A Novel Hydrogen Sulfide-releasing N-Methyl-\(\text{D}\)-Aspartate Receptor Antagonist Prevents Ischemic Neuronal Death*\(^{\text{†*}}\)

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Background: Hydrogen sulfide (\(\text{H}_2\text{S}\)) exerts neuroprotective effects, whereas \(\text{H}_2\text{S}\) may cause neurotoxicity via \(N\)-methyl-\(\text{D}\)-aspartate receptor (NMDAR) activation.

Results: A newly-synthesized \(\text{H}_2\text{S}\)-releasing NMDAR antagonist S-memantine exerted lower neurotoxicity and prevented ischemic neuronal death more markedly than conventional \(\text{H}_2\text{S}\)-releasing compounds or memantine alone.

Conclusion: S-memantine prevents ischemic brain injury without neurotoxicity.

Significance: \(\text{H}_2\text{S}\)-releasing NMDAR antagonists may prevent neurodegeneration of various causes.

Physiological levels of \(\text{H}_2\text{S}\) exert neuroprotective effects, whereas high concentrations of \(\text{H}_2\text{S}\) may cause neurotoxicity in part via activation of NMDAR. To characterize the neuroprotective effects of combination of exogenous \(\text{H}_2\text{S}\) and NMDAR antagonism, we synthesized a novel \(\text{H}_2\text{S}\)-releasing NMDAR antagonist \(N\-((1r,3R,5S,7r)-3,5\text{-dimethyladamantan-1-yl})-4-(3\text{-thioxo-3H-1,2-dithiol-4-yl})\text{-benzamide (S-memantine)} and examined its effects in vitro and in vivo. S-memantine was synthesized by chemically combining a slow releasing \(\text{H}_2\text{S}\) donor 4-(3-thioxo-3H-1,2-dithiol-4-yl)-benzoic acid (ACS48) with a NMDAR antagonist memantine. S-memantine increased intracellular sulfide levels in human neuroblastoma cells (SH-SY5Y) 10-fold as high as that was achieved by ACS48. Incubation with S-memantine after reoxygenation following oxygen and glucose deprivation (OGD) protected SH-SY5Y cells and murine primary cortical neurons more markedly than did ACS48 or memantine. Glutamate-induced intracellular calcium accumulation in primary cortical neurons were aggravated by sodium sulfide (Na\(_2\)S) or ACS48, but suppressed by memantine and S-memantine. S-memantine prevented glutamate-induced glutathione depletion in SH-SY5Y cells more markedly than did Na\(_2\)S or ACS48. Administration of S-memantine after global cerebral ischemia and reperfusion more robustly decreased cerebral infarct volume and improved survival and neurological function of mice than did ACS48 or memantine. These results suggest that an \(\text{H}_2\text{S}\)-releasing NMDAR antagonist derivative S-memantine prevents ischemic neuronal death, providing a novel therapeutic strategy for ischemic brain injury.

Hydrogen sulfide has been proposed as a gaseous signaling molecule along with nitric oxide and carbon monoxide (1). A number of studies suggested therapeutic potential of \(\text{H}_2\text{S}\)-donating compounds and \(\text{H}_2\text{S}\) gas itself for a number of animal models of human disease including ischemic brain injury (2, 3).

Gaseous \(\text{H}_2\text{S}\), however, may be difficult to be used clinically because of its characteristic odor and toxicity at high concentrations (1, 4). Na\(_2\)S and sodium hydrosulfide (Na\(_2\)HS) have been used as \(\text{H}_2\text{S}\) donor compounds in the majority of experimental studies (2, 3). However, because the half-lives of these sulfide salts are very short in biological fluid, plasma sulfide levels rapidly increase after bolus administration of Na\(_2\)S or Na\(_2\)HS and then return to baseline instantaneously (5). To sustain “physiological” levels of sulfide in circulation after bolus administration, many slow-releasing \(\text{H}_2\text{S}\) donor compounds, including ACS48, have been developed (2, 6).

While it has been reported that low and physiological levels of \(\text{H}_2\text{S}\) protect neurons, \(\text{H}_2\text{S}\) also exhibits neurotoxicity especially at high concentrations (4). Some investigators have suggested that \(\text{H}_2\text{S}\)-induced neurotoxicity may be mediated via enhancement of \(N\)-methyl-\(\text{D}\)-aspartate receptor (NMDAR) activity (7–9), because toxicity of \(\text{H}_2\text{S}\) was abolished by NMDAR antagonist in vitro and in vivo (8, 9). Based on these observations, we hypothesized that a hybrid NMDAR antagonist that is capable of slowly releasing \(\text{H}_2\text{S}\) in circulation is more effective in protecting neurons than \(\text{H}_2\text{S}\) donor compounds alone.

