Neuroprotective Effect of Demethylsuberosin, a Proteasome Activator, against MPP+-induced Cell Death in Human Neuroblastoma SH-SY5Y Cells

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Abstract

Demethylsuberosin isolated from the roots of *Cudrania tricuspidata* demonstrated a potent proteasome activator by enhancing all three chymotrypsin-like, trypsin-like, and caspase-like proteasome activities in a 20S proteasome activity assay. It also attenuated the 1-methyl-4-phenylpyridinium-induced dysfunction of the chymotrypsin-like and caspase-like activities of proteasome in SH-SY5Y cells with EC_{50} values of 0.76 µM and 0.82 µM, respectively. Additionally, demethylsuberosin protected neuronal cells against 1-methyl-4-phenylpyridinium-induced cell death with an EC_{50} value of 0.17 µM, while the EC_{50} value of betulinic acid was 4.29 µM. We are reporting that demethylsuberosin is a potent proteasome activator with a neuroprotective effect, suggesting a possible candidate for the protection or treatment of neurodegenerative diseases such as Parkinson's disease.

Key words

Cudrania tricuspidata · Moraceae · demethylsuberosin · neuroprotection · proteasome activator · 1-methyl-4-phenylpyridinium (MPP+)

Supporting information available online at http://www.thieme-connect.de/products

Cudrania tricuspidata (Carr.) Bureau ex Lavallee, belonging to the Moraceae family, is a small thorny tree widely distributed in Korea, Japan, and China, and its neuroprotective [1], anti-inflammatory [2], and antioxidant [3] effects have been reported. Recently it was reported that xanthones from the root bark [4] and isoflavones from the fruit [5] of *C. tricuspidata* reveleaed neuroprotective effects against 6-hydroxydopamine (6-OHDA)-induced cell death in SH-SY5Y cells. Demethylsuberosin, a prenylated coumarin, was isolated from the root of *C. tricuspidata*, and it was reported that demethylsuberosin showed a significant feeding deterrence effect against instars of *Spodoptera exigua* [6] and anti-inflammatory activity [7]. While many naturally occurring or synthetic proteasome inhibitors like epoxomicin and carfilzomib [8,9] have been substantially reported, proteasome activators have been rarely reported except for betulinic acid. Betulinic acid, as such, is a naturally occurring compound and it activates the chymotrypsin-like property of proteasome [10]. Neurodegenerative diseases such as Parkinson's disease are characteristic of the failure of the ubiquitin proteasome system (UPS) [11]. It was reported that proteasome activator 700 (PA700) and proteasome activator 28 (PA28), the cellular proteasome activators, showed decreased activity in the pars compacta of the substantia nigra in sporadic Parkinson's diseases [12]. 1-Methyl-4-phenylpyridinium (MPP+) is a Parkinsonism-inducing neurotoxin and it causes specific cell death in dopaminergic neurons. The rapid accumulation of MPP+ in the mitochondrial matrix inhibits mitochondrial respiratory chain complex I (NADH : ubiquinone oxidoreductase), resulting in the depletion of adenosine triphosphate (ATP) synthesis [13]. The depletion of ATP synthesis generates reactive oxygen species (ROS) and inhibits the function of ATP-dependent UPS, leading to the disruption of the unfolded protein response (UPR), and finally causes neuronal cell death [14]. We are reporting that demethylsuberosin potently activates proteasome and it has a neuroprotective effect.

Demethylsuberosin (**Fig. 1**) (> 95% purity) was isolated from the root of *C. tricuspidata*, and its structure was characterized by previously reported spectroscopic data [15]. As shown in **Fig. 2**, demethylsuberosin dose-dependently activated the chymotrypsin-like, trypsin-like, and caspase-like proteasome activities in a 20S proteasome activity assay, whereas the control compound, betulinic acid (> 97% purity, Enzo Life Science), revealed a less potent proteasome activity than demethylsuberosin. As shown in **Fig. 3**, demethylsuberosin attenuated the MPP+-induced dysfunction of the chymotrypsin-like and caspase-like activities of proteasome in SH-SY5Y cells (Human neuroblastoma; ATCC No. CRL-2266) with EC_{50} values of 0.76 µM and 0.82 µM, respectively, but not the trypsin-like activity. Betulinic acid revealed a less potent proteasome activity with an EC_{50} value of 3.56 µM (chymotrypsin-like activity) and 3.66 µM (caspase-like activity). As shown in **Fig. 4**, demethylsuberosin protected neuronal cells against MPP+-induced cell death with an EC_{50} value of 0.17 µM, while the EC_{50} value of betulinic acid was 4.29 µM.

