Na⁺/H⁺ Exchanger Inhibitor, SM-20220, Is Protective Against Excitotoxicity in Cultured Cortical Neurons

Yuji Matsumoto, MS; Seiji Yamamoto, MD, PhD; Yasuhiro Suzuki, PhD; Takashi Tsuboi, PhD; Susumu Terakawa, MD, PhD; Naohito Ohashi, PhD; Kazuo Umemura, MD, PhD

Background and Purpose—Recently, it has been reported that Na⁺/H⁺ exchanger (NHE) inhibitors demonstrated protective effects on ischemia/reperfusion brain injury in animal models. However, the mechanisms by which the neurons were protected against ischemic insult remain unclear. To reveal the cellular mechanism of the NHE inhibitor on the neuronal death, we examined the effects of a selective NHE inhibitor, SM-20220 (N-[aminoiminomethyl]-1-methyl-1H-indole-2-carboxamide methanesulfonate), on glutamate-induced neuronal death in rat cortical culture.

Methods—Cortical neurons were prepared from 1-day old rats, and cultured on the glass-based dishes. Glutamate-induced neuronal death was assessed by staining the cells with propidium iodide. Morphological changes in the neurons were observed with a video-enhanced contrast-differential interference contrast microscope. The intracellular calcium concentration ([Ca²⁺]i) and the intracellular pH (pH i) were measured by fluorescence imaging with a confocal laser microscope using fluo-3-acetoxymethyl ester (AM) and 2′, 7′-bis-2-carboxy-ethyl-5(6)-carboxyfluorescein (BCECF)/AM as a fluorescent dye, respectively.

Results—SM-20220 (0.3 to 30 nmol/L) dose-dependently attenuated glutamate (300 μmol/L)-induced neuronal death over a period of 6 hours, and inhibited the acute cellular swelling following glutamate (500 μmol/L) exposure. Dual peaks of [Ca²⁺]i rise were observed at 5 and 12 minutes after glutamate (500 μmol/L) exposure, followed by a persistent rise. SM-20220 suppressed the persistent [Ca²⁺]i increase. SM-20220 inhibited intracellular acidification following glutamate (500 μmol/L) exposure. All of the events induced by glutamate were also inhibited by the N-methyl-D-aspartate receptor antagonist, MK-801, indicating the death process was excitotoxicity.

Conclusions—NHE inhibitor is neuroprotective through inhibition of both persistent [Ca²⁺]i increase and acidification in excitotoxicity. (Stroke. 2004;35:185-190.)

Key Words: excitotoxicity ■ glutamates ■ Na⁺/H⁺ exchanger ■ neuronal death ■ rats

B rain neurons are vulnerable to hypoxia/ischemia, which often occurs during stroke and seizure. In neurons, ischemia causes the release of the excitatory amino acid neurotransmitter, glutamate, which activates the receptor-dependent Ca²⁺ channel, resulting in Ca²⁺ overload. The increase in intracellular Ca²⁺ concentration ([Ca²⁺]i) leads to a cascade of events, which can precipitate necrosis and/or apoptosis of susceptible neurons. Further, ischemia causes cellular acidosis by disturbing the energy metabolism. Glutamate also produces intracellular acidosis in cultured neurons.

In the brain, the Na⁺/H⁺ exchanger (NHE), a ubiquitous protein, is essential for intracellular pH (pH i) regulation under both physiological and pathological conditions. While glutamate receptors effectively increase intracellular Na⁺, the intracellular acidosis activates the NHE to extrude the proton from the cell, resulting in the Na⁺ influx followed by cellular swelling. The increase in Na⁺ influx reverses the Na⁺/Ca²⁺ exchange system to protrude Na⁺, and then leads to Ca²⁺ overload. This can deteriorate the process of neuronal death, whereas it remains controversial. Thus, inhibition of the NHE should improve not only Na⁺-mediated cellular swelling but also Ca²⁺ overload, and thereby, cell death. Although recent reports have indeed demonstrated the protective effects of NHE inhibitors on ischemia/reperfusion brain injury in animal models, the mechanism underlying the neuroprotection remains unclear.

