament through the internal carotid artery for 2 h, and then recirculated for 6 h, and the effect of NS-7 on the cerebral infarction, edema, and mortality caused by the transient MCAO was investigated. We also examined the effect of an osmotic agent glycerol on both the cerebral edema and infarction to determine whether the inhibition of edema formation is associated directly with the reduction of the infarct size. In addition, the minimal effective concentration of plasma NS-7 for producing the cerebroprotective action was also determined to compare with the concentrations that had been shown to block Na\(^+\) and Ca\(^{2+}\) channels in a variety of neurochemical and electrophysiological studies.

**Materials and Methods**

**Chemicals.** NS-7 and its analog 4-(4-fluorophenyl)-2-methyl-6-(5-piperidinoxy) pyrimidine hydrochloride were synthesized in our laboratories. Other chemicals and drugs were obtained from commercial sources: halothane (FLOSEN; Takeda Pharmaceutical, Osaka, Japan), 2,3,5-triphenyl tetrazolium chloride (TTC; Nalacal Tesque, Kyoto, Japan), and glycerol (glycerin; Wako Pure Chemical, Osaka). NS-7 was dissolved in physiological saline (Otsuka Pharmaceutical Co., Ltd., Tokushima, Japan) and injected intravenously in a volume of 1 ml/kg of body weight. Glycerol was diluted with physiological saline to the concentration of 4 g/5.3 ml, and infused intravenously over 3 min using the syringe pump (Harvard 22 syringe pump; Harvard Apparatus, Holliston, MA).

**Animals.** Male Sprague-Dawley rats (Japan SLC, Hamamatsu, Japan) of 7 weeks of age at the time of MCAO were used in the experiment. Animals were housed in groups in a room whose environment was maintained at 21–25°C, 45–65% humidity, and 12-h light/dark cycle (lights automatically on at 8:00 AM). They had free access to pellet chow and water. Experiments were all carried out in a light/dark cycle (lights automatically on at 8:00 AM). They had free

**MCAO.** The right MCA was transiently occluded, according to the method of Koizumi et al. (1986). Briefly, rats were anesthetized by inhalation of 2% halothane (a mixture of 70% nitrogen and 30% oxygen) and placed on a stereotaxic apparatus in a supine position. Anesthesia was maintained thereafter with 1% halothane. After median section of the neck, the right carotid artery branch was carefully exposed so as not to injure the vagal nerves. Focusing on the right carotid artery branch, the common carotid artery and external carotid artery were retracted from the peripheral connective tissue, and ligated by a suture. Then, the internal carotid artery was isolated and its origin was fixed with a suture to prepare for ligation and fixation after insertion of a nylon filament. Both ends of 4-0 surgical nylon filament (Ethilon; Ethicon, Tokyo, Japan) of 18-mm length was rounded by heat to a globe of about 0.2 mm in diameter, and used as an embolizer. Subsequently, the common carotid artery was resected and a nylon suture was inserted into the internal carotid artery to a distance of 16 to 18 mm. The proximal end of nylon suture was ligated and fixed to the internal carotid artery. By the above-mentioned procedure, the tip of nylon filament passed through the MCA branch and entered the origin of anterior cerebral artery for a distance of 0.5 to 1.0 mm so that the origin of MCA was completely occluded. After 2 h of MCAO, the blood was recirculated for 6 h by withdrawing the nylon filament. In our preliminary study, the cerebral infarction was variable in size and was not reproducible after MCAO for less than 2 h followed by 6 h of reperfusion.

**Measurement of Infarct Size.** At 6 h after recirculation, animals were sacrificed by decapitation and the brain was quickly removed. Seven serial coronal brain sections of 2-mm thickness were prepared starting from the frontal edge of the cerebrum. The infarct area was determined by TTC staining, in which each brain section was transferred to a tube containing 2% TTC saline solution and incubated at 37°C for 30 min. Subsequently, the images of TTC-stained sections were captured into the computer. The infarct area, which was not stained red, was measured both in the cerebral cortex and striatum by using the image-processing software Win ROOF (Mitani Co., Chiba, Japan). In a set of experiments where the effective plasma concentration of NS-7 for reducing the infarct size was determined, the total (both cortical and striatal) infarct area in the ipsilateral brain was measured. The infarct volume was calculated from the infarct area (mm\(^2\)) in each brain section by using a trapezoidal approximation method.

