Research report

Protection of neuronal cells from excitotoxicity by disrupting nNOS-PSD95 interaction with a small molecule SCR-4026

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Article info

Article history:
Received 29 February 2016
Received in revised form 21 June 2016
Accepted 12 July 2016
Available online 13 July 2016

Keywords:
Cerebral ischemia
nNOS
PSD95

Abstract

Stroke is a major public health problem leading to high rates of death and disability in adults. Coupling of postsynaptic density protein-95 (PSD-95) and neuronal nitric oxide synthase (nNOS) plays an important part in neuronal damage caused by stroke. Recent studies suggest the possibility of alleviating post ischemia neuron damage by blocking ischemia-induced nNOS-PSD-95 association. Here, we report a small-molecular inhibitor of nNOS-PSD-95 interaction, SCR-4026, which exhibits neuroprotective activities in NMDA-induced or Oxygen and glucose deprivation (OGD)-induced neuronal damage in primary cortical neurons cultures, and ameliorated focal cerebral ischemic damage in rats subjected to middle cerebral artery occlusion (MCAO) and reperfusion. Furthermore, we found that SCR-4026 was also able to promote neural stem cells to differentiate into neurons-like cells, which is potentially of great significance for neural protection. Taken together, SCR-4026 is identified as a novel small molecule that shows great potential in treating stroke.

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1. Introduction

Transient cerebral ischemia can cause delayed neuronal damage days after blood flow is restored. One mechanism is neuronal excitotoxicity induced by glutamate, a principal excitatory neurotransmitter in the central nervous system. One of the subtypes of glutamate receptors is N-methyl-D-aspartate receptor (NMDAR). Activation of NMDAR by massive glutamate released upon cerebral ischemia leads to calcium overload in neuron cells which contributes to neuronal death (Arundine and Tymianski, 2004). NMDAR therefore becomes an interesting target for cerebral ischemia treatment. Evidences from both in vitro and in vivo ischemic models have shown the efficacy of NMDAR antagonists (Lai et al., 2014). However, so far none of those molecules demonstrated their usefulness in clinical trials. One major reason is inhibition of NMDAR without selectivity causes severe side effects for that NMDAR is involved in many normal physiological processes (Lai et al., 2014).

Many studies have been focusing on the downstream events of abnormally activated NMDAR under cerebral ischemia condition. One mechanism of particular interest is the neuronal death mediated by PSD95-nNOS signaling pathway (Garthwaite et al., 1988; Aarts et al., 2002; Cao et al., 2005). PSD95 is a scaffolding protein that contains three PDZ domains which allow it to bind to various PDZ ligand proteins, including the subunits of NMDARs (Kornau et al., 1993) and nNOS protein (Brenman et al., 1996). During neuronal excitation, PSD95 binds both NMDARs and nNOS, bringing them proximate to each other. NMDAR efficiently activates nNOS in a calcium/calmodulin-dependent manner (Garthwaite et al., 1988). Under cerebral ischemia condition, abnormally activated nNOS contributes to neuronal death by increasing intracellular free-radical forms of nitric oxide (Lipton et al., 1993; Xia et al., 1996) and consequently leads to the downstream cytotoxicity events such as DNA and protein damages, activation of PARP-1 (Goto et al., 2002; Mandir et al., 2000) and protein S-nitrosylation (Takahashi et al., 2007). On the other hand, inhibition of nNOS activity alleviates NMDAR-mediated excitotoxicity in cultured neurons and animal models (Dawson et al., 1996, 1981, 1993; Huang et al., 1994). Particularly, mutant mice with nNOS deficiency are resistant to ischemic stroke model

Abbreviations: PSD-95, postsynaptic density protein-95; nNOS, neuronal nitric oxide synthase; NMDA, N-methyl-D-aspartate; OGD, Oxygen and glucose deprivation; MCAO, middle cerebral artery occlusion; BBB, blood-brain barrier; TTC, 2,3,5-Triphenyltetrazolium chloride; NSC, Neural stem cell
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http://dx.doi.org/10.1016/j.brainres.2016.07.012
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(Huang et al., 1994). These evidences lead to the hypothesis that disrupting PSD95-nNOS interaction might be a promising strategy in treating NMDAR-mediated neuronal damage.

