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Short communication

Neuroprotective effect of *S*-allyl-L-cysteine derivatives against endoplasmic reticulum stress-induced cytotoxicity is independent of calpain inhibition

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ABSTRACT

S-allyl-L-cysteine (SAC) is known to have neuroprotective properties. We synthesized various SAC derivatives and tested their effects on endoplasmic reticulum stress-induced neurotoxicity in cultured hippocampal neurons (HPNs). Among the compounds tested, *S*-propyl-L-cysteine (SPC) exhibited the strongest neuroprotective activity in HPNs, followed by *S*-ethyl-L-cysteine (SEC) and *S*-methyl-L-cysteine (SMC). Unlike SAC and SMC, SPC and SEC did not have inhibitory activity on μ-calpain, suggesting that the mechanism underlying the protective activity of SPC and SEC differs from that of SAC.

S-allyl-L-cysteine (SAC), a sulfur-containing amino acid present in aged garlic extract, has long been used as a common dietary supplement and traditional medicine. SAC has been shown to exert multiple biological effects, including antioxidant (1), anti-inflammatory (2), and neurotrophic (3) activities. SAC is reportedly absorbed in the gastrointestinal tract after oral administration, and has already been used to treat patients with hypertension without any obvious signs of toxicity (4). These results strongly suggest that SAC can be a beneficial therapeutic agent with few adverse effects.

The endoplasmic reticulum (ER) is an important organelle required for cell survival and normal cellular processes, such as regulation of protein synthesis, appropriate folding of protein, and calcium storage. Conditions such as glucose deprivation, calcium depletion and exposure to free radicals induce accumulation of unfolded proteins in the ER, a condition known as ER stress (5). ER stress is involved in neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, Huntington's disease, and amyotrophic lateral sclerosis (6). Therefore, amelioration of ER stress-induced toxicity could be an effective approach for the treatment of these neurodegenerative disorders. Previously, we have demonstrated that SAC exerts protective activity against ER stress-induced neurotoxicity in cultured rat hippocampal neurons (HPNs) (7,8) and organotypic hippocampal slice cultures (9). We have also demonstrated that the neuroprotective effect of SAC is at least partly attributable to direct inhibition of μ-calpain, a family of Ca^{2+}-dependent cysteine proteases, through binding of SAC to the Ca^{2+}-binding domain of this enzyme (10). However, structurally related compounds with antioxidant activity, L-cysteine (CYS) and *N*-acetylcysteine (NAC), had no effect on calpain activity, suggesting that the sulfhydryl group does not play a role in the inhibition of μ-calpain activity.

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In the present study, in order to explore SAC-related compounds that might have stronger neuroprotective activity, we synthesized various structurally related derivatives of SAC and examined their effects on tunicamycin (TM)-induced neuronal death in HPNs and μ-calpain activity using an *in vitro* assay system.

A list of SAC derivatives is shown in Table 1. Using thin-layer chromatography (TLC) and ^{1}H NMR (proton nuclear magnetic resonance), we confirmed that all of the SAC derivatives described above were pure forms. SAC and its derivatives were dissolved in distilled water or dimethyl sulfoxide (DMSO).

Experiments were carried out in accordance with the Guidelines for Animal Experiments at Nihon University. HPNs were prepared as described previously (7). Briefly, hippocampi were isolated from the brains of embryonic day 18 Wistar rats and treated with TrypLE Express™ (Thermo Fisher Scientific, Waltham, MA, USA) and 0.01% deoxyribonuclease (Sigma–Aldrich, St Louis, MO, USA). The cells were plated at a density of 5.0×10^5 cells/cm^2 on poly-L-lysine coated 48-well culture plates (Asahi Techno Glass, Tokyo, Japan). They were cultured at 37 °C in humidified 5% CO_2/95% air for 7 days.

Cell viability was determined using a [3-(4, 5)-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium (MTT) reduction assay as described previously (10). Briefly, the cells were incubated with MTT (0.25 mg/mL, Sigma) for 4 h at 37 °C, and the reaction was stopped by adding a solution of 50% dimethylformamide and 20% SDS, pH 4.8. The next day, the amount of MTT formazan product was determined by measuring its absorbance with a microplate reader.

