Protective Effects of Quinic Acid Derivatives on Tetrahydropapaveroline-Induced Cell Death in C6 Glioma Cells

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Protective effects of quinic acids from *Aster scaber* on tetrahydropapaveroline (THP) -induced cell toxicity were evaluated in rat C6 glioma cells. Among 4 quinic acid derivatives tested, (−) 4,5-dicaffeoyl quinic acid (QA3) exhibited the highest protective effect against THP-induced cell toxicity. C6 cells treated with THP exhibited the decrease in the survival rate and activities of glutathione peroxidase and catalase, but increased the level of malondialdehyde and superoxide dismutase activity. Staining C6 cells with propidium iodide and Hoechst 33342 revealed that 10 μM of THP treatment caused to necrotic and apoptotic cell death. However, preincubation of cells with QA3 prior to THP exposure recovered the cell survival rate and activities of antioxidant enzymes to control level. Taken together, the results indicate that QA3 might be a potential agent for treating or preventing diseases with oxidative stress.

Key words tetrahydropapaveroline; quinic acid; antioxidant enzyme

Aster species have been used in traditional alternative medicine for the treatment of bruises, headache and dizziness. However, the mechanism for the observed pharmacological activities remains relatively unknown. Triterpene glycosides and volatile compounds were isolated from the *Aster scaber*.[1,2] Recently, structures of quinic acid derivatives and monoterpene glycosides from *Aster scaber* and their biological activities were being investigated.[3,4] Quinic acid derivatives inhibited human immunodeficiency virus-1 integrase[4] and exhibited the neuroprotective effect against β-amyloid peptide and neurotrophic activity in PC12 cells.[5]

Tetrahydropapaveroline (THP), a 1-benzyl isoquinoline alkaloid, is synthesized in plant by Pictet-Spengler condensation of dopamine with dopaldehyde.[6,7] It can be also synthesized in mammals including humans. THP has been detected in the urine of Parkinsonian patients on L-dopa medication and in the urine and brain region of rats after L-dopa or acute ethanol administration.[8] In addition, THP was reported to inhibit mitochondrial respiration and thought to be a contributing factor for Parkinson's disease.[9,10] THP and related 1-benzyl-catecholisoquinolines were proposed to be candidates of dopaminergic neurotoxines to cause Parkinsonism. However, there is a controversy on the role of THP as a pro-oxidant or anti-oxidant. Compounds such as L-DOPA and dopamine exert pro-oxidant actions as well as anti-oxidant properties. THP was reported to undergo redox cycling in the presence of certain transition metals such as Cu^{2+} to produce reactive oxygen intermediate.[11,12] THP has a relatively high redox potential and is likely to form highly electrophilic tautamers via oxidative conversion to 3,4-dihydroisoquinolines.[13] Simiar to 1-methyl-4-phenylpyridinium ion (MPP^+), THP inhibited dopamine uptake through dopamine transporter.[14] However, the biochemical and molecular mechanisms of cytotoxicity on neuron derived cells exerted by THP remain poorly understood.

Recently, astrocytes have been drawing more attentions in brain research since they are involved in the etiology of numerous brain diseases. The aim of this study was to investigate the protective effect of 4 quinic acid derivatives isolated from *Aster scaber* on THP-mediated toxicity toward rat C6 glioma cells, which have been largely used for studying the properties of astrocyte, more resistant to oxidative stress than neuronal cells.[15] Quinic acids tested here (Fig. 1) are (−) 3,5-dicaffeoyl quinic acid (QA1), (−) 3,5-dicaffeoyl-mucoquinic acid (QA2), (−) 4,5-dicaffeoyl quinic acid (QA3), and (−) 5-caffeoyl quinic acid (QA4) identified by Kwon *et al*.[4]

MATERIALS AND METHODS

Materials C6 rat glioma cells were purchased from the American Type Culture Collection (Rockville, MD, U.S.A.). Hoechst 33342 was from Molecular Probes, Inc. (Eugene, OR, U.S.A.). Propidium iodide, Glutathione peroxidase assay kit, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium

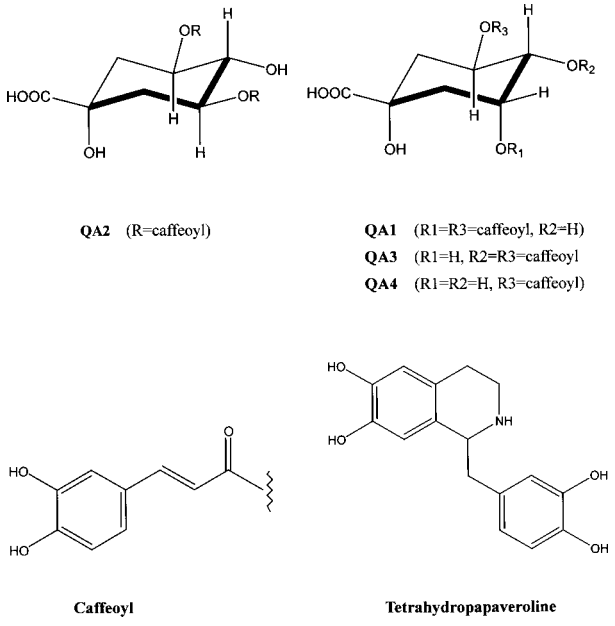

Fig. 1. Chemical Structure of QA1, QA2, QA3, QA4 and Tetrahydropapaveroline

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bromide (MTT), and THP were purchased from Sigma (St. Louis, MO, U.S.A.). Cell culture media was purchased from GIBCO-BRL (Gaithersburg, MD, U.S.A.). Quinic acid derivatives (diluted in DMSO, 0.1% (v/v) as a final concentration) were kindly supplied by Dr. K.R. Lee. Other reagents not listed here were as same as described elsewhere.[16)]

Cell Culture C6 cells were grown on plastic microtiter plates or culture dishes in DMEM containing 10% heat inactivated fetal bovine serum, and antibiotics (100 units/ml penicillin G, 100 μg/ml streptomycin, and 100 units of fungizone) at 37 °C in 5% CO_2 and 95% air in a humidified incubator.

Measurement of THP-Induced Cytotoxicity C6 glioma cells (2×10^4 cells/well) were grown in 96-well microtiter plates for 2 d in regular serum containing media. After cells were exposed to low serum containing media (1% fetal bovine serum) for 16 h, varying concentrations of THP (diluted in ethanol, 0.05% as a final concentration) were added to the culture media. After incubation of C6 cells for indicated times, THP-containing media was aspirated from the plates and each well was washed twice with equal volume of phosphate buffered saline. Viability of remaining C6 cells was determined by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) reduction assay.[17)] The optical density at 495 nm was determined using an automatic ELISA reader.

Fluorescence Microscopy Cells were incubated with 10 μM THP in the presence or absence of quinic acid QA3 for 18 h and morphological changes in the cells were observed by phase-contrast microscopy and fluorescence microscopy after staining with propidium iodide (50 μM) and Hoechst 33342 (50 μM). Cells stained with propidium iodide or Hoechst 33342 for 30 min were observed fluometrically with emission at 580 or 420 nm and excitation at 520—550 nm or 330—385 nm, respectively.

Enzyme Assays Catalase activity was determined at 25 °C according to Claiborne and Fridovich.[18)] Decomposition of hydrogen peroxide by catalase was followed by ultraviolet spectroscopy at 240 nm. The reaction was performed using a solution of 20 mM hydrogen peroxide in 50 mM KH_2PO_4 containing 100 μg of total cellular protein in a final volume of 1 ml. Specific acitvity of catalase was calculated from the equation: specific activity (units/mg of protein/min)$=\Delta A_{240\,nm}$ (1 min)$\times$1000/43.6$\times$mg protein. Glutathione peroxidase activity was determined by using the commercially available kit (Sigma, St. Louis, MO, U.S.A.). The following solutions were added to a cuvette: 700 μl of solution containing 50 mM Tris/HCl, 5 mM EDTA (pH 7.4), 1.6 mM glutathione, 0.32 mM NADPH, and 0.8 units of glutathione reductase; 70 μl of sample (0.3—1.0 mg of protein); and 350 μl of 0.0007% (v/v) *t*-BOOH. The decrease in absorbance at 340 nm was followed for 4 min. Results are expressed as units of specific activity defined as the amount of enzyme that consumes 1 μmol of NADPH/min/mg of protein. Total superoxide dimutase (SOD) activity was measured according to Oyanagui.[19)] Briefly, the sample (0.1 ml), reagent A (0.2 ml, 0.5 mM hypoxanthine, 10 mM hydroxylamine HCl, pH 7.0) and water or 1 mM of KCN (0.5 ml) were mixed. The reaction was started by adding reagent B (0.2 ml, 5 mU/ml xanthine oxidase in EDTA-phosphate buffer). This mixture was incubated for 30 min at 37 °C without shaking and received reagent C (2 ml, 300 μg/ml sulfanilic acid, 5 μg/ml *N*-1-naphthylethylenediamine and 16.7% acetic acid). This mixture was incubated for 20 min at room temperature and absorbance was measured at 550 nm. One uint of SOD activity was defined as the amount of enzyme resulting in one half of maximal inhibition.