UPS is critical for the degradation of damaged and aberrant proteins, and the accumulation of ubiquitinated proteins is a hallmark of many neurodegenerative diseases, such as Parkinson's disease. Recent studies suggest that proteasomal impairment plays an important role in these diseases [14,16]. In dopaminergic neurons, the rapid accumulation of MPP+ occurs in the mitochondrial matrix and it inhibits NADH dehydrogenase, resulting in the depletion of ATP synthesis and decreasing the function of ATP-dependent UPS [17]. It was reported that MPP+ upregulates and aggregates α-synuclein by dysfunction of the UPS and, finally, causes neuronal cell death [18]. It was also reported that an antioxidant, a keto-cartenoid astaxanthin [19], and verbascoside [20] protected neuronal cells against MPP+-induced cell death in SH-SY5Y cells. Based on our data, the neuroprotective effect of demethylsuberosin is partly due to the activation of proteasome, even though further research is needed in relation to the neuroprotective effect of demethylsuberosin and the UPS.

Materials and Methods

The root bark of *C. tricuspidata* was collected by the Korea Forest Research Institute, Southern Forest Research Center, Jinju, Korea, in September 2008 and authenticated by Dr. Hak Ju Lee (Korea

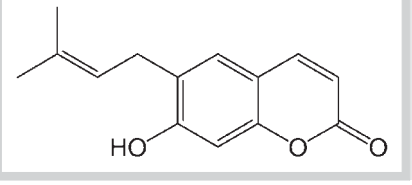

Fig. 1 Chemical structure of demethylsuberosin.

