Cytotoxic Effects of Gallic Acid and its Derivatives Against HIV-I-infected Microglia

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In the previous study, we found that flavonoids and ginsenosides exhibited high eliminate rates of human immunodeficiency virus type 1 (HIV-1) D3-transfected macrophages. Based on these findings, here we synthesized the derivatives of gallic acid, including methyl gallate, methyl 4-O-methyl gallate, methyl 3,4-O-dimethyl gallate, and methyl 3,4,5-O-trimethyl gallate and measured their cellular toxic effects against HIV-1-infected macrophages. Of these, treatment with methyl 4-O-methyl gallate in the presence of lipopolysaccharide (LPS) and cycloheximide (CHX) most effectively eliminated HIV-1-transfected cytoprotective human microglial CHME5 cells and HIV-1-D3-infected human primary macrophages. Furthermore, these strongly inhibited LPS/CHX-induced phosphorylation of phosphoinositide 3-kinase (PI3K), pyruvate dehydrogenase lipoamide kinase isozyme 1 (PDK1), Akt, and glycogen synthase kinase-3β (GSK-3β) in the Tat-transfected cells and HIV-1-D3-infected human primary macrophages. These findings suggest that methyl 4-O-methyl gallate may be a promising candidate for eliminating HIV-1 infected macrophages by blocking PI3K/Akt signaling pathway.

Key Words: HIV-1; Macrophage; Gallic acid; Methyl 4-O-methyl gallate

INTRODUCTION

The infection of human macrophages by human immunodeficiency virus type 1 (HIV-1) activates the phosphoinositide 3-kinase (PI3K)/Akt cell survival pathway, resulting in macrophages resisting to cytotoxic attacks, e.g., some HIV-1 antigens such as Tat protein and gp120 activate PI3K/Akt signaling pathway in macrophages (1, 2). PI3K/Akt signaling pathway regulates the expression of mammalian target of rapamycin (mTOR) and glycogen synthase kinase-3β (GSK-3β), resulting in the potentiation of cell survival and growth (3). In addition, the transfection of HIV-1 Tat into microglial CHME5 cells activates the PI3K/Akt signaling pathway by suppressing the expression of phosphatase and tensin homolog (PTEN), which is acting as a negative regulator. The expression of Tat protein from HIV-1 in the human microglial cell line, CHME5 as well as primary human macrophages, activates the PI3K/Akt pathway under the cellular stresses by reducing the level of PTEN, leading to a strong resistance to extracellular stresses such as LPS or nitric oxide (1, 4, 5). The Tat-transfected cells become resistant to extracellular cytotoxic insults such as LPS (6). HIV-1 Tat-expressed microglia in humans becomes long-
lived HIV-1 reservoirs in the central nervous system (4, 6, 7), and causes neuronal death and neurodegenerative diseases (8, 9). Therefore, to control these diseases, many studies have been conducted to eliminate the HIV-1-infected macrophages by searching for synthetic chemicals such as miltefosine (3), and phytochemicals such as arctigenin (5), oroxylin A and tectorigenin (10). These eliminated the HIV-1-infected macrophages by inhibiting PI3K/Akt cell survival signaling pathway.

Based on these findings, in the present study, we synthesized the derivatives of gallic acid and confirmed their anti-HIV-1 mechanism in CHME5 cells and primary macrophages.

MATERIALS AND METHODS

Reagents and antibodies

Cycloheximide (CHX), propidium iodide (PI), polybren, LPS purified from Escherichia coli O26:B6 and miltefosine were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Antibodies for p-PI3K p85α (Tyr 508), PI3K C2γ (M-228), p-Akt1/2/3 (Ser 473), Akt 1 (G-5), p-GSK-3β (Ser 9), GSK-3β (L-17), and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies for p-PDK1 (Ser 241), PDK1, mTOR, and p-mTOR (Ser 2448) were purchased from Cell Signaling Technology (Beverly, MA, USA). Human recombinant GM-CSF was purchased from R&D Systems (Minneapolis, MN, USA). Live/Dead Viability/Cytotoxicity Kit and Calcein AM and lipofectamine 2000 transfection reagent were purchased from Invitrogen (Carlsbad, CA, USA). HIV-1 vectors (D3) pseudotyped with the p-CMV-VSV-g envelope proteins (11) were kindly donated by Dr. Baek Kim (Emory University, Atlanta, GA, USA).