Radical Scavenging Effects of Compounds on 1,1-Diphenyl-2-picrylhydrazyl (DPPH) Radical Radical scavenging activity of quinic acids was measured according to Yoshida *et al.*[20)] Quinic acids at four different concentrations in MeOH (4 ml) were added to a solution of 0.15 μM DPPH (1 ml) with vigorous shaking. After keeping these mixtures at room temperature for 30 min, the remaining amounts of DPPH were determined by the absorbance at 520 nm. The radical scavenging activity of each compound was expressed by the ratio of the reduced absorption of DPPH (%) relative to that (100%) of the DPPH in the absence of compounds.

Lipid Peroxidation Levels of lipid peroxidation were detected with thiobarbituric acid. Typically, total cellular extract was mixed with 2 volumes of cold 10% (w/v) trichloroacetic acid to precipitate. A standard curve was prepared using malondialdehyde bisdimethylacetal as the source of malondialdehyde (MDA). After centrifugation at 12000$\times$***g*** for 10 min, an aliquot of supernatant was reacted with an equal volume of 0.67% (w/v) thiobarbituric acid in a boiling water bath for an hour. An absorbance was read at 532 nm and the concentration of MDA was calculated based on an ε value of 153000.

Statistical Analysis All the experimental data shown were repeated at least three times, unless otherwise indicated. The data were analyzed by Mann-Whitney *U* test and $p<0.05$ was considered significant.

RESULTS

All the experimental data shown were repeated at least three times, unless otherwise indicated. To determine the cytotoxic effects of THP on C6 cells, cells were exposed with varying concentrations of THP for 24 h and measured cell death with the MTT assay. A significant portion of cells died after THP treatment in a dose-dependent manner; approximately 9.7, 25.8, 51.6, 66.1, and 79.5% of cells died on treatment with 1, 5, 10, 20, and 50 μM THP, respectively. When cells were exposed with THP, cell number significantly decreased and cells lost processes (*vide infra*). Quinic acid derivatives were shown to exhibit protection against β amyloid-induced PC12 cell toxicity and neurotrophic effect mimicking nerve growth factor.[9)] To test the potential protection by quinic acid derivatives against THP-induced cytotoxicity, C6 cells were pretreated with 4 compounds (QA1—QA4) isolated from *Aster scaber* and their survival rates were assessed by MTT assay (Fig. 2A). Among the 4 compounds tested, QA3 showed the highest protection against THP-induced cell damage. Pretreatment of QA3 ranging from 0.005 μM to 5 μM prior to10 μM THP treatment showed the highest protective effect of QA3 at 0.5 μM concentration (Fig. 2B).

The radical scavenging activity of 4 quinic acids isolated from *Aster scaber* was measured by use of DPPH radical (Table 1). The potency of radical scavenging activity of the compounds was in the following order: QA1>QA3>QA2>