Similar to NMDAR, nNOS also bears important physiological roles in brain and therefore is inappropriate for direct inhibitory targeting for cerebral ischemia treatment. However, one interesting hypothesis is that disruption of PSD95-nNOS interaction without affecting nNOS’s enzymatic activity could alleviate cerebral stroke condition with minimum impact on the normal function of brain. NA-1(Tat-NR2B9c) is a small peptide which fuses the membrane transduction of HIV-Tat domain to 9 amino acid residues from the C-terminal domain of GluNR2B (Aarts et al., 2002). This small peptide exhibited strong activity in disrupting NMDAR-nNOS interaction in vitro (IC_{50}=200 nM) (Cui et al., 2007) and neuron protection activities in various in vivo stroke models, without affecting NMDAR-mediated normal calcium current (Lai et al., 2013).

Recent result from a Phase II clinical trial, has demonstrated its safety and efficacy of NA-1 in preventing procedurally-induced strokes in patients with aneurysm and subjected to endovascular repair in a double-blind, randomized, controlled clinical study. In this clinical trial, patients in the NA-1 group sustained fewer ischemic infarcts than did patients in the placebo group. Encouraged by this result, we speculate that development of a small molecule targeting for cerebral ischemia with minimum impact on the normal function of brain. NA-1(Tat-NR2B9c) is a small peptide which fuses the membrane transduction of HIV-Tat domain to 9 amino acid residues from the C-terminal domain of GluNR2B (Aarts et al., 2002). This small peptide exhibited strong activity in disrupting NMDAR-nNOS interaction in vitro (IC_{50}=200 nM) (Cui et al., 2007) and neuron protection activities in various in vivo stroke models, without affecting NMDAR-mediated normal calcium current (Lai et al., 2013).

2. Material and methods

2.1. Antibodies

Mouse anti-PSD95 antibody and Rabbit anti-nNOS antibody for Western blots were purchased from Millipore and BD Biosciences, respectively. Goat HRP-conjugated secondary antibodies were purchased from Sigma. Anti-His-hrp antibody used in NOS–PSD95 interaction assay was purchased from MBL (Japan). Monoclonal mouse anti-PSD95 antibody for co-immunoprecipitation assay was obtained from Santa Cruz.

2.2. Plasmid construction and protein expression

cDNA fragments encoding PSD95(aa1–435) and nNOS(aa1–299) were amplified from plasmids encoding full-length human PSD95 and nNOS (GeneCopoeia), and were subsequently inserted into pGEX 4T-1 and pET-25b (+) vectors, respectively. The obtained plasmids were then transformed into E. Coli BL21 bacteria respectively and IPTG was used to induce the expression of GST-PSD95(aa1–435) and nNOS-His(aa1–299). GST-PSD95(aa1–435) was purified using GSTrap 4B affinity chromatography (GE healthcare Life Science). nNOS-His(aa1–299) was purified using HisTrap excel (GE healthcare Life Science).

2.3. In vitro nNOS–PSD95 interaction assay

96-well plates (Corning) were coated with 30 nM of recombinant GST-PSD95(aa1–435) for 12 h at 4 °C, followed by 30 min blocking with 10% BSA (Sigma) and then washed for 3 times with PBS containing 0.05% Tween 20. 100 μL of nNOS-His (aa1–299) at a concentration of 12.5 nM was added to the wells in the presence or absence of testing compounds. The plates were incubated at room temperature for 1 h, followed by washing with PBS/0.05% Tween 20 for 3 times. 100 μL of 1:1000 diluted anti-His-HRP antibody was added and incubated for 1 h at room temperature, followed by washing with PBS/0.05% Tween 20 for 3 times. The formation of GST-PSD95(aa1–435)/nNOS(aa1–299)–his complex was detected by addition of 100 μL of TMB (Thermo Fisher Scientific). NA-1 (YGRLLRQRRKLSIESDV, GL Biochem, ShangHai, China) and IC87201 were used as positive references in this assay.