Gallic acid and its methyl derivatives

Gallic acid derivatives were obtained by following the commonly known procedure available in relevant literature (5). In briefly, gallic acid (compound 1) was transformed to its methyl ester (compound 2) by heating at reflux in methanol in the presence of a catalytic amount of c-H2SO4. The compound 2 was methylated by treating excess methyl iodide in the presence of K2CO3 in dimethylformamide (DMF) to yield its 4-O-methyl, 3,4-O-dimethyl, and 3,4,5-O-trimethyl ethers (compound 3, 4, and 5), respectively (Fig. 1).

Cell and cell culture

A human microglial cell line, CHME5, transfected with plasmid stably expressed full-length Tat, and a control cell line, transfected with plasmid pcDNA3.1-Hygro, were cultured in DMEM medium containing 10% fetal bovine serum (FBS). Human monocytes were isolated from the blood of volunteer donors, which was donated from Korea Red Cross Blood Donation Center (approved by the Committee for the Care and Use of Clinical Study in the center) (2, 10).
The peripheral blood mononuclear cells were collected by Ficoll density gradients and peripheral monocytes were isolated by the immunomagnetic selection using with anti-CD14 antibody-conjugated magnetic beads (Miltenyi Biotech, Bergisch Gladbach, Germany). The monocytes were incubated for four days in a RPMI 1640 medium containing 20% human AB serum (Sigma-Aldrich) and then cultured for an additional three days in the absence of recombinant GM-CSF to differentiate macrophages.

### Production of HIV-1-D3 particles

Primary macrophages were infected with a HIV-1-D3. First, D3 and pCMV-VSV-g vectors were transfected in 293-FT cells with lipofectamine 2000 transfection reagent for three days. Viral production was determined by measuring the level of p24 by enzyme linked-immunosorbent assay (Perkin Elmer Life Sciences, Boston, MA, USA) in the supernatant of the cell culture according to the manufacturer's protocol. The supernatant was treated in primary macrophages in the presence of polybrene (2 μg/ml) for five days. Virus-expressed primary macrophages were stained with green fluorescence and then detected by a fluorescent microscope because HIV-1-D3 contains green fluorescent protein (GFP) (Axio Vert.A1, Carl Zeiss, Oberkochen, Germany).

### Cytotoxicity assay

Tat-transfected CHME5 cells were stressed by treatment with CHX (10 μg/ml) and LPS (50 μg/ml) in the absence or presence of test compounds (5, 10, and 20 μM) for 48 h and then trypsinized and stained with Trypan blue solution (12). The dead and live cells were counted. To analyse the cytotoxicity of test compounds by flow cytometer, cells were trypsinized, stained with calcein AM (0.25 μM) / PI (1 μg/ml), and analyzed by a flow cytometer (C6 Flow Cytometer® System, BD, Ann Arbor, MI, USA). To check cytotoxicity of test compounds using fluorescent microscope, the cells were washed with PBS, stained with calcein AM (0.25 μM) / PI (1 μg/ml), and analyzed by a fluorescent microscope. The primary macrophages were infected with a GFP-conjugated HIV-1-D3 vector and stressed by treatment with CHX (10 μg/ml) and LPS (50 μg/ml) in the absence or presence of test compounds (5, 10 and 20 μM) for 48 h, then stained with PI (1 μg/ml), and analyzed by fluorescent microscopy (10). To detect cytotoxicity of test compounds in HIV-1-D3 infected macrophages, cells were stressed by treatment with CHX (10 μg/ml) and LPS (50 μg/ml) in the absence or presence of test compounds (5, 10 and 20 μM) for 48 h and trypsinized cells were stained with PI (1 μg/ml), and analyzed by flow cytometer. When treatment with LPS/CHX alone induced a significant amount of the untransduced/control cell death within 24 h, as previously reported (4), the analysis of the untransduced cells in the present experiment was not indicated. Test compounds were dissolved in dimethyl sulfoxide (a final concentration, 0.2%).

### Immunoblotting assay

Tat-transfected CHME5 cells (5 × 10^5 cells/well) or primary macrophages (1 × 10^6 cells/well) were treated with LPS/CHX in the presence or absence of test compounds for 2 h and then lysed (10). The supernatant of the lysates was applied to a 10% SDS polyacrylamide gel electrophoresis, followed by transferring to a nitrocellulose membrane. Protein Levels of PI3K, p-PI3K, PDK-1, p-PDK-1, Akt, p-Akt, GSK-3β, p-GSK-3β, mTOR, p-mTOR and β-actin were assayed as previously described (10). Immunodetection was carried out using an enhanced chemiluminescence detection kit.