In the current study, we sought to characterize the efficacy of a novel \(\text{H}_2\text{S}\)-releasing NMDAR antagonist derivative, S-memantine, in cerebral ischemia and reperfusion injury using in vitro and in vivo approaches. We also compared neurotoxicity of Na\(_2\)S, ACS48, and S-memantine in a human neuroblastoma cell line and murine primary cortical neurons. Here, we report

\(^{2}\) The abbreviations used are: NMDAR, \(N\)-methyl-\(\text{D}\)-aspartate receptor; S-memantine, \(N\-((1r,3R,5S,7r)-3,5\text{-dimethyladamantan-1-yl})-4-(3\text{-thioxo-3H-1,2-dithiol-4-yl})\text{-benzamide; OGD, oxygen and glucose deprivation; DMF, N,N\'-dimethylformamide; HATU, O-(7-azabenzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate; LDH, lactate dehydrogenase; SSA, S-sulfosalicile acid; MTV, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyazetridol bromide; CV, crystal violet; TTC, 2,3,5-triphenyltetrazolium chloride; BCAO, bilateral carotid artery occlusion.
that S-memantine exhibits high therapeutic potential with low toxicity against ischemic neuronal death.

**EXPERIMENTAL PROCEDURES**

**Materials**—All reagents were purchased from Sigma-Aldrich unless otherwise specified.

**Synthesis of H2S-releasing NMDAR Antagonist S-memantine**—4-(3-thioxo-3H-1,2-dithiol-4-yl)-Benzoic acid (ACS48) was synthesized as described previously (Fig. 1A) (6). N-((1r,3R,5S,7r)-3,5-Dimethyladamantan-1-yl)-4-(3-thioxo-3H-1,2-dithiol-4-yl)-benzamide (S-memantine) was synthesized using the following two steps as shown in Fig. 1B: First, under nitrogen atmosphere, at room temperature, 4-(propan-2-yl)-benzoic acid (0.5 g; 3.05 mmol) and memantine (0.82 g; 4.57 mmol) were mixed in 5 ml of anhydrous N,N-dimethylformamide (DMF), and N,N-diisopropylethylamine (2.12 ml; 12.19 mmol) was added. After cooling to room temperature, O-(7-azabenzotriazol-1-yl)-N,N,N',N’-tetramethyluronium hexafluorophosphate (HATU) (1.045 g; 2.75 mmol), dissolved in 5 ml of DMF was gradually added, followed by an overnight stirring at room temperature. After evaporation of DMF, extraction and purification, N-((1r,3R,5S,7r)-3,5-dimethyl-adamantan-1-yl)-4-isopropylbenzamide (Amide 1) was obtained. In the second step, under nitrogen atmosphere, sulfur (0.05 g, 158 mmol) was melted at 140 °C and amide-1 (2.16 g; 13.2 mmol) was added. The reaction mixture was stirred for 24 h at 190 °C to form a layer of reddish brown solution. After cooling the reaction mixture back to 140 °C, 100 ml toluene was added followed by further cooling to room temperature. Acetone was added to form a suspension that was filtered and the filtrate was concentrated. The purity of the final product was greater than 98%. The structure of the final product was confirmed by mass spectrometry (Finnigan LCQ Advantage, ESI+) and 1H NMR spectroscopy. C22H25NOS3: m/z calculated: [M + H]+ 415.11; found: 415.64. 1H NMR (400 MHz, DMSO-d6) 9.21 (s, 1H), 7.82 (d, J = 6.8 Hz, 2H), 7.68 (s, 1H), 7.63 (d, J = 8.4 Hz, 2H), 2.12–2.14 (m, 1H), 1.92–1.93 (m, 2H), 1.71–1.78 (m, 4H), 1.16–1.39 (m, 6H), 0.86 (s, 6H).

**FIGURE 1. Synthesis of ACS48 (A) and S-memantine (B).**

**H2S-releasing NMDA Receptor Antagonist and Neuronal Ischemia**

**Cell Culture**—Human neuroblastoma SH-SY5Y cells were cultured in Eagle’s medium/Ham’s F-12 50/50 Mix (DMEM/F12, Cellgro by Mediatech, Inc.) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. The cells were seeded into 96 well plates (2 × 10^4 cells per well) for OGD and measurements of toxicity, 6 cm dishes (5 × 10^5 cells per dish) for measurements of sulfide and reduced glutathione (GSH), or 10 cm dishes (1 × 10^6 cells per dish) for measurements of intracellular calcium. Cell culture medium was replaced every 2 days, and the cultures were maintained at 37 °C in 95% air/5% CO2 in a humidified incubator. Cells were used after reaching 80% confluent.

Primary neuronal cultures were prepared from the cortex of embryonic day 15 C57BL/6J mice. In brief, brains were harvested and the hemispheres were dissected under a microscope. The cortical neurons were dissociated in Neurobasal medium (Invitrogen) with B27 supplement (antioxidant plus, Invitrogen). The cells were seeded into 24 well plates coated with poly-d-lysine (Becton Dickinson Labware, 2 × 10^5 cells per well), followed by the medium-change with fresh one on the next day. The half of culture medium was replaced with Neurobasal medium with B27 supplement (antioxidant minus) every other day, and the cultures were maintained at 37 °C in 95% air/5% CO2 in a humidified incubator. Cells were used for experiments 11 days after seeding.