2.4. Primary cortical neuron culture

Cortical neurons were prepared from 18-day SD rat embryos as described Terasaki et al. (2010). After isolation, cells were suspended in high-glucose DMEM (Gibco) containing 10% fetal bovine serum (Gibco) and 1% penicillin–streptomycin (Gibco), and plated on poly-D-lysine (Sigma–Aldrich) coated plates or 100 mm culture dish at a density of 2 x 10^5 cells/cm^2. Starting from 24 h post seeding, the medium was replaced with serum-free Neurobasal medium (Gibco) supplemented with 2% B27 (Gibco), 0.5 mM L-glutamine (Beyotime, China) and 1% penicillin–streptomycin every other day. Cells were cultured for 7–9 days at 37 °C in a humidified incubator supplemented with 5% CO_{2} before use.

2.5. Toxicity of the compounds

The toxicity of the compounds was determined by MTT (Sigma) assay. In brief, Primary neuronal cells on 96-well plate which have been cultured for 7 days were treated with compounds at different concentrations (0–50 μM) in complete neurobasal medium for 48 h. After the treatment, 10 μL of 5 mg/mL MTT was added to each well followed by 4 h of incubation at 37 °C. The formazan granules obtained were then dissolved in 100% dimethyl sulfoxide, and absorbance at 570 nm was detected with a multiscanner autoreader (M200, TECAN, Switzerland).

2.6. NMDA-induced excitotoxicity model

NMDAR-dependent excitotoxic injury was induced as described previously Zhou et al. (2010) with minor modification. Briefly, primary neuronal cells cultured for 9 days were used in the assay. The cells were pretreated with the testing compounds at different concentrations (0–25 μM) in incomplete neurobasal medium for
1 h, and then treated with 2.5 mM of NMDA (Sigma) for 4 h. The medium was then replaced with fresh neurobasal medium supplemented with 2% B27, 0.5 mM l-glutamine and 1% penicillin-streptomycin. The neurons were cultured for additional 24 h before subjected to CytoTox-Glo™ Cytotoxicity Assay (Promega).

2.7. Oxygen–glucose deprivation (OGD) injury model

Primary neuronal cells which have been cultured for 7 days were preincubated in complete neurobasal medium supplemented with compounds at different concentrations (0–25 μM) for 1 h. The neurons were then rinsed twice. Glucose-free DMEM medium (Tianhang Biological Technology, HangZhou, China) supplemented with or without the compounds was then added and the culture plates were placed into a humidified chamber filled with 85%N2, 10%H2 and 5%CO2, cultured at 37 °C for 2 h. At the end of the incubation, the medium was replaced with normal complete neurobasal medium and the cells were cultured under normal condition for additional 24 h. In this assay, control cells were incubated with high glucose DMEM medium (Gibco) and cultured under normal condition during the period of OGD treatment.

2.8. Co-immunoprecipitation assay

The co-immunoprecipitation assay was performed as described previously Zhou et al. (2010) with minor modifications. Cortical neurons were treated with vehicle or indicated concentrations of the compounds, then challenged with 200 μM glutamate and 10 μM Glycine in neurobasal medium (Gibco) for 1.5 h. The neurons were then washed twice with cold PBS and lysed with RIPA lysis buffer (Beyotime, China). The lysates were centrifuged at 12,000 rpm for 10 min at 4 °C and the supernatants were collected. Protein concentration in the supernatants was determined by BCA assay (Thermo Fisher Scientific). 1 μg of proteins were used to incubate with monoclonal mouse anti-PSD95 antibody for 12 h at 4 °C, followed by addition of 60 μL of protein A/G agarose beads (Beyotime, China) and incubation for another 4 h. The beads were washed three times with PBS and the co-immunoprecipitated nNOS was analyzed by Western blot analysis.