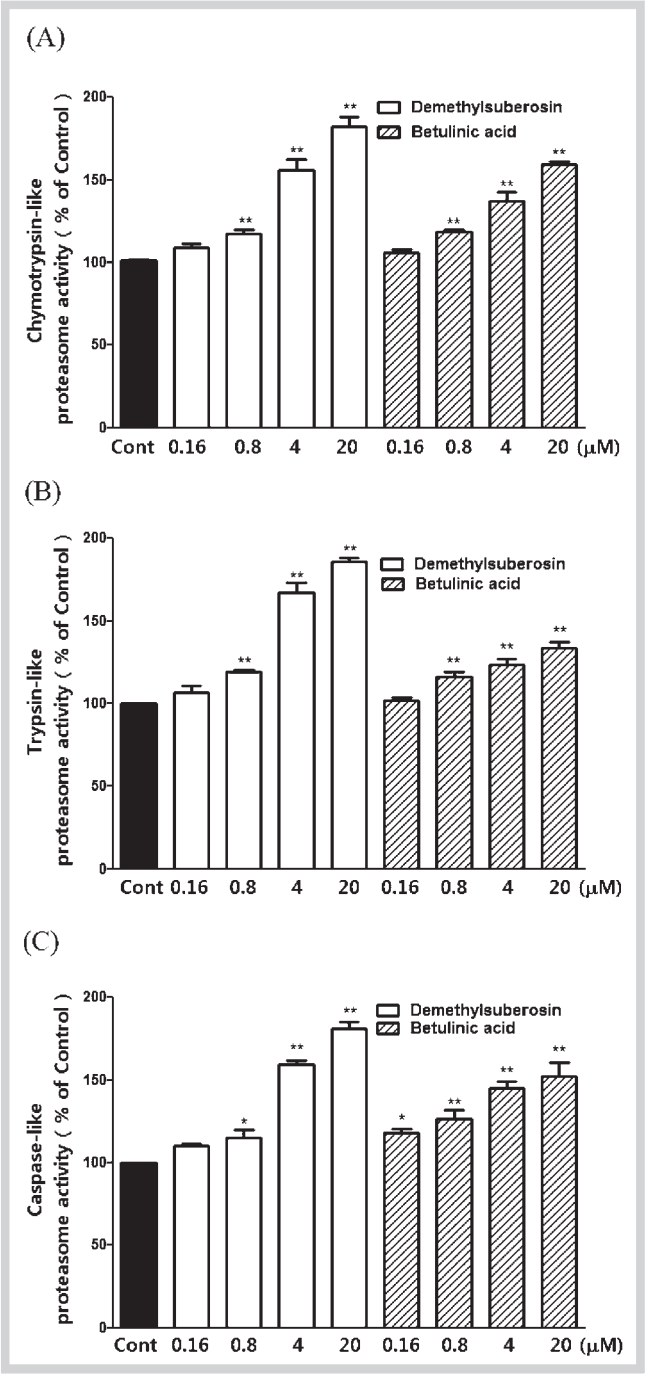

Fig. 2 Effects of demethylsuberosin on proteasome activities. Fluorogenic peptides used as substrates were Suc-LLVY-AMC (at 40 μM), Boc-LRR-AMC (at 40 μM), and Z-LLE-MCA (at 80 μM) for chymotrypsin-like (**A**), trypsin-like (**B**), and caspase-like (**C**) proteases activities, respectively. Betulinic acid was used as a control compound. The activity is given as a percentage of that of the control, and data represent the mean ± SD of three independent experiments; $p < 0.01$ and $^{**}p < 0.005$, compared with the control group, respectively.