We therefore examined the effects of a newly synthesized selective NHE inhibitor, SM-20220, (N-[aminoiminomethyl]-1-methyl-1H-indole-2-carboxamide methanesulfonate), on glutamate-induced neuronal death, Ca²⁺ overload, cellular swelling, and intracellular acidification in rat-cultured cortical neurons. SM-20220 has very little effect against the Na⁺, K⁺, and Ca²⁺ (L and N type) channels, and α-amino-3-hydroxy-5-methyl-4-isoxazolepropoionate (AMPA), kainate, and N-methyl-D-aspartate (NMDA) receptors even at a con-
Materials and Methods

Primary Culture of Cortical Neurons

All experiments were performed according to the guidelines for the care and use of animals established by the Physiological Society of Japan. The experimental protocol was approved by the Committee on Ethics of Animal Experimentation in Hamamatsu University School of Medicine.

Cortical neurons were prepared from 1-day-old Wistar rats as described in our publication.18 In brief, after ether anesthesia, the cortices were removed, dissected out, dissociated, and plated on collagen-coated culture dishes (Iwaki Glass Base Dish, Asahi Techno Glass Co). The culture medium was a mixture of Dulbecco modified Eagle medium (50%; Gibco No. 31600, Invitrogen Japan K.K.), Hank’s balanced salt solution (25%; Gibco No. 11201), and horse serum (25%). Glucose and 2-[4-(2-hydroxyethyl)-1-piperaziny1]ethanesulfonic acid (HEPES) were added at a final concentration of 36.1 mmol/L and 23.7 mmol/L, respectively. The pH of the medium was adjusted to 7.2. Penicillin-G, streptomycin, and 10 μmol/L cytosine arabinoside were added to the culture medium. The cells were maintained at 37°C with a 5% CO₂-containing atmosphere for 10 to 14 days, then used for experiments.

Induction of Excitotoxicity and Assessment of Neuronal Death

Glutamate excitotoxicity was induced in primary cultures as described.19 After washing the cultures with artificial cerebrospinal fluid (aCSF)—NaCl, 140 mmol/L; KCl, 5 mmol/L; MgCl₂, 1.2 mmol/L; CaCl₂, 2mmol/L; glucose, 10 mmol/L; and HEPES, 10 mmol/L (pH 7.2)—they were incubated at 37°C for 6 hours with aCSF-containing glutamate (300 μmol/L). In some experiments, SM-20220 or MK-801, an NMDA receptor antagonist, was added to the aCSF 20 minutes before glutamate exposure.

The mortality of the cultures was assayed by using propidium iodide (PI),19 which does not penetrate viable cells but enters injured cells, yielding the red PI staining. At the end of glutamate exposure, the cultures were incubated with aCSF-containing PI (3 μmol/L) at 37°C for 15 minutes. To assess the neuronal death, we employed a differential interference contrast (DIC) microscopy (Axiovert 10, Zeiss) with a 20× DIC objective lens and fluorescence optics (excitation at 515 nm, emission at 620 nm). The DIC image of the cells and the fluorescence image of PI were obtained with a 3-chip colored charge-coupled device (CCD) camera (C5810, Hamamatsu Photonics.). The percentage of neuronal death was calculated by counting the number of all neurons (in the DIC mode) and that of the neurons stained with PI (in the fluorescence mode). Under the microscope, 3 fields (30 to 50 neurons per field) were chosen at random in each dish.

Morphological Observation

The morphological changes of the neurons were examined under the DIC microscope. The DIC image was obtained with a CCD camera (C6489, Hamamatsu Photonics), contrast-enhanced digitally in real time by using an image processor (ARGUS 20, Hamamatsu Photonics), then monitored, and recorded on a videocassette. A dish containing cells was superfused with aCSF (34°C) for 5 minutes (0.5 mL/min), followed by superfusion with aCSF-containing glutamate (500 μmol/L) combined with drugs or vehicles. To analyze the cellular swelling, the area of the cell was measured before application of glutamate to establish the baseline value, and subsequently calculated as a percentage of the baseline.