**Treatment of Cells with H2S Donor Compounds**—ACS48 and S-memantine were dissolved in dimethyl sulfoxide (DMSO), then, diluted to desired concentration with culture medium. The final concentration of DMSO was adjusted to 1%. We confirmed that 1% DMSO did not affect cell viabilities of SH-SY5Y and primary cortical neurons using lactose dehydrogenase (LDH) method (data not shown).

**Measurement of Sulfide Levels in SH-SY5Y Cells, Culture Medium, and Murine Plasma and Brain**—Concentration of free sulfide in SH-SY5Y cells was measured using high performance liquid chromatography (HPLC) (10). Briefly, SH-SY5Y cells were seeded into 6 cm dishes (5 × 10^5 cells per dish). After being 80% confluent, 20 μM H2S donor was added to the dish and incubated at 37 °C. Cells were washed with ice-cold Tris-HCl (100 mM, pH 9.5, DTPA 0.1 mM) buffer, scraped, transferred to Eppendorf tubes, and centrifuged. MBB (10 mM in acetonitrile, 50 μl) was added to 100 μl of supernatant. After 30 min of incubation at room temperature in dark, 50 μl of 200 mM 5-sulfosalicylic acid (SSA) was added. After centrifugation, supernatant was analyzed by HPLC. For sulfide levels in medium, after centrifugation, supernatant was used for MBB reaction as above.

Plasma and brain sulfide levels were measured 90 min after intraperitoneal administration of Na2S, ACS48, or S-memantine. Blood was drawn from left ventricle and centrifuged to collect the plasma. After perfusion with Tris-HCl buffer via left ventricle, brain was harvested, homogenized in Tris-HCl, and centrifuged. Plasma and supernatant of brain homogenate were derivatized with MBB and analyzed by HPLC.

**Oxygen-Glucose Deprivation (OGD)—OGD for SH-SY5Y**—was performed by placing cells in a hypoxia chamber (STEM-CELL Technologies Inc.) for 15 h, followed by 24 h of reoxygenation as previously reported (Fig. 2A) (11). Briefly, medium was
replaced with glucose-free RPMI 1640 with 1-glutamine (Cellgro by Mediatech, Inc), deoxygenated with anaerobic gas mixture (93% N₂-5% CO₂-2% H₂) for 30 min before using. Cells were then placed in a hypoxia chamber, flushed with anaerobic gas mixture (93% N₂-5% CO₂-2% H₂) and incubated at 37 °C. After 15 h of hypoxia, medium was replaced with DMEM/F12 supplemented with glucose and incubated for 21 h at 37 °C in 95% air/5% CO₂, then, used for viability experiment.

**LDH Assay**—Microtiter plate containing cells was centrifuged at 250 × g for 10 min, and the supernatant was used for LDH measurement with LDH Cytotoxicity Detection Kit (Roche). After aspirating medium, remaining cells were washed with PBS, then, 100 μl of 1% Triton-X was added to each well, followed by incubation at 37 °C for 30 min. Medium and lysates were used for LDH measurement at wavelength 492 nm. Percentage of released LDH was calculated with following formula: [LDH (medium)/LDH (medium + cell) × 100]. The average value of control (cells without OGD) was deducted as background.

3-(4,5-dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium bromide (MTT) Assay—10 μl of thiazolyl blue tetrazolium bromide solution (5 mg/ml in pH 7.4, PBS) was added to each well containing 100 μl of medium and cells, followed by incubation at 37 °C for 4 h in the dark. Isopropanol (100 μl, 0.04N HCl) was added to dissolve the blue dye. After dissolving completely, absorbance was measured with a plate-reader (Synergy 2, BioTek Instrument) at test wavelength 570 nm and reference wavelength 670 nm. Cell viability was determined by absorbance at 570 nm and reported as ratio to control cells (without OGD).

**Crystal Violet (CV) Assay**—After aspirating culture medium, cells were fixed and stained by 0.5% CV in 95% (v/v) ethanol for 5 min, then washed by tap water several times. After taking photographs, 1% sodium dodecyl sulfate solution was added to each well to elute blue dye. Absorbance was measured with a plate reader at 595 nm of wavelength. Values were shown as ratio to control (cells without OGD).

**Measurement of Intracellular Calcium Level in Murine Primary Cortical Neurons**—Intracellular calcium level was measured by a previously described method using Fura-2/AM with some modifications (12). Briefly, cells were trypsinized, pelleted, resuspended in the medium, and incubated with 5 μM Fura-2/AM (Invitrogen) in HEPES buffer (pH 7.4, NaCl 110 mM, KCl 2.6 mM, MgSO₄ 1 mM, CaCl₂ (Fisher Scientific) 1 mM, HEPES 25 mM, and glucose 11 mM) at 37 °C for 40 min, and then washed twice. Cells were resuspended in HEPES buffer and transferred to a cuvette. Na₂S, ACS48, memantine or S-memantine at 20 μM was added to the cuvette with or without glutamate (100 μM), respectively. Final cell concentration was 1 × 10⁵ cells/ml. We were not able to examine the effects of higher concentration of S-memantine than 20 μM because of its poor solubility to HEPES buffer. The fluorescence intensity ratio was measured with Spectra Max M5 ( Molecular Devices, CA) at a wavelength of λex = 340/380 nm and λem = 510 nm.