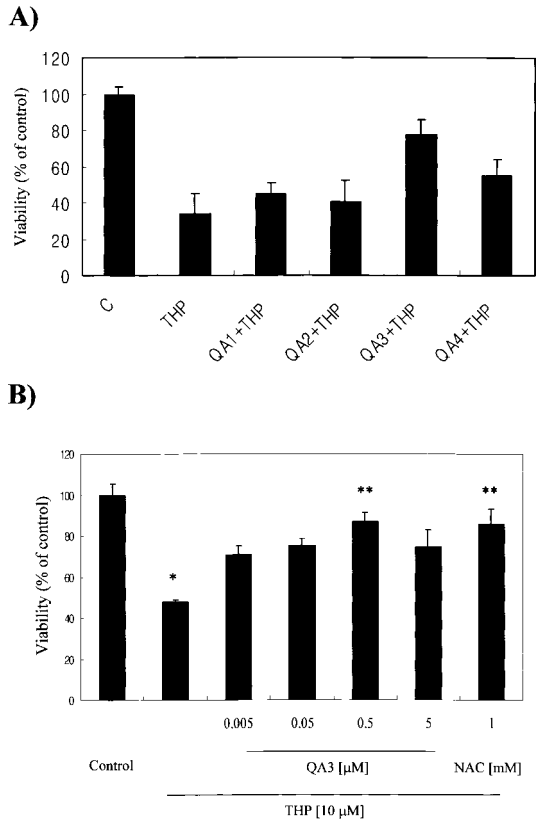

Fig. 2. Effect of Quinic Acid Derivatives on THP-Mediated Toxicity in C6 Cells

A, C6 cells were pretreated with 5 μM of QA1—QA4 for 2 h and then treated with 10 μM THP for additional 48 h. B, Cell were incubated with 10 μM THP for 48 h. Various concentrations of QA3 were added in the culture medium for 2 h before THP exposure. Cell viability was assessed by MTT assay in triplicate. The data are expressed as percentage of control. $*p<0.01$ compared with control: $**$ $p<0.01$ compared with THP.

Table 1. Radical Scavenging Effects of Quinic Acid Compounds[a)]

Sample[a)]	ED_{50} (μg)
Butylated hydroxyanisole	9.5±0.2
Compound QA1	14.2±0.1
Compound QA2	15.0±0.5
Compound QA3	14.9±1.3
Compound QA4	23.6±0.2

a) Radical scavenging activity of quinic acids was measured by use of 0.15 μM 1,1-diphenyl-2-picrylhydrazyl (DPPH) according to Yoshida *et al.*[20] Median effective dose (ED_{50}) represents the amount of a compound (μg) required for 50% decrease in DPPH radicals. *b*) Effect of each compound was determined with four different concentrations in triplicate.

QA4. Even though QA3 gave the highest protection against THP-induced cell toxicity, its radical scavenging activity was not as good as QA1.

Glutathione peroxidase (GSH-Px), catalase, and SOD are the most important antioxidant enzymes in cells. In our experiments, GSH-Px, catalase, and SOD activities in C6 cells were 0.12±0.01 (U/mg protein), 14.7±1.3 (U/mg protein), and 2.1±0.2 (U/mg protein), respectively. Treatment with QA3 alone did not have any significant effects on antioxidant enzyme activities (Table 2), while THP (10 μM) reduced the activities of GSH-Px and catalase by 55 and 45%, respectively, and increased SOD activities by 45%. Pretreatment with QA3 attenuated the changes in GSH-Px, catalase, and

Table 2. Effects of QA3 on Antioxidant Enzyme Activities and Level of MDA in C6 Cells after Treatment with THP

	GSH-Px	Catalase	SOD	MDA
Control	100±9.8 (0.12±0.01) (U/mg)	100±11.2 (14.7±1.6) (U/mg)	100±8.3 (2.1±0.2) (U/mg)	100±9.0 (0.23±0.02) (nmol/ml)
QA3 (0.5 μM)	111.5±13.0	114.3±12.4	104.4±9.5	110.3±7.6
THP (10 μM)	45.2±5.7*	54.8±4.7*	145.2±19.6*	153.7±16.3*
THP+QA3 (0.005 μM)	60.7±5.8	67.8±6.5	126.1±10.6	133.5±12.5
THP+QA3 (0.05 μM)	85.0±7.9	76.7±8.2	114.7±8.8	115.7±9.5
THP+QA3 (0.5 μM)	97.4±9.5**	90.3±7.4**	106.6±9.2	103.0±7.2 b
THP+QA3 (5.0 μM)	73.8±4.5	79.6±7.2	117.3±11.0	109.5±12.0

a) QA3 was treated 2 h prior to addition of 10 μM THP to C6 cells. The activities of Glutathione peroxidase (GSH-Px), catalase, superoxide dismutase (SOD) and the level of MDA were determined 48 h later and described in parenthesis as units/mg of cell protein for enzymes and nmol/ml for MDA, respectively. Data represented are % value of control and the average±S.D. (n=4). $*p<0.05$ compared with control. $**$ $p<0.05$ compared with THP.