Measurement of [Ca²⁺] and pH

[Ca²⁺] was measured by fluorescence imaging with fluo-3 or Calcium Orange-5N, and pH with BCECF (2’,7’-bis-2-carboxy-ethyl-5-(6)-carboxyfluorescein).20 Neurons were rinsed 3 times and incubated at 37°C with aCSF-containing fluo-3/aceotoxymethylene (AM) (4.4 μmol/L) for 20 minutes or Calcium Orange-5N/AM (5 μmol/L) for 30 minutes, or with BCECF/AM (1 μmol/L) for 10 minutes. After 3 rinses with aCSF, the cells were observed with a microscope (IX 70, Olympus) equipped with a confocal scanner of a microscopes-adjacent Nipkow-disk (CSU-10, Yokokawa Electric Co). After recording the control images, the neurons were exposed to aCSF (34°C)-containing 500 μmol/L, and the fluorescence images were taken for 20 minutes. Since the continuous perfusion under the confocal microscope interfered the focus, the neurons were exposed to glutamate by dropping aliquot (1 μL) out of the observation field. The pilot study revealed that the time for diffusion was about 2 minutes. After recording the control data, fluorescence images of the neurons were taken for 20 minutes. In some experiments, drugs or vehicles were added to the medium 20 minutes before glutamate application.

Chemicals

MK-801 and PI were purchased from Sigma. Fluo-3/AM and BCECF/AM were obtained from Dojindo, and Calcium Orange-5N/AM obtained from Molecular Probe. SM-20220 synthesized at the Sumitomo Pharmaceutical Research Center was dissolved in 8% polyethylene glycol 400 (PEG) just before the experiments, and used at the final concentration of 0.008% PEG.

Statistical Analysis

Data are presented as the mean ± SD and evaluated using Student’s t test or 1-way analysis of variance (ANOVA). Group comparisons were made using Dunnett’s multiple comparison test. A P value <0.05 was considered significant.

Results

SM-20220 Reduces Glutamate-Induced Neuronal Death

Under the fluorescence microscope, PI stained dead neurons red. Exposure to 300 μmol/L glutamate resulted in neuronal

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Inhibitory Effect of SM-20220 on Radioligand Bindings for Ca²⁺ Channels and Glutamate Receptors

<table>
<thead>
<tr>
<th>Ion Channels/Receptors</th>
<th>IC₅₀ (μmol/L)</th>
<th>Sources</th>
<th>Radioligands</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-type Ca²⁺ channel</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Benzothiazepine-sensitive</td>
<td>5.6×10⁻⁶</td>
<td>Rat cortex</td>
<td>[³H] diltiazem</td>
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<tr>
<td>Dihydropriidine-sensitive</td>
<td>&gt;10⁻⁴</td>
<td>Rat cortex</td>
<td>[³H] nitrendipine</td>
</tr>
<tr>
<td>Phenylalkylamine-sensitive</td>
<td>&gt;10⁻⁴</td>
<td>Rat brain</td>
<td>[³H] (-)-D8888†</td>
</tr>
<tr>
<td>N-type Ca²⁺ channel</td>
<td>&gt;10⁻⁴</td>
<td>Rat brain</td>
<td>[¹²⁵I] ω-conotoxin</td>
</tr>
<tr>
<td>Glutamate receptors</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AMPA</td>
<td>&gt;10⁻⁴</td>
<td>Rat cortex</td>
<td>[³H] AMPA</td>
</tr>
<tr>
<td>Kainate</td>
<td>&gt;10⁻⁴</td>
<td>Rat brain</td>
<td>[³H] Kainate</td>
</tr>
<tr>
<td>NMDA, agonism</td>
<td>&gt;10⁻⁴</td>
<td>Rat cortex</td>
<td>[³H] CGP 39653§</td>
</tr>
<tr>
<td>NMDA, glycine</td>
<td>&gt;10⁻⁴</td>
<td>Rat cortex</td>
<td>[³H] MLY 105519</td>
</tr>
<tr>
<td>NMDA, phencyclidine</td>
<td>&gt;10⁻⁴</td>
<td>Rat cortex</td>
<td>[³H] TCP¶</td>
</tr>
<tr>
<td>NMDA, polyamine</td>
<td>&gt;10⁻⁴</td>
<td>Rat cortex</td>
<td>[³H] ifenprodil#</td>
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<tr>
<td>Nonsensitive</td>
<td>&gt;10⁻⁴</td>
<td>Rat brain</td>
<td>[³H] L-glutamate</td>
</tr>
</tbody>
</table>