**Measurement of Intracellular GSH Levels in SH-SY5Y**—Intracellular GSH level of SH-SY5Y was measured using HPLC method as previously reported (13). Briefly, cells were seeded into 6-cm dishes (5 × 10⁵ cells per dish) and treated with 50 μM H₂S donors or memantine w/wo 2 mM glutamate for 8 h, followed by washing with ice-cold PBS. Cells were scraped and transferred to an Eppendorf tube and sonicated. Some fraction of lysate was used for protein assay. After centrifugation, 75 μl of supernatant, 26 μl of 2-(cyclohexylamino)ethanesulfonic acid (CHES, 0.5 M, pH 8.4) and 4 μl of 50 μM MBB were mixed and incubated at room temperature in dark for 30 min. Acetic acid (100 μl, 30% v/v) was added, followed by centrifugation at 15,000 × g for 10 min after 5 min incubation of the tube on ice. The supernatant was analyzed using HPLC at wavelength of λex = 370 nm and λem = 486 nm.

**Measurement of Protein Levels and Phosphorylation**—Protein levels in SH-SY5Y were determined by standard immunoblot techniques using primary antibodies (1:1,000, Cell Signaling Technology Inc., Danvers, MA) against cleaved caspase-3, caspase-3, phosphorylated Akt at threonine 308, Akt, phosphorylated ERK1/2 at threonine 202, and tyrosine 204, Erk1/2, and β-tubulin. Bound antibody was detected with a horseradish peroxidase-linked antibody directed against rabbit IgG (1:10,000–1:25,000; Cell Signaling Technology Inc.) and was visualized using chemiluminescence with ECL Advance kit (GE Healthcare).

**Global Cerebral Ischemia and Reperfusion**—After approval by the Massachusetts General Hospital Subcommittee on Research Animal Care, all animal experiments were performed in accordance with the guidelines of the National Institutes of Health. Male mice (C57BL/6), 8–9 weeks old) were purchased from the Jackson Laboratory (Bar Harbor, ME) and given access...
to food and water *ad libitum* in our animal facility until the time of experiments. Mice were anesthetized with ketamine (80 mg/kg, intraperitoneal) and xylazine (12 mg/kg, intraperitoneal). Body temperature was kept at 37 ± 0.5 °C during whole procedure. Cerebral ischemia was induced by 40 min of bilateral common carotid artery occlusion (BCAO) with microsurgical clips. Na₂S, ACS48, S-memantine, or memantine at 25 μmol/kg or vehicle was intraperitoneally administered 1 min after the initiation of reperfusion. After reperfusion and recovery from anesthesia, mice were intraperitoneally given 1 ml of 5% dextrose-enriched lactated Ringer’s solution daily for 1 week. Neurological score was evaluated as described previously (14). The next eight items were checked and scored to evaluate neurological function: 1) Grasping movement reflex (inducing the catching reflex by running a little rod over the plantar surface of the paw): 0 ~ 4 points, 2) stop at the edge of a table: 0 or 1 point, 3) turning the head (turning the head when touching the ear from behind with a little rod): 0 ~ 2 points, 4) falling reflex (lifting the mouse at the tail and lowering with the front legs toward the ground): 0 or 1 point, 5) spontaneous motility (moving behavior on a flat surface): 0 ~ 2 points, 6) circling behavior (moving behavior on a flat surface): 0 or 2 points, 7) pelt property (appearance of the coat): 0 or 1 point, 8) flight reaction (spontaneous behavior on a flat surface): 0 or 1 point. Total 14 points. The higher score means worse neurological function.

*Treatment of Mice with H₂S Donor Compounds—*ACS48 and S-memantine were dissolved in the corn oil/DMSO (v/v, 95/5) suspension. Na₂S was dissolved in saline 5 min before administration. Mice were intraperitoneally given 4 μl/g of these solutions 1 min after reperfusion following 40 min of BCAO.

*Measurement of Cerebral Infarct Volume after BCAO—*Mice were decapitated and brains were harvested 24 h after BCAO and reperfusion. Coronal sections (2 mm thickness) of the cerebrum were then soaked into 1% 2,3,5-triphenyltetrazolium chloride (TTC) solution in PBS at 37 °C for 30 min. After taking photographs under the same condition, infarct volume was calculated with Image J software ver.1.44. Photographs were gray-scaled, then, brighter area than threshold determined using Image J software was calculated as infarct area. Values were shown as ratio of cerebral infarct volume to total volume. The average value of the brighter region volume in control mice was deducted from calculated area as background.