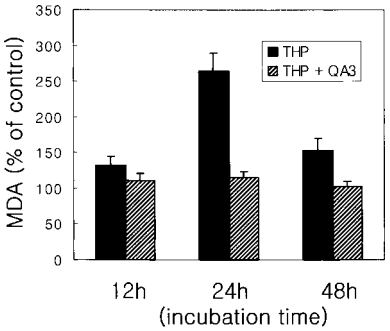

Fig. 3. Effect of QA3 on MDA Production by THP at Various Time Points

C6 cells were treated with 10 μM of THP in the presence or absence of 0.5 mM QA3 for 12 h, 24 h and 48 h and then the level of MDA was measured in triplcate as described in Materials and Methods. The data are expressed as percentage of control.

SOD activities in THP-treated C6 cells. The toxicity caused by THP was accompanied by the increase of lipid peroxides. Intracellular concentration of MDA, a product of lipid peroxidation, was raised by 54% 48 h after the C6 cells exposed to 10 μM THP. However, pretreatment with QA3 significantly attenuated the level of MDA (Table 2). The results demonstrated that cellular toxicity caused by THP was associated with the disturbance between the oxidant and antioxidant balance, and the QA3 protected cells from THP insult. QA3 also prevented the reduction in GSH-Px and catalase activities and inhibited overproduction of MDA.

To examine whether MDA production increased before the cell death or after the cell death, MDA was measured at 12h, 24h and 48h after the compound treatment in presence or absence of 0.5 μM QA3 (Fig. 3). The MDA reached the highest level at 24 h post THP treatment and then decreased. However, the level of MDA at indicated time points was close to that of control when QA3 was pretreated to cells before THP. The results indicated that QA3 could prevent the increase of MDA by THP before the cell death.

C6 cells preincubated with THP for 18 h exhibited marked morphological changes showing condensed cell body with retarded process under phase contrast microscopy (Fig. 4). In contrast, pretreatment with QA3 recovered the shape of cell similar to control. Cells positively stained with propidium iodide (PI) were determined to be dead, whereas cells with