**Measurement of Cerebral Edema.** Rats were decapitated at 6 h after recirculation and brains were quickly removed. A coronal brain section whose anterior border is the optic chiasma and posterior edge is the caudal part of the mammillary body, and the cerebral cortex and striatum were bilaterally dissected from the section. Each brain tissue was placed on a piece of aluminum foil and separately weighed. It took approximately 30 s for this procedure. During this time, the reduction in tissue weight due to desiccation was less than 0.2%, which was considered to be negligible. Each hemisphere was subjected to freeze-drying for 72 h to obtain the dry weight. The tissue water content was expressed as the percentage of the wet tissue weight as follows: % water = (wt wet – dry weight) / wet weight.

**Determination of NS-7 in Plasma.** In another set of experiments where the plasma concentration of NS-7 was determined during and after its intravenous infusion, rats were subjected to MCAO for 2 h, followed by recirculation for 6 h. NS-7 (0.1, 0.2, or 0.3 mg/kg) was infused over 2 h through the femoral vein using the syringe pump (Harvard 22 syringe pump), starting immediately after recirculation. Blood specimens of 0.8 ml each were taken from the cervical vein at 1, 2, and 3 h after the start of NS-7 infusion. At 6 h, rats were deeply anesthetized with ethyl ether and blood was taken from abdominal aorta. Plasma NS-7 was determined by HPLC with electrochemical detection. Briefly, to 0.2-ml aliquot of plasma, 0.8 ml of 100 mM Na\(_2\)HPO\(_4\), 0.02 ml of methanol containing 4-(4-fluorophenyl)-2-methyl-6-(5-piperidinoxy) pyrimidine hydrochloride (400 ng/ml) as the internal standard, and 8 ml of n-hexane were added. The mixture was shaken for 10 min, and then centrifuged at 3000 rpm for 10 min. The resultant n-hexane layer was taken and dried under vacuum. The residue was dissolved in 0.05 ml of the solvent used as the mobile phase for HPLC, and 0.03-ml aliquot of the solution was injected into HPLC. The HPLC system was composed of a working processor (Class-LC10; Shimadzu, Kyoto, Japan), solvent-delivery pump (LC-10AD; Shimadzu), automatic sample injection system (SIL-10A; Shimadzu), coulometric electrochemical detector (Coulochem II, ESA Inc., Bedford, MA), and the separation column (Inertsil ODS-3, 4.6 mm inside diameter × 150 mm; GL Sciences Inc., Tokyo, Japan). The column temperature was set to 40°C. Mobile phase was the mixture of 0.03 M KH\(_2\)PO\(_4\)/acetonitrile (2:1, v/v) and delivered at the flow rate of 1.0 ml/min. The electrochemical detector consisted of three flow cell units, including guard cell, first, and second cells, the working electrodes of which were set to 950, 400, and 850 mV, respectively. The sensitivity was set to 500 nA in full scale and the gain of electrical signals was 100 mV.

**Statistical Analysis.** Statistical analysis was performed by using SAS system (SAS Institute Inc., Cary, NC). Unless otherwise indicated, the size of cerebral infarction was compared between control and NS-7-treated groups and data were statistically evaluated by Dunnett’s test for multiple comparison or by Student’s t test for comparison between two groups (control versus drug-treated). In other sets of experiments where the effects of combined treatment
with NS-7 and glycerol on the cerebral infarction and cerebral edema were examined, the differences in values among experimental groups were statistically analyzed by Tukey’s multiple range test. Data on mortality were compared between control and NS-7-treated groups, and the statistical analysis was performed by Fisher’s exact test.

Results

**Effect of NS-7 Treated Immediately after MCAO on the Cerebral Infarction.** The infarct size was measured at 6 h after start of recirculation. The infarction was noted in the ipsilateral cerebral cortex and striatum corresponding to the region dominated by MCA. In particular, a large portion of the striatum was injured. NS-7 (0.0625–1 mg/kg), when injected i.v. immediately after the recirculation, reduced the size of the infarction in a dose-dependent manner both in the cerebral cortex and striatum, and the significant effect was observed at 0.125 mg/kg ($P < 0.05$ both in the cerebral cortex and striatum) and higher doses ($P < 0.01$ in both regions) (Fig. 1).