*Data Analysis—*All data are presented as means ± S.E. Data were analyzed by ANOVA using Sigmapstat 3.01a (Systat Software Inc., Chicago, IL) and Prism 5 software package (GraphPad Software, La Jolla, CA). Newman-Keuls multiple comparison post hoc test or Bonferroni post hoc test were respectively performed for One-way Anova or Two-way Anova as required. Smaller p values than 0.05 were considered significant.

**RESULTS**

*S-memantine Released H₂S in the Medium—*To determine the timing and levels of sulfide release by different sulfide donors, sulfide concentrations after addition of Na₂S, ACS48 or S-memantine to the Dulbecco’s modification of DMEM/F12 with 10% FBS (without cells) was measured using HPLC as reported (10). Fig. 3A shows time-dependent changes of sulfide concentrations in the medium after addition of 20 μM of each compounds at time 0 at pH 7.4. Although Na₂S raised sulfide levels immediately, sulfide levels induced by Na₂S decreased rapidly and became lower than sulfide levels induced by ACS48.
and S-memantine at 1.5 h and 8 h after addition to the medium, respectively ($p < 0.01$ by two-way ANOVA with Bonferroni post-test). ACS48 and S-memantine increased sulfide levels to 3.6 μM and 5.1 μM after 24 h, respectively. Sulfide levels in the medium were higher after addition of S-memantine than ACS48 at all time points examined ($\sim 2.1$-fold, $p < 0.01$ by two-way ANOVA with Bonferroni post-test). Interestingly, both ACS48 and S-memantine released very little sulfide in PBS whereas ACS48 released more sulfide than did S-memantine in Tris-HCl (pH 9.5) and in DMEM/F12 without FBS (supplemental Fig. S1).

S-memantine Increased Intracellular Sulfide Levels—Incubation of SH-SY5Y cells with Na$_2$S, ACS48, and S-memantine increased intracellular sulfide levels with different magnitude and time course. Intracellular sulfide levels peaked around 1.5 h after addition of Na$_2$S and ACS48 to the medium that disappeared by 8 h (Fig. 3B). In contrast, incubation of SH-SY5Y cells with S-memantine increased intracellular sulfide level more markedly than incubation with ACS48 at all time points after addition ($\sim 10$-fold at 4 h, $p < 0.001$ by two-way ANOVA with Bonferroni post-test). In a separate experiment, we examined whether or not incubation with memantine itself or incubation with ACS48 and memantine would affect intracellular sulfide levels in SH-SY5Y cells. We found that memantine itself did not affect intracellular sulfide levels in SH-SY5Y cells incubated with or without ACS48 (Fig. 3C). Hence, it was indicated that chemical bonding between ACS48 and memantine would be important for the high intracellular sulfide levels achieved after addition of S-memantine.

Na$_2$S Failed to Improve Viability of SH-SY5Y Cells after Oxygen-Glucose Deprivation—We examined the effect of Na$_2$S since it has been widely used as a therapeutic compound against neuronal ischemia in vitro. Fifteen hours of OGD followed by 24 h of reoxgenation induced cell death in SH-SY5Y cells as indicated by increased LDH release into the medium (Fig. 4). Addition of Na$_2$S to the culture medium at various time points (pre-OGD or 0.5, 2, 5, and 8 h after the end of OGD) and concentrations (10 and 50 μM) failed to improve cell viability (Fig. 4).

S-memantine Improved Viability of SH-SY5Y Cells and Murine Primary Cortical Neurons after Oxygen-Glucose Deprivation and Reoxygenation—We examined whether or not ACS48, memantine, and S-memantine improves viability of SH-SY5Y cells subjected to 15 h of OGD followed by 24 h of reoxygenation. Based on our time- and dose-ranging studies, we determined that 50 μM and at 8 h after the end of OGD were the most effective dose and time point to add ACS48 or S-memantine to improve viability of SH-SY5Y cells after OGD (Fig. 5, A and B). Addition of S-memantine to the medium at 50 μM at 8 h after the end of OGD improved the viability of SH-SY5Y cells more markedly than did addition of ACS48 or memantine at the same dose and time point, as indicated in LDH release, MTT, and CV assays (Fig. 6E).

We also examined whether or not ACS48, memantine, and S-memantine improves survival of murine primary cortical neurons after 2.5 h of OGD followed by 21 h of reoxygenation. Based on our dose- and timing-ranging studies (Fig. 5, A and B), we added ACS48 and S-memantine at 50 μM at 30 min after the end of OGD. S-memantine exhibited more robust neuroprotective effects compared with ACS48 or memantine, as assessed by LDH release assay (Fig. 6E).

