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# Involvement of heme oxygenase-1 in neuroprotection by sanguinarine against glutamate-triggered apoptosis in HT22 neuronal cells

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ABSTRACT

Sanguinarine is a natural compound isolated from the roots of *Macleaya cordata* and *M. microcarpa*, has been reported to possess several biological activities such as anti-inflammatory and anti-oxidant effects. In the present study, we demonstrated that sanguinarine markedly induces the expression of HO-1 which leads to a neuroprotective response in mouse hippocampus-derived neuronal HT22 cells from apoptotic cell death induced by glutamate. Sanguinarine significantly attenuated the loss of mitochondrial function and membrane integrity associated with glutamate-induced neurotoxicity. Sanguinarine protected against glutamate-induced neurotoxicity through inhibition of HT22 cell apoptosis. JC-1 staining, which is a well-established measure of mitochondrial damage, was decreased after treatment with sanguinarine in glutamate-challenged HT22cells. In addition, sanguinarine diminished the intracellular accumulation of ROS and $Ca^{2+}$. Sanguinarine also induced HO-1, NQO-1 expression via activation of Nrf2. Additionally, we found that si RNA mediated knock-down of Nrf2 or HO-1 significantly inhibited sanguinarine-induced neuroprotective response. These findings revealed the therapeutic potential of sanguinarine in preventing the neurodegenerative diseases.



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## 1. Introduction

Glutamate is an endogenous excitatory neurotransmitter, which is widely distributed in the central nervous system. However, at high concentrations, glutamate is neurotoxic and induces neuronal cell death, which contributes to the progression of neurodegenerative diseases (Chen et al., 2011). One main pathway that has always been described to explain the oxidative toxicity caused by glutamate is the inhibition of cystine/glutamate antiporter system by high concentrations of extracellular glutamate. Such inhibition prevents the uptake of cystine by cells, leading to depletion of intracellular glutathione and accumulation of ROS (Jeong et al., 2010b). ROS are major contributors of neuronal cell death and to a variety of pathological process within the nervous system (Melo et al., 2011; Jeong et al., 2010a). In addition to ROS production, glutamate-induced oxidative injury of neuronal cells has also been ascribed to an increase in calcium ($Ca^{2+}$) influx (Henke et al., 2013; Ha and Park, 2006). Increases in intracellular $Ca^{2+}$ can lead to mitochondrial dysfunction and apoptosis, and result in cell death by ROS generation (Kumar et al., 2012). Moreover, accumulation of mitochondrial $Ca^{2+}$ can initiate the apoptotic process through activation of the members of the pro-apoptotic Bcl2 family, leading to the formation of mitochondrial pores through which cytochrome c is released and the mitochondrial membrane potential (MMP) collapses (Kumar et al., 2012; Grebinyk et al., 2012; Tobaben et al., 2011). Currently, a widespread experimental model to study the pernicious effects of glutamate oxidative injury is the mouse hippocampus-derived neuronal cell line HT22. Because this cell line does not express functional ionotropic glutamate receptors, cell death excitotoxicity can be excluded when using HT22 cells (Gliyazova et al., 2013). Moreover, HT22 cells are being used as a tool to screen for novel neuroprotective reagents. Naturally occurring compounds such as alkaloids and limonoids may act as new therapeutic agents because of their anti-oxidant and ROS-scavenging abilities (Jeong et al., 2010a).

Heme oxygenase-1 (HO-1) is an inducible enzyme that degrades heme into three byproducts: carbon monoxide (CO), biliverdin, and free iron (Son et al., 2013). Due to their anti-oxidative effects, HO-1 and its enzymatic products play important roles in regulating the biological oxidative stress system, including reduction of ROS production (Jeong et al., 2010b; Son et al., 2013). Recent studies revealed that when HO-1 induced by natural compounds, it could generate CO, which, by itself, was effective in reducing ROS production (Terazawa et al., 2013; Bijjem et al., 2013). HO-1 expression is mainly regulated by the critical transcription factor Nrf2, which, in turn, is negatively regulated by Kelch-like ECH-associated protein 1 (Keap1). Upon stimulation, Nrf2 translocates to the nucleus, binds to the anti-oxidant response elements (AREs), and activates the promoter regions of many genes encoding phase II detoxification enzymes and anti-oxidants, including HO-1, and NAD(P)H quinine oxidoreductase 1 (NQO1) (Tsai et al., 2013).

Sanguinarine (SA) is a benzophenanthridine alkaloid, which is documented to have antimicrobial, anti-inflammatory, anti-oxidant, and anticancer properties (Han et al., 2013). Previous research has mainly focused on the anticancer effects of SA and its pro-apoptotic properties (Han et al., 2013). In present study, however, we focused on the neuroprotective effects of SA on glutamate-induced HT22 cell death and their dependency on HO-1 expression.

## 2. Materials and methods

### 2.1. Chemicals and reagents

Sanguinarine (purity ≥98%: HPLC) and other reagents were purchased from Sigma–Aldrich (St. Louis, MO, USA). Antibodies for TBP and α-tubulin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Dulbecco's modified Eagle medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco BRL (Invitrogen Corporation, Carlsbad, CA, USA).

### 2.2. Cell culture

Mouse HT22 hippocampal cells were a generous gift from Prof. Youn-Chul Kim (Wonkwang University, Iksan, Korea). Cells were cultured in DMEM supplemented with 5% heat-inactivated FBS and 0.1% penicillin–streptomycin (BioSource International, Camarillo, CA, USA) at 37 °C in a humidified atmospheric chamber with 5% $CO_2$.

### 2.3. Cell viability assay

Cells were incubated in 24-well plates at a density of $5 \times 105$ cells per well. MTT solution (5 μL at 5 mg/mL) was added to each well (final concentration, 62.5 μg/mL). After a 3-h incubation at 37 °C in 5% $CO_2$, the supernatant was removed and the formazan crystals produced by the viable cells were solubilized in 150 μL of dimethylsulfoxide (DMSO). The absorbance of each well was then read at 570 nm by using a microplate reader (Wallac 1420, Boston, MA, USA).

### 2.4. Measurement of intracellular ROS

To evaluate intracellular ROS levels, the cells were treated with CM-H2DCFDA, an indicator of general oxidative stress, for 1 h at 37 °C under 5% $CO_2$. The cells were then harvested and washed 3 times with PBS. Fluorescence intensity was measured using flow cytometry, at an excitation wavelength of 488 nm and an emission wavelength of 525 nm. Data analyses were performed using CXP software 2.0 (Beckman Coulter, Brea, CA, USA).

### 2.5. Western blot analysis

Cells were harvested in ice-cold lysis buffer consisting of 1% Triton X-100, 1% deoxycholate, and 0.1% sodium dodecyl sulfate (SDS). Protein content in the cell lysates was determined using the Bradford assay (Bio-Rad, Hercules, CA, USA). Total protein in each sample (50 μg) was resolved using 7.5% SDS-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to a polyvinylidene difluoride membrane, and incubated with the appropriate antibodies. The proteins were visualized using