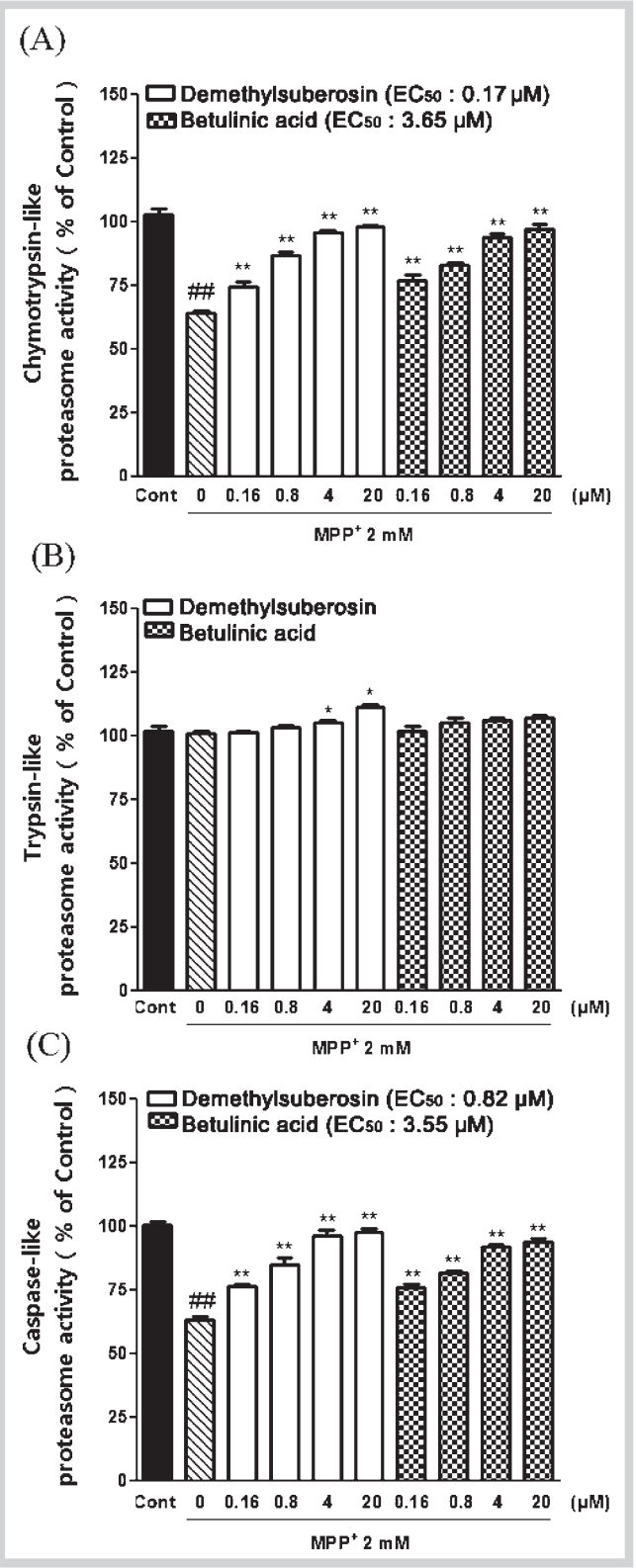

Fig. 3 Effects of demethylsuberosin against MPP^+-induced dysfunction of proteasome activities in SH-SY5Y cells. Cells were cultured in 48-well plates for 24 h and samples were simultaneously treated with MPP^+ (2 mM) for 48 h. Betulinic acid was used as a control compound. The activity is given as a percentage of that of the control, and data represent the mean ± SD of three independent experiments; $^{\#\#}p < 0.005$, compared with the control group; $^{*}p < 0.01$ and $^{**}p < 0.005$, compared with the MPP^+-induced group, respectively.

Forest Research Institute, Seoul, Korea). A voucher specimen (accession number KH1–4–090814) was deposited at the Department of Biosystems and Biotechnology, Korea University, Seoul, Korea.

The dried root bark of *C. tricuspidata* (13.0 kg) was ground and extracted with MeOH (48 L, 20 L, and 18 L) at room temperature,

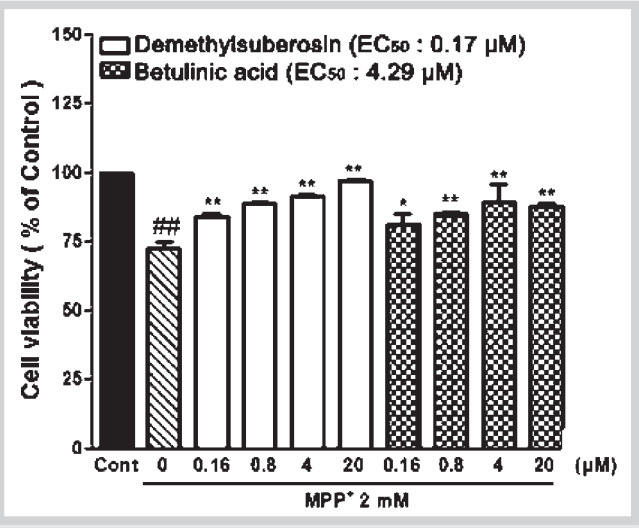

Fig. 4 Neuroprotective effects of demethylsuberosin against MPP^+-induced cell death in SH-SY5Y cells. Cells were cultured in 96-well plates for 24 h and samples were simultaneously treated with MPP^+ (2 mM) for 48 h. Betulinic acid was used as a control compound. The cell viability is given as a percentage of that of the control, and data represent the mean ± SD of three independent experiments; $p < 0.005$, compared with the control group; $^*p < 0.01$ and $^{**}p < 0.005$, compared with the MPP^+-induced group, respectively.

and the extracts were concentrated *in vacuo* at 35 °C. The dark brown residue (702.1 g) was suspended in H_2O (4.0 L) and partitioned with *n*-hexane (4.0 × 5 L) and EtOAc (4.0 × 6 L), sequentially. The EtOAc-soluble fraction (213.0 g) was applied to a silica gel column (15 × 60 cm, mesh 230–400) using $CHCl_3$/MeOH (1 : 0 to 1 : 1, 6 L for each eluent) to afford seven fractions (F1, 6 L; F2, 6 L; F3, 6 L; F4, 12 L; F5, 18 L; F6, 24 L; F7, 18 L). F4 (36.0 g) was fractionated on a silica gel column (10 × 60 cm, mesh 230–400) with *n*-hexane/EtOAc (30 : 1 to 0 : 1, 4 L for each eluent) to give eight fractions (F4.1, 7 L; F4.2, 3 L; F4.3, 4 L; F4.4, 4 L; F4.5, 3 L; F4.6, 7 L; F4.8, 9 L). F4.5 (7.2 g) was chromatographed on a silica gel column (10 × 60 cm, mesh 230–400) using $CHCl_3$/MeOH (1 : 0 to 5 : 1, 2 L for each eluent) to afford seven fractions (F4.5.1 to F4.5.7, each 2 L), and F4.5.2 (4.6 g) was then subjected to a C_{18} reversed-phase silica gel column (6 × 70 cm, 75 μm) with MeOH/H_2O (4 : 1 to 1 : 0, 5 L for each eluent) to give 15 fractions (F4.5.2.1 to F4.5.2.15; each 1 L). F4.5.2.2 (37.1 mg) was purified by preparative HPLC (YMC Pack ODS-A, 250 × 20 mm i.d., 5 μm, 50–85% MeOH in H_2O, flow rate 8.0 mL/min) to afford demethylsuberosin (2.9 mg, > 95%).