Enzymatic activities of purified μ-calpain (Calbiochem, San Diego, CA, USA) was measured according to the protocol provided with the Calpain-Glo™ protease assay kit (Promega, Madison, WI, USA) as described previously (10). Briefly, 50 μL of luminogenic succinyl-calpain substrate (Suc-LLVY-aminoluciferin) was added to 50 μL of sample containing SAC derivative with 1 μM Ca^{2+} and purified recombinant human μ-calpain (100 μM) in each well of a white 96-well plate. Samples were analyzed using a plate-reading luminometer (FlexStation3, Molecular Devices, CA, USA). The measured value minus the background without drug was normalized to calpain activity from vehicle-treated samples (control; 100%) and expressed as a percentage of the control.

Data are expressed as mean ± SEM. Significance was determined by one way ANOVA as appropriate by applying Tukey's multiple range tests. A value of <5% was considered significant.

A previous study from our laboratory has shown that exposure of HPNs to TM, a typical ER stress inducer, resulted in a decrease of MTT reduction in a concentration-dependent manner (1–10 μg/mL), and that the maximal protective effect of SAC on TM (10 μg/mL)-induced neuronal death was observed at 1 μM in the HPNs (7). We also reported previously that the results of MTT reduction assay were comparable to those of propidium iodide/Hoechst33258 double staining and phase-contrast microscopy in the HPNs (7). In order to investigate the protective effect of SAC derivatives against ER stress-induced neuronal cell death, HPNs were treated with TM (10 μg/mL) in the absence or presence of 1 μM SAC derivatives. Exposure to TM for 24 h resulted in a significant decrease (43.0%) of MTT reduction activity in the HPNs (Fig. 1A). Consistent with previous results (8), this decreased MTT reduction activity was restored significantly by simultaneously applied SAC (Compound 1). Among all the compounds tested, a series of SAC derivatives with 3-alkylthio groups (Compounds 4, 7 and 10) also exhibited a neuroprotective effect. Among these three compounds, *S*-propyl-L-cysteine (SPC, Compound 10) exhibited the strongest neuroprotective activity with a recovery of up to 84.8%, followed by *S*-ethyl-L-cysteine (SEC, Compound 7) and *S*-methyl-L-cysteine (SMC, Compound 4). In contrast, a sequence of compounds with a bulky substituent (Compounds 3, 5, 8, 13 and 14) showed cytotoxic effects. A number of compounds with a modified carboxyl group or amino group (Compounds 6, 11 and 15) were found to be as equally effective as SAC. The bioactivity of compounds sharing the same basic skeleton is influenced by the substituents attached to the skeleton. Indeed, the cytoprotective effect of sodium 4-phenylbutyrate, a chemical chaperone, against ER stress-induced toxicity increased with the number of carbons in the terminal aromatic substituted fatty acids (11). These results suggest that the number of linear carbon atoms in the 3-alkylthio group of SAC derivatives plays a key role in potentiating the protective activity against ER stress-induced neurotoxicity.

In order to address the potential role of calpain in TM-induced neuronal cell death in the HPNs, the effect of Z-Leu-Leu-Tyr-CH2F, a typical μ-calpain inhibitor (12), on TM-induced cell death was investigated. When various concentrations of Z-Leu-Leu-Tyr-CH2F (1–10 μM) was applied simultaneously with TM, it attenuated the TM-induced decrease of MTT reduction in a concentration-dependent manner in the HPNs, and significant protective effect was seen at concentrations of 5 and 10 μM (Fig. 1B). These results

Table 1
List of SAC derivatives used in this study.