### Statistical analysis

Data are indicated as the means ± a standard deviation (S.D.) of at least three replicates. One-way variance analysis and student's t-test were used. A p-value < 0.05 was statistically significant.

## RESULTS

The cytotoxic effects of gallic acid derivatives against HIV-1 Tat-transfected CHME5 cells

LPS-induced cell death in macrophages and microglial
cells requires blockade of protein synthesis, whereas HIV-1-infected cells were not died by LPS/CHX (12). Therefore, to evaluate the cytotoxic effects of gallic acid derivatives against HIV-1-infected human macrophages, we measured the effect of the synthetized compounds (1) through (5) against Tat-transfected CHME5 cells (Fig. 2). Treatment with LPS/CHX in the presence of compound 3 eliminated Tat-transfected cytoprotective CHME5 cells most effectively, followed by compounds 1 and 2. However, treatment with LPS/CHX in the absence of gallic acid derivatives did not exhibit any cytotoxicity against HIV-1-infected CHME5 cells.

**Figure 2.** The cytotoxic effects of gallic acid derivatives against cytoprotective HIV-1 Tat-transfected CHME5 cells. HIV-1 Tat-transfected CHME5 cells were treated with test compounds (0, 5, 10, and 20 μM) or miltefosine (MF, 20 μM) for 48 h. (Compound 1), gallic acid; (compound 2), methyl gallate; (compound 3), methyl 4-O-methyl gallate; (compound 4), methyl 3,4-O-dimethyl gallate; (compound 5), methyl 3,4,5-O-trimethyl gallate in the absence or presence of LPS/CHX. Cell death was determined by the trypan blue staining assay. All values are mean ± S.D. (n = 4). *, p < 0.05 compared with 0 μM treatment group.

To confirm the cytotoxic effects of compounds 1 and 3 against HIV-1-infected macrophages, HIV-1-D3 was transfected into the primary human macrophages, and then the cytotoxicity of which was measured by Trypan blue staining and PI staining with FACS using Live/Dead assay kit (Fig. 4). Treatment with compound 1 or 3 in the presence LPS/CHX showed the strong cytotoxicity in a dose-dependent manner. Compound 3 showed cytotoxic effect against the HIV-1-D3-infected primary human macrophages more potently than gallic acid (compound 1). However, compound 1 or 3 at a dose of 20 μM did not show the cytotoxicity (< 5%) under the experimental condition.

To investigate the cytotoxic mechanism of compounds 1 and 3 in HIV-1-D3-infected human macrophages, their effects on the PI3K/Akt cell survival signaling pathway were measured (Fig. 5). Treatment with LPS/CHX significantly increased the phosphorylation of PI3K, PDK1, Akt, GSK-3β, and mTOR. However, compounds 1 and 3 inhibited LPS/CHX-induced phosphorylation of PI3K, PDK1, Akt, GSK-3β, and mTOR.