SH-SY5Y cells in Dulbeco's modified Eagle's medium (DMEM) media were cultured in 96-well plates (5×10^4 cells/200 μL/well) for 24 h, and samples were simultaneously treated with MPP^+ (2 mM) for 48 h. Cell viability was performed using the MTT assay by measuring at 540 nm using a microplate reader (SpectraMax Plus 384, Molecular Devices), as described by Carmichael [21].

The proteolytic activities of the proteasome were measured by using a 20S proteasome activity kit (APT 280, Millipore), as described by the manufacturer's instructions. Briefly, 10 μL of test compounds (final 1% DMSO) were incubated in a provided buffer (final 100 μL) containing 40 μg of 20S proteasome and a substrate for 2 h at 37 °C. Fluorogenic peptides used as substrates were succinyl-Leu-Leu-Val-Tyr-7-amido-4-methylcoumarin (Suc-LLVY-AMC at 40 μM), *t*-butyloxycarbonyl-Leu-Arg-Arg-7-amido-4-methylcoumarin (Boc-LRR-AMC at 40 μM), and benzyloxycarbonyl-Leu-Leu-Glu-4-methyl-coumaryl-7-amide (Z-LLE-MCA at 80 μM) for chymotrypsin-like, trypsin-like, and caspase-like proteases activities, respectively. Suc-LLVY-AMC included in the kit was used and Boc-LRR-AMC and Z-LLE-MCA were purchased from Enzo Life Sciences. Reaction mixtures containing substrates and test samples without 20S proteasome were used as blanks, and proteasome activities were measured by quantification of relative fluorescent units from the release of the fluorescent-cleaved products aminomethylcoumarin (AMC) (at 380/460 nm) or methylcoumarylamide (MCA) (at 380/440 nm) using a microplate reader (SpectraMax Plus 384, Molecular Devices).

Cell-based proteasome activity was determined using MPP^+-treated SH-SY5Y cells, as described by Henrik Lovborg and coworkers [22]. Briefly, cells (1.0×10^5 cells/300 μL/well) were cultured in 48-well plates for 24 h in DMEM media supplemented with 10% FBS. Samples and MPP^+ (2 mM) were simultaneously treated for 48 h in DMEM media supplemented with 5% FBS. The proteolytic activity of the proteasome was evaluated in cell lysates by using a proteasome activity kit (APT 280; Millipore). In brief, 40 μg of cell lysate were incubated for 2 h at 37 °C in the provided buffer with fluorophore-linked peptide substrates. We used Suc-LLVY-AMC, Boc-LRR-AMC, and Z-LLE-MCA as the same substrates. Reaction mixtures without cell lysates were used as blanks and AMC or MCA fluorescence was measured at excitation/emission wavelengths of 380/460 and 380/440 nm, respectively.

Data obtained are expressed as the mean ± standard deviation (SD). Statistical significance was determined using GraphPad Prism (GraphPad Software). The differences among groups were evaluated by one-way analysis of variance (ANOVA) with Bonferroni's multiple comparison method. A p value less than 0.05 was considered to be statistically significant. All the data were obtained from at least three independent experiments.

Supporting information

Spectroscopic data of demethylsuberosin including an HPLC chromatogram and microscopy images of the cells are available as Supporting Information.

Acknowledgements

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Conflict of Interest

The authors declare no conflict of interest.

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