2.9. Neural differentiation assay

NSCs were prepared from the hippocampus of 17-day SD rat embryos as described in Ahmed (2009). After isolation, cells were suspended in complete NSC culture medium (Wei Kai Biological Engineering Co., Ltd., TianJin, China) and then maintained at 37 °C in a humidified incubator supplemented with 5% CO2. The NSCs were ready to use when neural microspheres grew to the appropriate size. NSCs were suspended in neurobasal medium (Gibco), neurobasal supplemented with 5% FBS (Gibco), 2% B27 (Gibco) or 2 μM SCR-4026 respectively, and plated on poly-0-ly-sine coated 24-well plates (Corning). The cell morphology was observed on different time points (Day 0, 3 and 7).

2.10. Assessment of drug permeability across the blood–brain barrier

BBB penetration property of SCR-4026 was evaluated by intravenous administration of 1 mg/kg of SCR-4026 to the rats. The concentration of SCR-4026 in brain tissue and plasma 5, 20, 60 min after drug administration were determined by LC-MS-MS (Agilent 6410). There were three SD rats in each time point.

2.11. MCAO model in rats

Adult male SD rats were subjected to transient middle cerebral artery occlusion (MCAO) for 2 h following a modified Longa method (Longa et al., 1989). In brief, following overnight fasting, the rats were anesthetized with 7% chloral hydrate. The right common carotid artery, external carotid artery (ECA), and internal carotid artery were exposed. A 4/0 monofilament nylon suture, with its tip rounded by heating near a flame, was advanced ~20 mm (determined by animal weight) from the ECA into the lumen of the internal carotid artery until it blocked the origin of the MCA (middle cerebral artery). Rectal temperature was controlled at ~37 °C with a heating pad during surgery. After surgery, the animals were kept in a temperature controlled incubator to maintained body temperature at 37 °C to recover from anesthesia and were allowed food and drink. Two hours after induction of ischemia, the filament was withdrawn. Animals were treated by a single intravenous bolus injection with saline, the Edaravone, or the testing compound SCR-4026 immediately after ischemia reperfusion.

To evaluate the extent of cerebral infarction, neurological functional scores were recorded at 24 h post reperfusion. Bederson neurological scoring assessment was performed by the observer blinded to the experimental groups (Rating scale: no deficit; 1, forelimb flexion deficit on left side; 2, decreased resistance to lateral push and torso turning to the ipsilateral side when held by tail; 3, very significant circling to affected side and reduced capability to bear weight on the affected side; 4, animal rarely moves spontaneously and prefers to lay down or stay at rest).

After finishing neurological functional scores evaluation, all the rats were then euthanatized by an overdose of 7% chloral hydrate i.p. injection. The brain was removed rapidly and frozen at −20 °C for 5 min. Then the brain tissues were cut into six equally spaced (2 mm) coronal sections. The sections were immersed in PBS containing 2% (w/v) TTC (Sigma) at 37 °C for 20 min. With TTC staining, the area without staining was determined to be the infarct area. The infarction volume is presented as a volume percentage of the infarct area compared with contralateral hemisphere. The percentage of TTC-stained tissue with respect to the whole piece of tissue was analyzed by Image J software (NIH, USA).

3. Results

3.1. Rational design of small molecules disruption PSD95–nNOS interaction

Two structurally related compounds, IC87201 and ZL006, to our knowledge, are the only small molecule compounds reported to have activity in direct disruption of PSD95–nNOS interaction (Florio et al., 2009; Lee et al., 2015). ZL006 has been demonstrated to decrease PSD95–nNOS association in cultured cortical neurons (Zhou et al. 2010). However, IC87201’s activity is relatively low (IC50 ≈ 14.2 μM) as shown in an ELISA assay (Fig. 2b). To develop small molecules with stronger PSD95–nNOS-disrupting activity and better potential in treating cerebral ischemia, we designed a series of small molecules based on the structure of IC87201 and ZL006 (Fig. 2a). These molecules were subjected for an ELISA screening for their ability to decrease the association between PSD95(1–435aa) and nNOS(1–299aa), which comprise the domains required for the binding between the two proteins (Florio et al., 2009) (Fig. 1). Many of these molecules can block the PSD95–nNOS interaction in a dose-dependent manner. Fig. 2b shows SCR-4026 exhibited comparable or better activities than IC87201 in the ELISA assay. As a positive control, NA-1 was also tested and exhibited a much lower IC50 in the assay (24 nM), which is consistent with previous report (Cui et al., 2007) because it contains amino acid sequence that direct binds to the PZD domain 1 and 2 of PSD95 (Bach et al., 2008).