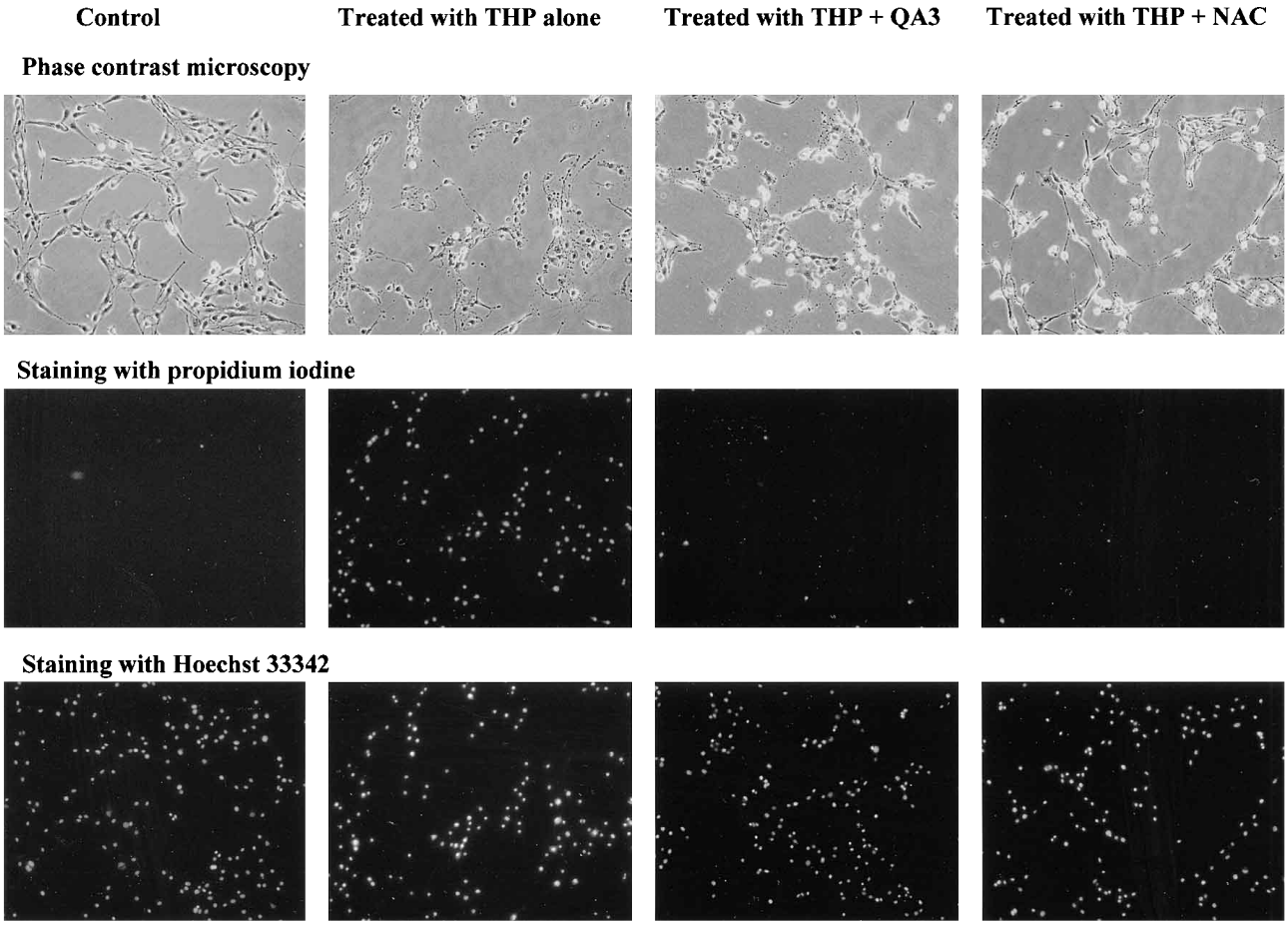

Fig. 4. Effects of QA3 on C6 Cell Damage Induced by THP

C6 cells were incubated with 0.5 μM QA3 or 1 mM *N*-acetyl-L-cysteine (NAC) prior to exposure to 10 μM THP for 18 h, and then examined by phase contrast microscopy and fluorescence microscopy after staining with PI or Hoechst 33342. Necrotic cells are stained with PI, whereas apoptotic cells are stained with Hoechst 33342 with condensed and fragmented nuclei.

condensed and fragmented nuclei after staining with Hoechst 33342 were assessed to be apoptotic. Almost all the cells treated with THP were stained with PI or positively stained with Hoechst 33342, showing condensed and fragmented nuclei typical for necrosis and apoptosis. In contrast, C6 cells exhibited the significantly reduced number of cells stained with either PI or Hoechst 33342 when pretreated with QA3 prior to exposure to THP. The data suggest significant protective effects of QA3 against THP insults.

DISCUSSION

Exposure of THP to C6 cells significantly decreased the GSH-Px and catalase activities, but elevated the level of malondialdehyde. Interestingly, an increase in SOD activity was observed after the exposure of cells to THP, which might reflect a direct induction or otherwise a compensatory reaction against THP insult. Oxidative stress decreases the level of GSH. Therefore, the decrease in the GSH-Px and catalase activities might be secondary consequence due to the cell death caused by THP. The involvement of oxidative stress is further confirmed by formation of MDA in treated cells since MDA represents an index of oxidative damage to membrane lipids. The specific activities of GSH-Px, catalase and SOD in C6 cells were in agreement with those in cultured rat astrocytes and PC12 cells.[21,22] Maruyama *et al.* reported that THP induced necrosis and oxidized papaverolines caused apoptosis in human doparminergic neuroblastoma SH-SY5Y cells.[23] Since 100 μM or higher concentrations of papaverolines were treated in SH-SY5Y cells, it would not be surprising to see the necrotic cell death. At present study, C6 cells with 10 μM THP caused both apoptosis and necrosis (Fig. 4).

Quinic acid derivatives exhibited protective effect against THP-induced cell damage. In the present study, submicromolar concentrations (5 nM—5 μM) of QA3 were required to prevent THP-induced cell death. This cytoprotective response could be a consequence of antioxidative capacity of QA3 to enhance a mitogenic/proliferative response within these cells, as seen in PC12 cells.[5] Our unpublished data showed that quinic acid activated extracellular signal regulated kinase and phosphatidyl inositol 3-kinase which were involved in cell proliferation. In addition, QA3 significantly attenuated THP-induced cell toxicity and redox desequilibrium by elevating activities of catalase and GSH-Px as well as the cell viability. As seen in Fig. 2 and Table 1, QA3 exerted the best neuroprotective effect against THP-induced cell death, whereas QA1 was better in radical scavenging activity than QA3. Therefore, it could be postulated that QA3 may have protective functions at various cellular levels besides antioxidative properties.

In conclusion, the data presented here demonstrate that quinic acid (QA3) could alleviate the damage induced by THP and be a potential agent for treating or preventing diseases with oxidative stress.

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