**Time-Window Study on the Cerebroprotective Effect of NS-7.** Figure 2 shows the effect of delayed treatment with NS-7 (0.25 mg/kg) after recirculation on the infarct size caused by a transient MCAO. The delayed injection of NS-7 at 0.5 to 1 h after recirculation produced an almost equivalent reduction in the infarct size as that observed by the injection immediately after recirculation. NS-7, when injected at 2 h after recirculation, was still effective ($P < 0.01$ in both brain regions), although to a lesser extent, on the cerebral infarction, but was no longer effective in case it was treated at 3 h after recirculation.

**Inhibitory Effect of NS-7 on Cerebral Edema.** A marked increase in the water content was observed in the ipsilateral cerebral cortex and striatum at 6 h after recirculation. NS-7 injected at a dose of 0.25 mg/kg immediately after recirculation significantly lowered the water content both in the occluded cerebral cortex ($P < 0.05$) and striatum ($P < 0.01$) without changing the water content in the nonoccluded brain regions (Fig. 3). Glycerol (4 g/kg) also significantly inhibited the cerebral edema in both brain regions ($P < 0.05$ in the cerebral cortex; $P < 0.01$ in the striatum), but significantly lowered the water content in nonoccluded cerebral cortex ($P < 0.01$). It was noteworthy that the combined treatment with NS-7 and glycerol further decreased the water content in the occluded cerebral cortex, which was significant compared with either NS-7 alone ($P < 0.01$) or glycerol alone ($P < 0.01$).

**Effects of Glycerol, NS-7, and Their Combination on the Cerebral Infarction.** The effect of glycerol injected alone or in combination with NS-7 on the cerebral infarction was examined. As shown in Fig. 4, NS-7 at 0.25 mg/kg again significantly ($P < 0.01$) reduced the infarct volume in the cerebral cortex induced by a transient MCAO. In contrast, glycerol (4 g/kg) did not reduce the size of infarction in either brain regions, nor did it have any influence on the inhibitory
Effect of NS-7 on the cerebral infarction when injected in combination.

Effect of NS-7 on the Mortality Induced by a Transient MCAO. More than 80% of animals died within 24 h after the recirculation. After the recovery from anesthesia, these animals exhibited neurological symptoms, such as circling and hemi-paralysis of the contralateral forelimbs. However, the paralysis of forelimbs disappeared several hours after the recirculation. Tonic convulsion was noted before death. Figure 5 shows the effect of NS-7 on the mortality induced by a transient MCAO. NS-7, when injected immediately after recirculation at doses of 0.125 or 0.25 mg/kg, significantly decreased the mortality observed up to 10 days after a transient MCAO.

Estimation of the Effective Plasma Concentration for Producing a Neuroprotective Action. To determine the effective plasma concentration of NS-7 for producing a cerebroprotective action, the effect of the continuous infusion of NS-7 at doses of 0.1 to 0.3 mg/kg over 2 h, starting immediately after recirculation, on the cerebral infarction was examined in rats with a transient MCAO. As shown in Fig. 6B, NS-7 at 0.3 mg/kg/2 h significantly reduced the size of cerebral infarction, although the compound at lower doses (0.1–0.2 mg/kg/2 h) produced no significant protective action (Fig. 6A). Subsequently, the plasma concentration during and after NS-7 infusion was monitored. The average of the plasma concentrations during 1 to 3 h after recirculation were 5.1 to 8.1 ng/ml at 0.1 mg/kg/2 h, 11.0 to 19.4 ng/ml at 0.2 mg/kg/2 h, and 14.5 to 28.5 ng/ml at 0.3 mg/kg/2 h (Fig. 7). Therefore, the minimal pharmacologically effective concentration of plasma NS-7 is assumed to be 14.5 to 28.5 ng/ml (36.9–72.3 nM) in the present transient MCAO model.