All of the compounds were subjected to toxicity test using
cultured rat cortical neurons. A serial dilution of the compounds solution starting from 50 μM were used to treat the neurons and wait for 48 h before cellular damage measured as indicated by cell viability (Supplementary Table 1). Compounds which have no significant impact on cell (> 85% cell viability) were selected for further functional tests on primary rat neuronal cells. In this assay, compound SCR-4026 also exhibited minimal toxicity.

3.2. Blocking NMDA-induced injury in cultured cortical neurons

NMDA-induced injury model was used to evaluate compounds’ ability to alleviate cell damage on cultured rat cortical neurons (Fig. 3). Upon NMDA stimulation, activated NMDAR lead to the overload of calcium in cultured cortical neurons and eventually cell death. Addition of an NMDAR antagonist, Memantine, strongly suppressed the cell death caused by NMDAR stimulation. IC87201 also exhibited relatively weak, but dose-dependent and statistically significant rescue effect. Interestingly, SCR-4026 also blocked NMDA-induced cell injury efficiently, suggesting that their ability to disrupt PSD95-nNOS interaction in the ELISA can be translated into cellular efficacy.

3.3. Alleviation of cell damage in an OGD model

The neuron protection activities of SCR-4026 were further validated in oxygen and glucose-deprived (OGD) model, another widely used cerebral ischemia model. Similarly, the compounds also exhibited a dose-dependent neuron protection effect (Fig. 4). Particularly, 66.67% protection effect was achieved at highest concentration (25 μM) of SCR-4026 in this experiment, which was comparable or better than NA-1, for which 16.69% protection rate was seen in the same experiment. Statistically significance was seen at higher concentration for both compounds.

3.4. Inhibiting PSD95-nNOS interaction in neuron cells

To verify whether SCR-4026 actually blocked PSD95-nNOS interaction in neurons, co-immunoprecipitation was used to determine the binding between PSD95 and nNOS in the presence or absence of the compounds in the NMDA-induced injury model. As shown in Fig. 5, treatment with the compounds significantly decreased the binding between PSD95 and nNOS. SCR-4026 almost completely inhibited the elevated binding between PSD95 and nNOS induced by NMDA stimulation. Of note, although we have shown that SCR-4026 could directly inhibit the interaction between PSD95 and nNOS in the ELISA experiment (Fig. 2), the possibility that the compound interfere with PSD95-nNOS interaction indirectly remains.

3.5. BBB penetration property of the SCR-4026

The brain-blood ratio of the compound was 0.75 five minutes after i.v. administration and still remained at 0.27 at one hour (Table 1). Thus the compound showed a promising BBB penetration property which is critical for small molecules designed for treating stroke.

Fig. 1. Purification of nNOS-His and GST-PSD95. (A) nNOS-His (aa1–299) was purified by using HisTrap HP(GE Healthcare, USA) column. GST-PSD95(aa1–435) was purified by using GST rap HP(GE Healthcare, USA). (B) The purified proteins were stained with Kaumas blue after SDS-polyacrylamide gel electrophoresis.
3.6. **SCR-4026 alleviates brain injury in MCAO model**

MCAO for 2 h followed by reperfusion for 24 h led to severe infarct, and SCR-4026 administered immediately after reperfusion significantly decreased infarct volume (Fig. 6). The inhibitory percent of infarct volume was about 36.9%. Consistent with the effect on the infarct volume, SCR-4026 significantly reduced the neurological deficit scores comparing to the saline control group (Fig. 6).

4. **Discussion**

Selective inhibition of NMDAR-PSD95-nNOS signaling without disturbing the normal physiological functions of NMDAR and nNOS may be valuable in treating brain disorders because this strategy is anticipated to cause less severe side effects which are commonly seen for NMDAR or nNOS antagonist. A few studies have supported this hypothesis. Peptide NA-1 and small molecule ZL006 have been shown to be effective in treating ischemia in various *in vitro* and *in vivo* models. IC87201, was found to be useful in alleviating chronic and acute pain. Another small molecule, honokiol, showed good protection against ischemia–reperfusion injury in rats (Hu et al., 2013). These molecules were highly specific in disrupting PSD95-nNOS interaction, and have no impact on the normal functions NMDAR or nNOS, or the interaction between PSD95 and other proteins.