NMDAR Antagonist Suppressed Toxicity of H$_2$S in Murine Primary Cortical Neurons—To define the role of NMDAR in cytotoxicity of H$_2$S, we examined whether or not memantine suppresses toxicity of Na$_2$S and ACS48 to murine primary cortical neurons. LDH released from primary cortical neuronal was measured 24 h after addition of Na$_2$S or ACS48 with or without memantine (Fig. 7). Although incubation with Na$_2$S or ACS48 at 50 μM markedly increased LDH release in the murine primary cortical neurons, LDH release caused by Na$_2$S or ACS48 was abolished by co-incubation with 50 μM memantine, suggesting the critical role of NMDAR activation in the cytotoxicity of H$_2$S. Although S-memantine increased intracellular sulfide levels more robustly than Na$_2$S and ACS48, incubation with S-memantine at 50 μM did not induce LDH release from primary cortical neurons. These observations suggest that S-memantine retains the ability to antagonize NMDAR.

NMDAR Antagonist Prevented H$_2$S-induced Calcium Accumulation in Primary Cortical Neurons—Activation of NMDAR increases intracellular calcium concentration (15, 16). We examined the influence of 20 μM Na$_2$S, ACS48, S-memantine, and memantine on intracellular calcium levels ([Ca$^{2+}$]) in murine primary cortical neurons incubated with or without glutamate using previously reported approach with some modifications (17). Incubation with Na$_2$S or ACS48, but not memantine or S-memantine, without glutamate increased [Ca$^{2+}$] in primary cortical neurons (Fig. 8A). While incubation with Na$_2$S, ACS48 or S-memantine without glutamate increased [Ca$^{2+}$] in murine primary cortical neurons, magnitude of calcium accumulation induced by S-memantine was markedly smaller than that induced by Na$_2$S or ACS48. Similarly, S-memantine or memantine suppressed calcium accumulation in primary cortical neurons induced by 100 μM glutamate, whereas Na$_2$S or ACS48 augmented glutamate-induced calcium accumulation.
S-memantine Augmented Intracellular GSH Levels in SH-SY5Y Cells with or without Glutamate—Without glutamate, incubation with 50 μM Na₂S, ACS48, or S-memantine increased intracellular GSH levels in SH-SY5Y cells. While glutamate decreased GSH levels in cells incubated with medium alone (p < 0.001 by two-way ANOVA with Bonferroni post-test), incubation with Na₂S, ACS48, or S-memantine restored intracellular GSH levels. Further, the magnitude of increase of GSH levels after incubation with S-memantine was greater than with Na₂S or ACS48 (Fig. 8C). Memantine per se did not affect GSH levels.

S-memantine Inhibited Caspase-3 Activation and Dephosphorylation of ERK—S-memantine, but not memantine, at 50 μM added 8 h after the end of OGD prevented caspase-3 activation in SH-SY5Y subjected to 15 h of OGD followed by 24 h of reoxygenation. S-memantine or memantine did not affect Akt phosphorylation. S-memantine, but not memantine, attenuated dephosphorylation of ERK (Fig. 9).

S-memantine Increased Plasma and Brain Sulfide Levels—To define the impact of S-memantine on systemic sulfide concentrations, we measured plasma and cerebral sulfide levels 90 min after intraperitoneal administration of Na₂S, ACS48, or S-memantine at 25 μmol/kg in mice using HPLC (Fig. 10, A and B). ACS48 and S-memantine, but not Na₂S, increased plasma sulfide levels. Cerebral sulfide levels were increased after treatment with S-memantine, but not after Na₂S or ACS48.

S-memantine Attenuated Brain Damage and Improved Survival and Neurological Function after Global Cerebral Ischemia and Reperfusion in Mice—To validate the neuroprotective effects of S-memantine in vivo cerebral ischemia, we examined whether or not administration of Na₂S, ACS48, S-memantine, or memantine at 25 μmol/kg attenuated cerebral injury after BCAO in mice. Vehicle (corn oil/DMSO suspension) did not affect survival of mice after BCAO and reperfusion. Na₂S, ACS, or S-memantine at 25 μmol/kg did not affect body temperature. All vehicle- or Na₂S-treated mice died in 8 or 10 days (75% or 67% died in 2 days), respectively, whereas treatment with ACS48, memantine, or S-memantine enabled 1, 2, or 5 mice to survive for more than 60 days, respectively. S-memantine-treated mice exhibited markedly higher survival rate than vehicle, Na₂S, ACS48, or memantine-treated mice. (p < 0.05 by log-rank test). There was no significant difference in survival rate among Na₂S, ACS48, memantine, and vehicle groups (Fig. 11A). S-memantine and memantine improved neurological score on day 1–4 and day 2–3 after BCAO, respectively (p < 0.05 by two-way ANOVA with Bonferroni test, Fig. 11B). S-me-
mantine markedly decreased cerebral infarct volume compared with vehicle (Fig. 11C, \(p < 0.001\) versus vehicle by unpaired \(t\) test).

**DISCUSSION**

The current study demonstrated that a newly-synthesized H\(_2\)S-releasing NMDA receptor antagonist derivative S-memantine increases intracellular H\(_2\)S levels and protects neurons from OGD more robustly than conventional H\(_2\)S donor compounds Na\(_2\)S and ACS48 without cytoxicity. Our results showed that S-memantine retains the beneficial effects of memantine and prevents glutamate-induced intracellular calcium accumulation. We also validated that post-reperfusion treatment with S-memantine attenuates cerebral injury induced by global cerebral ischemia and reperfusion in mice. Taken together, these observations demonstrate that hybrid H\(_2\)S-releasing NMDAR antagonists may prevent ischemic brain injury.