Discussion

We have previously shown that NS-7 reduces the infarct size caused by permanent MCAO in rats (Aoki et al., 1997; Takagaki et al., 1997). In the present study, the cerebroprotective effect of NS-7 was evaluated in a transient MCAO model that was developed by Koizumi et al. (1986) as a simple and reproducible model of transient focal cerebral ischemia. In this model, the cerebral infarction was observed both in the cerebral cortex and striatum after recirculation, but it developed much earlier than that elicited after permanent MCAO, reaching approximately 80% of the maximal infarction at 6 h after recirculation. Moreover, the ischemic damage was so severe that more than 80% of animals died within 24 h after recirculation. It is likely that the death is attributable to the compression of the brain stem caused by the marked cerebral edema. The neurological symptoms such as circling and hemiplegia were also more serious than those observed after permanent MCAO (Aoki et al., 1997).

In the present study, NS-7 inhibited the development of the cerebral infarction as well as the cerebral edema caused by a transient MCAO in a dose-dependent manner. Moreover, a significant cerebroprotective action was still observed,
when it was injected later at 2 h after recirculation. The hyperosmolar substance glycerol also reduced the cerebral edema, although the substance markedly lowered the water content in nonoccluded brain, which was a contrast to the action of NS-7. Glycerol has been used for the alleviation of the cerebral edema in patients with acute cerebral infarction (Meyer et al., 1971). It has also been shown that glycerol improves the neurological status in patients with cerebral infarction by enhancing the regional cerebral blood flow in the ischemic brain secondary to the reduction of focal cerebral edema, and possibly by becoming an alternative source of brain energy (Meyer et al., 1972, 1975). In contrast, the deleterious effect of glycerol on the ischemic brain has been reported by Bralet et al. (1983) who showed in the rat microembolic MCAO model that glycerol causes the impairment of cerebral reperfusion by increasing brain lactate level. In the present study, glycerol did not affect the cerebral infarction caused by a transient MCAO, in spite of the marked reduction in the cerebral edema. Moreover, the anti-infarct action of NS-7 was not influenced by glycerol, although the inhibitory effect of NS-7 on the cerebral edema was significantly potentiated by glycerol. Therefore, it is unlikely that the anti-infarct action of NS-7 is attributable to the reduction of water content in the ischemic brain. The mortality was also dramatically lowered by NS-7. Taken together, the present findings indicate that NS-7 is an effective neuroprotective agent in the transient MCAO model.

Subsequently, the effective plasma concentration of NS-7 for producing the cerebroprotective action was determined to examine the involvement of Na\(^+\) and Ca\(^{2+}\) channel blockade that had been shown in a variety of neurochemical and electrophysiological studies in the cerebroprotective action of this compound. In the present study, NS-7 produced an almost similar reduction in the infarct size, when treated within 1 h after recirculation, and was still effective, although less markedly, in case it was injected at 2 h after recirculation. However, the delayed injection of NS-7 at 3 h after recirculation was no longer effective, thereby indicating that the therapeutic time-window is within the first 3 h after recirculation. In addition, it seems likely that the presence of this compound in plasma during 1 to 3 h after recirculation is necessary to produce a cerebroprotective action. In the present study, the continuous infusion of NS-7 over 2 h at a dose of 0.3 mg/kg caused a marked and significant reduction in the size of cerebral infarction. Its plasma concentration gradually increased up to 2 h after the start of its infusion, and the concentration during 1 to 3 h after recirculation was 14.5 to 28.5 ng/ml (36.9–72.3 nM). Thus, these values are assumed to be the minimal effective plasma concentrations of NS-7 for producing the cerebroprotective action in a transient MCA occlusion model.