ID	IUPAC name	Common name	Structure	M.W	Solvent
1	(*R*)-3-(Allylthio)-2-aminopropanoic acid	*S*-Allyl-L-cysteine (SAC)	$C_6H_{11}NO_2S$	161.222	Distilled water
2	(*S*)-3-(Allylthio)-2-aminopropanoic acid	*S*-Allyl-D-cysteine (*ent*-SAC)	$C_6H_{11}NO_2S$	161.222	Distilled water
3	(*S*)-2-Amino-3-(benzylthio)propanoic acid	*S*-Benzyl-D-cysteine (*ent*-SBC)	$C_{10}H_{13}NO_2S$	211.2807	DMSO
4	(*R*)-2-Amino-3-(methylthio)propanoic acid	*S*-Methyl-L-cysteine (SMC)	$C_4H_9NO_2S$	135.1848	Distilled water
5	(*R*)-2-Amino-3-(prop-2-ynylthio)propanoic acid	*S*-Propynyl-L-cysteine (SPC)	$C_6H_9NO_2S$	159.2062	Distilled water
6	(*R*)-2-Acetamido-3-(allylthio)propanoic acid	*N*-Acetyl-*S*-allyl-L-cysteine (NASAC)	$C_8H_{13}NO_3S$	203.2587	Distilled water
7	(*R*)-2-Amino-3-(ethylthio)propanoic acid	*S*-Ethyl-L-cysteine (SEC)	$C_5H_{11}NO_2S$	149.2113	Distilled water
8	(*R*)-2-Amino-3-(benzylthio)propanoic acid	*S*-Benzyl-L-cysteine (SBC)	$C_{10}H_{13}NO_2S$	211.2807	DMSO
9	(*R*)-2-Acetamido-3-(allylthio)propanamide	*N*-Acetyl-*S*-allyl-L-cysteine amide (NASACA)	$C_8H_{14}N_2O_2S$	202.274	Insoluble
10	(*R*)-2-Amino-3-(propylthio)propanoic acid	*S*-Propyl-L-cysteine (SPC)	$C_6H_{13}NO_2S$	163.2379	Distilled water
11	(*RS*)-4-(Allylthio)-2-aminobutanoic acid	*S*-Allyl-DL-homocysteine (*rac*-SAHC)	$C_7H_{13}NO_2S$	175.2486	Distilled water
12	(*R*)-Methyl 3-(allylthio)-2-aminopropanoate	Methyl *S*-allyl-L-cysteinate (MSAC)	$C_7H_{13}NO_2S$	175.2486	DMSO
13	(*R*)-2-Amino-3-(carboxymethylthio)propanoic acid	*S*-Carboxymethyl-L-cysteine (SCMC)	$C_5H_9NO_4S$	179.1943	DMSO
14	(*R*)-2-Amino-3-(tritylthio)propanoic acid	*S*-Trityl-L-cysteine (STC)	$C_{22}H_{21}NO_2S$	363.4726	DMSO
15	(*R*)-3-(Allylthio)-2-aminopropanamide	*S*-Allyl-L-cysteine amide (SACA)	$C_6H_{12}N_2OS$	160.2373	Distilled water
16	(*S*)-3-(Allyloxy)-2-aminopropanoic acid	*O*-Allyl-L-serine (OAS)	$C_6H_{11}NO_3$	145.1564	Distilled water
17	(*S*)-Methyl 3-(allyloxy)-2-aminopropanoate	Methyl *O*-allyl-L-serinate (MOAS)	$C_7H_{13}NO_3$	159.183	Distilled water
18	(*S*)-3-(Allyloxy)-2-aminopropanamide	*O*-Allyl-L-serine amide (OASA)	$C_6H_{12}N_2O_2$	144.1717	Insoluble
19	(*S*)-Methyl 3-(allyloxy)-2-(tert-butoxycarbonylamino)propanoate	Methyl *O*-allyl-*N*-Boc-L-serinate (MOANBS)	$C_{12}H_{21}NO_5$	259.2988	Insoluble
20	(*R*)-Methyl 3-(allylthio)-2-(nicotinamido)propanoate	Methyl *S*-allyl-*N*-nicotinoyl-L-cysteinate (MSANNC)	$C_{13}H_{16}N_2O_3S$	280.3427	Distilled water