Combining ACS48 and memantine by amide bonding apparently modified sulfide-releasing characteristics of ACS48. We observed that S-memantine released sulfide at \(2.1\) fold higher rate than did ACS48 in the cell culture medium (DMEM/F12) supplemented with \(10\%\) FBS (pH 7.4). On the contrary, ACS48 released greater amount of sulfide than did S-memantine in the cell culture medium without FBS (pH 7.4) or in Tris-HCl buffer (pH 9.5). Of note, both ACS48 and S-memantine released very little sulfide in PBS (pH 7.4). These results suggest that pH of the solution as well as unidentified proteins (e.g. enzymes) contained in FBS markedly modulate rate of sulfide release from
ACS48 and S-memantine. Detailed chemistry of sulfide-releasing kinetics of S-memantine remains to be determined in the future studies.

We found that S-memantine more robustly increased intracellular sulfide levels than did ACS48 (~10-fold), while sulfide level in the medium containing S-memantine was only ~2.1-fold higher than in the medium containing ACS48. This disproportional increase of intracellular sulfide levels with S-memantine was not caused by the presence of memantine, because memantine does not affect the intracellular sulfide levels of SH-SY5Y cells incubated with or without ACS48. We speculate that strong hydrophobicity of S-memantine makes it more membrane permeable than ACS48. ACS48 is a carboxylic acid while S-memantine is a carboxylamide. Of note, solubility of ACS48 and S-memantine to DMEM/F12 are greater than 1.5 mM and less than 10 μM, respectively.

While protective effects of H2S donors on cell viability have been extensively studied, the majority of the studies examined effects of pre-administration of H2S donors before ischemic/hypoxic insults. For example, Ren et al. reported that NaHS at 25 μmol/kg administered at 30 min before ischemia attenuated rat neuronal injury induced by global cerebral ischemia and reperfusion (18). Tay et al. reported that pretreatment with NaHS at 50 or 100 μM added at 15 min before Na2S2O4-induced hypoxia improved survival of SH-SY5Y cells (19). Nonetheless, effects of any treatment strategies are more clinically translatable if the treatment is effective when it is initiated after ischemic/hypoxic insults. The current study demonstrated that 10 or 50 μM of Na2S administered pre- or post-OGD did not improve cell viability of SH-SY5Y subjected to OGD. Similarly, administration of 25 μM of Na2S 1 min after reperfusion after BCAO failed to prevent brain injury in mice. In contrast, cell viability of SH-SY5Y cells or primary cortical neurons subjected to OGD were improved by incubation with S-memantine starting at any time between pre-OGD and up to 8 h after OGD or at 30 min or 2 h after OGD, respectively. Furthermore, these in vitro findings were confirmed by our in vivo studies in which administration of S-memantine 1 min after reperfusion after BCAO markedly improved survival rate and neurological outcomes in mice. Taken together, these results indicate that slow H2S-releasing compounds have higher therapeutic potential against neuronal ischemia than simple sulfide salts.

Although Na2S and ACS48 showed significant toxicity to primary cortical neurons, the cytotoxicity was abolished by co-incubation with memantine. Interestingly, S-memantine showed no toxicity to primary cortical neurons up to 50 μM while ACS48 was toxic at 10 μM. Memantine did not affect the intracellular sulfide levels of SH-SY5Y cells incubated with or without ACS48. We speculate that strong hydrophobicity of S-memantine makes it more membrane permeable than ACS48. ACS48 is a carboxylic acid while S-memantine is a carboxylamide. Of note, solubility of ACS48 and S-memantine to DMEM/F12 are greater than 1.5 mM and less than 10 μM, respectively.

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Although Na2S and ACS48 showed significant toxicity to primary cortical neurons, the cytotoxicity was abolished by co-incubation with memantine. Interestingly, S-memantine showed no toxicity to primary cortical neurons up to 50 μM (upper limit of solubility of this compound in the culture medium). While short application (<15 min) of relatively low concentration of NaHS (~130 μM) did not induce any current via NMDA receptor (20), overnight incubation with higher concentration of NaHS (200–1000 μM) alone caused neuronal death that is totally preventable by pretreatment with NMDAR antagonists (9). Our results may be in line with the latter study since we incubated primary cortical neurons with H2S donors for 24 h. On the other hand, H2S may activate other calcium channels and thereby indirectly activate NMDA receptors via releasing glutamate (21). Taken together, these observations support the hypothesis that cytotoxicity of H2S is at least in part mediated via enhancement of NMDAR activation.