Although the interference peptide NA-1 has exhibited efficacy and safety in brain aneurysm patients undergoing endovascular repair, disadvantages often associated with peptide-based drugs, such as BBB penetration property, may limit its potential in clinical practice. So we are particularly interested in developing small molecules with strong activity in blocking PSD95-nNOS association.

Based on the structure of IC87201 and ZL006, we designed and synthesized a serial of compounds, among which SCR-4026 are of particular interest due to its balanced properties in various assays (Supplementary Table 1). SCR-4026 has stronger activity in blocking PSD95-nNOS interaction than IC87201 (Fig. 2b) and ZL006 (data not shown), and showed better efficacy in preventing
neuron death in NMDA-induced or OGD-induced neuron injury models. Notably, although NA-1 showed magnitude higher potency than SCR-4026 in disrupting PSD95-nNOS association, it only displayed comparable protective activity as SCR-4026 in the NMDA-induced excitotoxicity model and the OGD injury model. The difference in BBB permeability between the small molecule compound and the small peptide may at least partially interpret the discrepancy. Interestingly, SCR-4026 was also found to be able to promote neuron stem cell growth and differentiation, which is important in brain damage recovery (Abe, 2000). As shown in Supplementary Fig 2, SCR-4026 and B27 showed similar abilities in promoting the differentiation of NSCs into neuron-like morphology. This interesting property might also contribute to the neuronal protection effect of SCR-4026.

Despite of the above progress, the ability of the small molecules, including IC87201, ZL006 and SCR-4026, to block PSD95-nNOS interaction is still weak. To observe definite blocking effect, high concentration of the compounds has to be used in biochemical assays, which may lead to unexpected interference with the assay readout in certain assay format. In fact, controversial result has been reported arguing the mechanism of action of IC87201, ZL006 and analogues (Bach et al., 2015). Thus, for potential clinical application, more improvement in the compound efficacy is demanded although it might be very challenging because the binding pockets of PDZ domains are shallow and narrow (Bach et al., 2015).

Another concern is that off-target effects, either beneficial or deleterious, could exist in the efficacy models. Although SCR-4026 could disrupt the interaction between PSD95 and nNOS in vitro, which aligns well with the cell-based efficacy models and the immunoprecipitation assay, we cannot exclude the possibility that SCR-4026 functions via indirect or even irrelevant mechanisms. Further investigation to elucidate the specificity of the compound is of great importance.

Although various in vitro and in vivo models have shown that blocking PSD95-nNOS is effective in treating cerebral ischemia, to validate this strategy in clinical trial is challenging. This is not only because patients recruited for clinical trial have complex physiological and pathological background and hospitalized circumstance, but also because PSD95-nNOS signaling pathway probably is not the only mechanism involved in delayed brain damage after stroke, and other NMDAR-related mechanisms may also be involved (Lai et al., 2013). Although we still do not have the full picture of what triggers the brain damage after cerebral stroke, it is almost certain that different mechanisms interwork together, generating the detrimental consequences. Thus, using a treatment cocktail including PSD95-nNOS blocker and other drugs might be more effective.


Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.brainres.2016.07.012.

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Cui, H., et al., 2007. PDZ protein interactions underlying NMDA receptor-mediated excitotoxicity and neuroprotection by PSD-95 inhibitors. J. Neurosci. 27 (37), 9901–9915.

Fig. 6. Neuronal protective effect of SCR-4026 was evaluated on rats MCAO model (n = 9–11). The SCR-4026 group (5 mg/kg, i.v.) and the Edaravone group (6 mg/kg, i.v.) showed 36.9% and 41.6% reduction in infarct volume, 23.0% and 29.6% reduction in neurological scores, respectively, in comparison to the saline group. Treatment group versus Vehicle group. *P < 0.05, **P < 0.01.