*Not tested at concentration >10⁻⁵ mol/L.
†[³H] phenylalkylamine (-)-desmethoxyverapamil.
§Sills et al. ²⁹
¶Siegel et al. ⁴⁰
#Goldman et al. ³⁰
#Schoemaker et al. ³¹

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Coadministration of 30 nmol/L SM-20220 or 20 μmol/L MK-801 produced protective effects against glutamate-induced neuronal death (Figure 1a, B and C, respectively). The cell death was quantitatively analyzed by counting dead neurons (Figure 1a, A through C, red neurons) under the fluorescence microscope and total neurons under the DIC microscope (Figure 1a, D through F), in 3 randomly chosen fields in each dish. Figure 1b shows the protective effects of SM-20220 and MK-801 against glutamate-induced neuronal death (neuronal death [%]: 300 μmol/L glutamate, 26.9±4.9; with 0.3, 1.0, and 30 nmol/L SM-20220, 23.0±4.3, 17.4±5.7, and 15.3±3.5, respectively; with 20 μmol/L MK-801, 9.1±3.1, n=4 cultures in a completely different preparation). SM-20220 showed a dose-dependent protection against glutamate-induced neuronal death, and reduced the rate by approximately 30% at the highest dose (30 nmol/L). MK-801, an NMDA receptor antagonist, completely inhibited neuronal death, indicating that the death process was excitotoxicity.

SM-20220 Inhibits Glutamate-Induced Cellular Swelling

After glutamate (500 μmol/L) exposure, cellular swelling developed over 15 minutes (Figure 2a). The area of the neuron gradually increased to 111.9±9.0% of the baseline in 15 minutes (Figure 2b). MK-801 and SM-20220 (30 nmol/L) completely suppressed cellular swelling (Figure 2b). Neither aCSF nor SM-20220 (30 nmol/L) alone induced morphological changes (data not shown).

SM-20220 Suppresses Glutamate-Induced Increase in [Ca^{2+}]_i

SM-20220 alone (up to 30 nmol/L) did not affect [Ca^{2+}]_i in the neurons (data not shown). Neurons stained with fluo-3 were exposed to 500 μmol/L glutamate by dropping aliquot (see Materials and Methods), and observed every 20 sec under a confocal microscope. Figure 3a shows the percent change in fluorescence intensity. Glutamate (500 μmol/L) alone induced a dual peak, ie, the first transient increase at 5 minutes and the second peak at 12 minutes, followed by a persistent rise lasting until the end of the observation. SM-20220 (30 nmol/L) did not suppress the first peak, whereas MK-801 (20 μmol/L) suppressed both (Figure 3a).

Since some studies have reported that fluo-3 (Kd=390 nmol/L), a high-affinity calcium indicator, underestimates increase in [Ca^{2+}]_i by glutamate,21,22 we also examined changes in [Ca^{2+}]_i using a low-affinity calcium indicator, Calcium Orange-5N (Kd=20 μmol/L). The result indicated a comparable time course to that using fluo-3 with respect to the persistent [Ca^{2+}]_i increase after glutamate application. However, the indicator did not achieve the first phase and fluctuation, seen with fluo-3, due to the low-affinity. SM-20220 significantly suppressed the persistent [Ca^{2+}]_i increase (Figure 3b).
SM-20220 showed significant and dose-dependent prevention of a rise in \([\text{Ca}^{2+}]\), examined with fluo-3 15 minutes after glutamate exposure (Figure 4).