Since NMDAR activation increases intracellular calcium concentration, we hypothesized that S-memantine prevents cytotoxicity of H2S by inhibiting intracellular calcium accumulation induced by H2S. We found that [Ca2+]i in S-memantine-treated neuronal cells was markedly lower than that of Na2S- or
ACS48-treated cells. Moreover, S-memantine markedly suppressed glutamate-induced calcium accumulation in primary cortical neurons. These observations suggest that S-memantine suppresses calcium accumulation induced not only by H₂S but also by glutamate. Glutamate-induced excitotoxicity contributes to ischemic cerebral injury. It is therefore possible that at least some of the beneficial effects of S-memantine are mediated via its inhibitory effects on NMDAR-induced excitotoxicity.

Another possible mechanism of beneficial effects of H₂S may include augmentation of intracellular GSH levels. For example, Kimura et al. reported that NaHS prevented the glutamate-induced GSH depletion of immortalized mouse hippocampal cell line or murine primary cortical neurons (13, 22). In the current study, we confirmed that Na₂S up to 200 μM dose-dependently increased intracellular GSH levels in SH-SY5Y cells (data not shown). We further demonstrated S-memantine more robustly prevents glutamate-induced GSH depletion in SH-SY5Y cells than Na₂S, ACS48, or memantine. The higher intracellular sulfide levels induced by S-memantine compared with Na₂S or ACS48 may have further augmented intracellular GSH levels. These observations are in line with the study by Kimura et al. that showed H₂S protects neurons by maintaining the activities of γ-glutamylcysteine ligase.
FIGURE 10. Plasma (A) and cerebral sulfide (B) levels of mice, measured 90 min after intraperitoneal administration of Na₂S, ACS48, or S-memantine at 25 μmol/kg. n = 4 each. *, p < 0.05; **, p < 0.01.

FIGURE 11. A, percent survival of mice subjected to 40 min of bilateral carotid artery occlusion (BCAO) and reperfusion and treated with S-memantine, memantine, ACS48, Na₂S, or vehicle 1 min after reperfusion. n = 12 each. *, p < 0.05 versus Na₂S, ACS48, memantine and vehicle by log-rank test. B, neurological score after BCAO. Vehicle and ACS48 (n = 12, 6, 3, 3, 3 on day 0, 1, 2, 3, 4, respectively), Na₂S (n = 12, 8, 4, 4, 4), memantine (n = 12, 8, 3, 3, 3), S-memantine (n = 12, 11, 8, 7, 7), Sham (n = 5). *, **, or ***, p < 0.05, 0.01, or 0.001 versus vehicle, respectively. C, cerebral infarct volume and representative photographs of TTC-stained brain of mice subjected to sham operation or BCAO and treated with vehicle or S-memantine. n = 5 each. ***, p < 0.001 versus vehicle by unpaired t test.
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Tamylcysteine synthetase (γ-GCS) and cystine transport thereby augmenting GSH levels (23).

Multiple molecular signaling pathways have been implicated in the mechanisms responsible for cell-protective effects of sulfide (24, 25). In the current study, S-memantine prevented caspase-3 activation in SH-SYSY subjected to OGD. The anti-apoptotic effects of S-memantine were associated with maintenance of ERK phosphorylation but not with changes in Akt phosphorylation (Fig. 9). These results suggest that anti-apoptotic effects of S-memantine may be mediated by attenuation of ERK-dephosphorylation (26, 27).

High doses of sulfide salts appear to be detrimental after cerebral ischemia in animal models. For example, Moore et al. reported that pre-treatment with Na$_2$S at 90 μmol/kg administered 10 min before middle cerebral artery occlusion (MCAO) did not affect, whereas at 180 μmol/kg, aggravated cerebral injury of rats (8). Ren et al. reported that NaHS at 25 μmol/kg administered 30 min prior to ischemia, but not at 90 or 180 μmol/kg, attenuated neuronal injury induced by global cerebral ischemia (18). To examine the therapeutic potential of H$_2$S donors at more clinically-relevant post-ischemic timing, we examined effect of Na$_2$S, ACS48, S-memantine or memantine at 25 μmol/kg administered 1 min after reperfusion following BCAO. Moore et al. reported that H$_2$S-releasing moiety 5-((4-hydroxyphenyl)-3H-1,2-dithiol-3-thione (ADT-OH), which has a similar chemical structure to ACS48, showed the highest conversion to H$_2$S 90 min after addition to the rat plasma in vitro (28).

In the current study, ACS48 or S-memantine at 25 μmol/kg, but not Na$_2$S, increased plasma sulfide levels in mice 90 min after intraperitoneal administration. Consistent with our observations in SH-SYSY cells (see Fig. 1B), administration of S-memantine, but not Na$_2$S or ACS48, increased cerebral sulfide levels. We found that S-memantine markedly improved neurological function and 60-day survival rate and decreased neurological function and 60-day survival rate and decreased sulfide levels. We found that S-memantine markedly improved neurological function and 60-day survival rate and decreased

and biological effects of hydrogen sulfide (H$_2$S): development of H$_2$S-releasing drugs as pharmaceuticals. J. Med. Chem. 53, 6275–6286