**DISCUSSION**

HIV-1-infected human macrophages, including microglial cells, extend their lifespans by activating the PI3K/Akt cell survival signaling pathway (4) and transforming these cells into HIV-1 reservoirs, which persistently release the HIV-1 virus (13). Moreover, HIV-1-infected microglia and macrophages secrete nitric oxide and toxic viral proteins such as Tat and gp120, which establish the cytotoxic environment around the HIV-1-infected cells (4). The excessive release of NO and toxic viral proteins from these HIV-1-infected cells can cause neuron cell death, resulting in HIV-1-associated neurodegenerative diseases (14). Therefore, in
Figure 3. The cytotoxic effects of gallic acid and methyl 4-O-methyl gallate against cytoprotective HIV-1 Tat-transfected CHME5 cells. (A) Cytotoxic effects of gallic acid (compound 1) and 4-methoxy methyl gallate (compound 3) were determined using trypan blue staining assay. (B) Cytotoxic effects of gallic acid (compound 1) and 4-methoxy methyl gallate (compound 3) by the calcein AM/PI using a flow cytometer (C6 Flow Cytometer® System) and a fluorescence microscope. HIV-1 Tat-transfected CHME5 cells were treated with LPS/CHX in the absence or presence of test compounds (0, 10, and 20 μM) or miltefosine (MF, 20 μM) for 48 h. Trypsinized cells stained with calcein AM/PI were measured by a flow cytometer. All values are the mean ± S.D. (n = 4). (C) Cytotoxicity of test compounds was measured using a fluorescence microscope. Cells were stained with calcein AM/PI to distinguish between dead (red) and live (green) cells. Images (merged red and green fields) are representatives of 3 experiments conducted in duplicate. *, p < 0.05 compared with LPS/CHX treatment group.
Figure 4. Cytotoxic effects of gallic acid and methyl 4-O-methyl gallate against HIV-1-D3-infected human primary macrophages. (A) Cytotoxic effects of gallic acid (compound 1) and 4-methoxy methyl gallate (compound 3) were determined using trypan blue staining assay. (B) HIV-1-D3-infected primary macrophages were treated with LPS/CHX in the absence or presence of gallic acid (compound 1) or methyl 4-O-methyl gallate (compound 3) (0, 5, 10, and 20 μM). Cytotoxic effects of gallic acid (compound 1) and methyl 4-O-methyl gallate (compound 3) by PI/FACS assay. Cells stained with PI were examined by a flow cytometer. All values are mean ± S.D. (n = 4). (C) Cytotoxicity of compounds was determined using a fluorescence microscope. Cells were stained with PI to distinguish between dead (red) and live (green) cells. Images (merged red and green fields) are representatives of 3 experiments conducted in duplicate. *, p < 0.05 compared with LPS/CHX treatment group.
order to diminish neuronal cell death and HIV-1-associated neurodegenerative diseases, it needs to eliminate HIV-1-infected macrophages.

Gallic acid and its methyl ester are known to have anti-inflammatory (15), anti-oxidant (16), and anti-tumor properties (17, 18). Moreover, Ahn et al. proposed that the galloyl moiety plays a major role in inhibiting these compounds’ 3'-processing of HIV-1 integrase of these compounds (19). Rivero-Buceta et al. reported that the 2,3,4-trihydroxybenzoyl moiety has better antiviral properties against HIV-1 than the galloyl (3,4,5-trihydroxybenzoyl) moiety that is present in natural green tea catechins (20).

**Figure 5.** Effects of gallic acid and 4-methoxy methyl gallate on the phosphorylation of PI3K, PDK1, Akt, GSK3β, mTOR and β-actin in LPS/CHX-stimulated HIV-1-D3-infected human primary macrophages. (A) Effects of gallic acid (compound 1). (B) Effect of methyl 4-O-methyl gallate (compound 3). HIV-1-D3-transfected primary macrophages were treated with LPS/CHX in the absence or presence of gallic acid (compound 1), methyl 4-O-methyl gallate (compound 3) (0, 5, 10, and 20 μM) for 120 min. Proteins were measured by immunoblotting. #, p < 0.05 compared with normal control; *, p < 0.05 compared with LPS/CHX treatment group.
Moreover, methyl gallate inhibited HIV-1 reverse transcriptase, integrase, and viral replication activities (21–23). Liu et al. reported that tea polyphenols including gallic acid could inhibit entry of HIV-1 into target cells by blocking envelope-mediated membrane fusion (24). However, the cytotoxic effects of gallic acid derivatives against HIV-1-infected macrophages have not been studied. In a previous study, we found that 5,7-dihydroxy-6-methoxyflavonoids, such as oroxylin A and tectorigenin, were shown to exhibit the cytotoxic activity against HIV-1-D3-infected macrophages. The eliminating activity was structurally dependent on the number of hydroxyl groups of flavonoids at the 5-, 6-, and 7-positions and methylation of the 6-hydroxyl group (10).

In the present study, we synthesized gallic acid derivatives and compared their cell toxicity and PI3K/Akt-inhibitory activities in HIV-1-D3-infected macrophages, which are the long-term cell survival phenotypes against LPS/CHX stress by activating PI3K/Akt signaling pathway. Of these compounds, methyl 4-O-methyl gallate (compound 3) exhibited the most potent cytotoxic effect. However, we should investigate how of gallic acid derivatives to inhibit the PI3K/Akt signaling pathway in HIV-1-infected macrophages in near future. These studies raised the possibility that the methyl 4-O-methyl gallate can be beneficial for AIDS patients. These findings suggest that methyl 4-O-methyl gallate (compound 3) is a potent PI3K inhibitor and delivers anti-HIV-1 effects by decrease the survival of HIV-1 infected macrophages.

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