**SM-20220 Improves Glutamate-Induced Acidification**

Glutamate (500 \(\mu\text{mol/L}\)) gradually evoked a decrease in the fluorescence intensity of BCECF, which reached 43.7\(\pm\)9.1\% of the basal value by the end of the observation (Figure 5a), indicating a decrease in pH. MK-801 significantly suppressed the reduction of fluorescence intensity during the observation period (Figure 5a). Although SM-20220 (30 \(\mu\text{mol/L}\)) similarly suppressed it until 10 minutes after glutamate exposure, as the observation period progressed, its suppressive effect was less than that by MK-801 (Figure 5a). Figure 5b shows that SM-20220 (0.3 to 30 \(\mu\text{mol/L}\)) significantly inhibited the decrease in fluorescence intensity 15 minutes after glutamate exposure. MK-801 (20 \(\mu\text{mol/L}\)) also significantly inhibited intracellular acidification (16.4\(\pm\)9.2\% change from basal value). Neither aCSF alone (Figure 5) nor SM-20220 (30 \(\mu\text{mol/L}\)) alone (data not shown) affected fluorescence intensity of BCECF, except for photobleaching.

**Discussion**

We sought to determine whether the NHE inhibitor, SM-20220, could exert a neuroprotective effect against excitotoxicity in rat cortical neurons. In the present study, SM-20220 suppressed that glutamate-induced (1) neuronal death, (2) cellular swelling, (3) \([\text{Ca}^{2+}]\) increase, and (4) intracellular acidification. All of the events induced by glutamate were also inhibited by the NMDA receptor antagonist. These results indicate that the NHE inhibitor is neuroprotective against excitotoxicity through inhibition of both \(\text{Ca}^{2+}\) influx and acidification in the neurons.

In the earlier study, the IC\(_{50}\) of SM-20220 for inhibition of the NHE in neurons and astrocytes was 5 and 20 \(\mu\text{mol/L}\), respectively.\(^{23}\) In our present study, the lower concentrations of SM-20220 significantly reduced the neuronal death and acidification, indicating that SM-20220 mainly affected the neurons and produced the neuroprotection. However, we

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**Figures**

**Figure 2.** a. DIC images of rat-cultured cortical neuron exposed to glutamate. Neuron was observed before (A), and 5 minutes (B), 10 minutes (C), and 15 minutes (D) after application of 500 \(\mu\text{mol/L}\) glutamate. Glutamate developed cellular swelling over 15 minutes. Scale bar=10 \(\mu\text{m}\). b. Time course of cellular swelling. Neurons were treated with 500 \(\mu\text{mol/L}\) glutamate (Glu) alone, Glu\(+30 \text{nmol/L SM-20220} (\text{SM})\), and Glu\(+20 \text{\mumol/L MK-801 (MK)}\). The cell size was defined as the area surrounded by the pericircular line of the neuron in the 2-dimensional image. SM and MK completely suppressed the cellular swelling induced by Glu. **\(p<0.01\) versus Glu (Student’s t test). Values represent mean\(\pm\)SD, \(n=6\).

**Figure 3.** a. Time course of \([\text{Ca}^{2+}]\), changes measured with fluo-3. Fluorescence images using fluo-3, a high-affinity dye, were recorded following an application of aCSF (artificial cerebrospinal fluid) alone, 500 \(\mu\text{mol/L}\) glutamate (Glu) alone, Glu\(+30 \text{nmol/L SM-20220} (\text{SM})\), and Glu\(+20 \text{\mumol/L MK-801 (MK)}\), in 4 respective observations of 3 to 6 neurons each. Note that Glu induced a dual peak, one at 5 minutes and another at 12 minutes, followed by the persistent increase. SM markedly suppressed the second peak and persistent increase in \([\text{Ca}^{2+}]\). MK suppressed both increases. \(p<0.05\) versus Glu from 4 minutes to 5 minutes 20 seconds, at 6 minutes 20 seconds, at 8 minutes 20 seconds, and from 9 minutes 20 seconds to the end (Student’s t test). #\(p<0.05\) versus Glu from 4 to 5 minutes, at 8 minutes 20 seconds, and from 9 minutes 40 seconds to the end (Student’s t test). Data represent mean\(\pm\)SD.
cannot exclude the possibility that some effects of SM-20220 may be mediated through astrocytes, since our culture contains 30% to 50% neurons and 50% to 70% astrocytes. For optimum imaging, we used different concentrations of glutamate, ie, 300 μmol/L for the excitotoxicity study and 500 μmol/L for imaging [Ca2+]i, and pH. Since, in the present study, the effects of glutamate even in the different concentrations were completely inhibited by MK-801, the glutamate-induced events, including neuronal death, cellular swelling, and change in pH induced by glutamate. Values represent mean±SD, n=4. aCSF indicates artificial cerebrospinal fluid.