Although the precise mechanisms of cerebroprotective action of NS-7 remain to be clarified, our previous data have shown in the in vitro hypoxic injury model that the blockade of both Na\(^+\) and Ca\(^{2+}\) channels contributes to the cerebroprotective action of NS-7 (Tatsumi et al., 1998). However, the effective plasma concentration determined here was much smaller than values for blocking Na\(^+\) and Ca\(^{2+}\) channels in vitro: NS-7 inhibits the tetrodotoxin-sensitive Na\(^+\) current in a whole-cell patch-clamp study using NG108-15 cells with an IC\(_{50}\) value of 7.8 \(\mu\)M determined at the holding potential of −80 mV (Suma et al., 1997), and this effect is dependent on the membrane potential, in which the inhibition is potentiated in case it is measured at the holding potential of −60 mV (IC\(_{50}\) value of 3.6 \(\mu\)M), and is also frequency- and use-dependent (M. Oka, S. Hayashi, and Y. Itoh, unpublished data). In addition, NS-7 displaces the binding of \([\text{H}]\)batrachotoxin, a specific ligand for the Na\(^+\) channel neurotoxin receptor site 2, which is located within the transmembrane segment of the channel moiety, with a \(K_i\) value of 1 \(\mu\)M (Shimidzu et al., 1997), thereby suggesting that the site of action of NS-7 is within the neuronal membrane. On the other hand, NS-7 blocks the Ca\(^{2+}\) currents through L-type and N-type Ca\(^{2+}\) channels with IC\(_{50}\) values of 4.5 and 7.3 \(\mu\)M, respectively, in a whole-cell patch-clamp configuration (Suma et al., 1997). Moreover, this compound inhibits the L-type Ca\(^{2+}\) channel- and P/Q-type Ca\(^{2+}\) channel-mediated nitric-oxide synthesis with IC\(_{50}\) values of 2.5 and 3.1 \(\mu\)M, respectively, in primary neuronal culture of the mouse cerebral cortex (Oka et al., 1999).

Like the action on Na\(^+\) channel, this Ca\(^{2+}\) channel blockade is dependent on the stimulus intensity, and remains after washout, suggesting that the site of action exists within the cell membranes. Therefore, the brain concentration but not plasma level of NS-7 should be taken into account for discussing the pharmacologically effective concentration in relation to the mechanisms of actions such as Na\(^+\) and Ca\(^{2+}\) channel blockade. We have previously reported that the brain concentrations of NS-7 are always approximately 20 times higher than that in plasma at various times measured from 5 min to 12 h after its intravenous injection in rats (Itoh et al., 1997). Moreover, the subcellular fractionation of brain tissues after intravenous injection of NS-7 has shown a predominant distribution to membrane-enriched synaptosomal P2 fraction (Shimidzu et al., 1997). Although we did not measure the brain concentration of NS-7 after its continuous infusion at a dose of 0.3 mg/kg/2 h in the present study, the minimum effective brain concentration of NS-7 is assumed to be 0.74 to 1.45 \(\mu\)M, based on the presumption that brain NS-7 concentration is approximately 20 times higher than the plasma concentration (Itoh et al., 1997). These values are comparable to \(K_i\) or IC\(_{50}\) values for blocking the above-described Na\(^+\) and Ca\(^{2+}\) channels. Therefore, it is suggested...
that the blockade of Na\(^+\) and Ca\(^{2+}\) channels indeed contributes to the in vivo cerebroprotective action of NS-7.

In conclusion, we investigated here the effect of a Na\(^+\)/Ca\(^{2+}\) channel blocker NS-7 on the cerebral infarction, cerebral edema, and mortality in rats with a transient MCAO. The pharmacologically effective concentration of this compound was subsequently determined. NS-7 reduced the infarct size, cerebral edema, and the mortality in transiently MCA-occluded rats in a dose-dependent manner. The protective effect of NS-7 was still observed after its delayed treatment at 2 h after recirculation. Glycerol markedly lowered the water content in both occluded and nonoccluded brains but had no influence on the cerebral infarction. Combined treatment with NS-7 and glycerol further decreased the water content in occluded brains, although the extent of the anti-infarct action was almost comparable to that induced by NS-7 alone. The effective plasma concentration was assumed to be 14.5 to 28.5 ng/ml (36.9–72.3 nM). Since the concentration in occluded brains, although the extent of the anti-infarct action was almost comparable to that induced by NS-7 alone. The effective plasma concentration was assumed to be 14.5 to 28.5 ng/ml (36.9–72.3 nM). Since the concentration in occluded brains, although the extent of the anti-infarct action was almost comparable to that induced by NS-7 alone. The effective plasma concentration was assumed to be 14.5 to 28.5 ng/ml (36.9–72.3 nM).

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