Figure 4. Effects of SM-20220 and MK-801 on Ca2+ increases 15 minutes after glutamate exposure. Vehicle (0.008% polyethylene glycol 400) did not affect the Ca2+ responses induced by glutamate. SM-20220 significantly inhibited the increase in [Ca2+]i, in a dose-dependent manner and MK-801 did the same as well. *P<0.01 versus Glu (Student’s t test). Data represent mean±SD, n=4. aCSF indicates artificial cerebrospinal fluid.

exposure, intracellular acidosis activates the NHE to extrude the proton from the cell, resulting in the Na+ influx.9,10 The accumulation of Na+ increases the intracellular osmolarity, causing water influx, ie, cell volume increases.24,25 In addition, Ca2+ influx through NMDA receptor activation may also play a role in the cellular swelling, since MK-801 inhibited it. Glutamate-induced cellular swelling initially occurred by Na+ and Ca2+ influx through the glutamate receptor,5 and Na+ and Ca2+ are mainly counteracted by the energy-consuming ATP-dependent Na+/K+ adenosine triphosphatase26 and by the ATP-dependent Ca2+ pump,27 respectively. However, the energy failure due to mitochondrial dysfunction induced by Ca2+ influx impairs these energy-consuming systems and leads to further cellular swelling.28 Since the activated NHE also induces Ca2+ influx as discussed below, it must contribute to the energy failure as well as the cellular swelling. Irrespective of the mechanism, the cellular swelling eventually leads to the rupture of the cell membrane and then provides the neuronal death. Thus, suppression of the cellular swelling via NHE can be neuroprotective.

It is well established that Ca2+ overload is a major mechanism of ischemic cell damage.5 In this study, glutamate induced a dual peak in [Ca2+]i, observed with fluo-3; one peak represented the first transient increase, the other the second increase followed by a persistent rise. The initial rise can be induced by NMDA receptor activation, because MK-801 suppressed it, and because the NMDA receptor is much more permeable to Ca2+ among the glutamate receptors.5 The second increase followed by persistent increase in [Ca2+]i, can be mediated not only by the NMDA receptor but also by the NHE, since both SM-20220 and MK-801 suppressed it. Conceivably the NHE activation due to intracellular acidosis results in the Na+ influx,9,10 which reverses the Na+/Ca2+ exchange to protrude Na+, may worsen the Ca2+ overload, and leads to persistent [Ca2+]i increase.9,11–13 Given that the NHE inhibitor could reduce neuronal death, the suppression of persistent increase in [Ca2+]i, demonstrated by using the different types of calcium dye, may be important in preventing the excitotoxic neuronal death.
In the brain, NHE is essential for intracellular pH, regulation under both physiological and pathological conditions. The intracellular acidosis activates the NHE system to extrude the protons from the cell. It is therefore expected that the NHE inhibitor may worsen acidosis following glutamate exposure. However, in our present study, the NHE inhibitor did not worsen but, rather, partially improved acidification following glutamate application. The possible explanations for the unexpected result are as follows: (1) the glutamate-induced acidification is mediated by energy failure; (2) the energy failure is caused by mitochondrial dysfunction through Ca\(^{2+}\) overload; and (3) the reduction of Ca\(^{2+}\) overload by NHE inhibition might restore the mitochondrial function that consequently improves intracellular acidification. This is supported by our results; the NMDA receptor antagonist inhibited both Ca\(^{2+}\) overload and intracellular acidosis following glutamate exposure, although it has no direct effect on pH, regulation.

In conclusion, SM-20220, an NHE inhibitor, suppressed neuronal death and cellular swelling induced by glutamate through inhibition of both Ca\(^{2+}\) influx and acidification in the neurons. The activation of the NHE system may enhance the progress of cerebral damage and edema after cerebral ischemia. The results in the present study can translate the beneficial effects of NHE inhibitors in vivo with respect to the reduction of cerebral infarction and brain swelling. Thus, the blockade of NHE is likely to be of therapeutic benefit in the treatment of ischemic stroke.

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