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# Effects of andrographolide and 14-deoxy-11,12-didehydroandrographolide on cultured primary astrocytes and PC12 cells

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ABSTRACT

*Aims:* To test the effects of andrographolide (AP1) and 14-deoxy-11,12-didehydroandrographolide (AP2) on pheochromocytoma cell line 12 (PC12) cells in an astrocyte-rich environment.
*Main methods:* The abilities of AP1 and AP2 to reduce the secretion of pro-inflammatory cytokines Interleukin (IL)-1, IL-6, and Tumor necrosis factor (TNF)-α from stimulated astrocytes were tested. In addition, the abilities of AP1 and AP2 to reduce oxidative stress in astrocytes were tested using an oxidative-sensitive fluorescent dye. The reduction of chondroitin sulfate proteoglycan (CSPG) in stimulated astrocytes was tested using the dot blot method. Reduction of $H_2O_2$-induced death was tested in PC12 cells. Astrocyte-conditioned medium (ACM) and TNF-α-stimulated astrocyte-conditioned medium (SACM) were used to assess the effects of AP2 on PC12 cells treated with $H_2O_2$.
*Key findings:* AP1 and AP2 reduced pro-inflammatory cytokines, reactive oxygen species (ROS), and CSPG in TNF-α stimulated astrocytes. AP1 protected $H_2O_2$-treated PC12 cells cultured in ACM. Co-incubation of PC12 cells in $H_2O_2$, and ACM collected from AP1 treated astrocytes did not prevent cell death.
*Significance:* AP1 and AP2 effectively ameliorated astrocytic pro-inflammatory reactions and prevented PC12 cell death with different efficacies. These compounds may be candidates for treatment of spinal-cord injury and neurodegeneration.



## Introduction

White-matter degeneration after spinal-cord injury (SCI) blocks nerve impulses, resulting in paralysis. Following SCI, surviving neurons in long tracts are capable of sprouting, but this process halts in the presence of inhibitory proteoglycans (Silver and Miller, 2004). An inflammatory response at the injury site stimulates astrocytes to form glial scars and secrete high concentrations of chondroitin sulfate proteoglycan (CSPG), which has been shown to be involved in axonal growth inhibition (McKeon et al., 1995; Rudge and Silver, 1990). Digestion of CSPG or blocking its synthesis results in targeted axonal re-growth and improved function (Bradbury et al., 2002; Grimpe and Silver, 2004), suggesting that CSPG is an important target for white-matter regeneration.

Macrophages rapidly infiltrate the central nervous system (CNS) injury site and secrete pro-inflammatory cytokines and reactive oxygen species to minimize injury and kill microbes. However, these actions also cause further inflammation and additional damage (Fitch et al., 1999). Free radicals and oxidative species generated by macrophages result in lipid peroxidation-mediated cell death (Hall, 2003). High doses of methylprednisolone (MP) that reduce reactive oxygen-induced lipid peroxidation have been used for treating SCI (Genovese et al., 2006; Hall, 2003). Administration of anti-inflammatory drugs to inhibit secondary degenerative reactions after SCI results in improved locomotion and reduced histopathological changes (Genovese et al., 2006; Koopmans et al., 2009; Lee et al., 2003; Mulcahy et al., 2003; Nesic et al., 2001). One way to improve axonal regeneration is to reduce macrophage-induced inflammatory reactions. However, SCI often cannot be treated within 24 h, which is the window when many macrophage-induced molecules are released. Several important astrocytic reactions, however, occur downstream of macrophage-released factors (Asher et al., 2000; Giulian et al., 1988). These delayed astrocytic reactions may be easier to arrest following SCI; therefore, agents that inhibit reactive gliosis and proteoglycans in astrocytes are of particular interest.

Andrographolide (AP1) and 14-deoxy-11,12-didehydroandrographolide (AP2) are derived from *Andrographis paniculata*. Both compounds are structurally similar, although AP1 present in larger quantities and contains a hydroxyl group and two additional hydrogen atoms. Besides its anticancer and antiviral effects (Geethangili et al., 2008), AP1 inhibits the innate immune response *via* its actions on macrophages, pro-inflammatory cytokines, and chemotaxis (Burgos et al., 2005; Qin et al., 2006; Tsai et al., 2004). It ameliorates hypoxia

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by decreasing nitric oxide (NO), inducible nitric oxide synthase (iNOS), and oxygen radicals. AP1 also upregulates glutathione. AP1 reduces mesencephalic neuronal death in mixed cultures by inhibiting macrophage-derived pro-inflammatory cytokines (Wang et al., 2004). AP2 rescues cortical neurons from amyloid-beta-induced apoptosis (Chen et al., 2006). However, the effects of AP1 and similar compounds have not been tested on astrocytes, and the effects of AP1 and AP2 have not been systematically compared. We used Tumor necrosis factor (TNF)-α as a macrophage-derived, acute inflammatory factor to activate gliotic phenotypes. We also investigated a possible protective effect in PC12 cells for $H_2O_2$-induced death.

## Materials and methods

### *AP1 and AP2 compounds*

AP1 and AP2 were isolated from the whole plant of *A. paniculata*, and their structures were identified, as previously described (Koteswara Rao et al., 2004).

### *PC12 cell culture*

Rat PC12 cells (adrenal gland; pheochromocytoma) were purchased from the Bioresource Collection and Research Center (BCRC, Food Industry Research and Development Institute, Taiwan, ROC) and maintained in Dulbecco's modified Eagle's medium (DMEM), which was supplemented with 10% heat-inactivated horse serum, 5% fetal bovine serum (FBS; Gemini, California, U.S.A.), 100 U/ml penicillin and 100 μg/ml streptomycin in a water-saturated atmosphere of 5% $CO_2$ at 37 °C.

### *$H_2O_2$ and AP compound treatment*

To induce apoptosis, PC12 cells were re-plated at a density of $2\times10^4$ cells/well in 96-well plates and allowed to recover for 1 d. Cultures were subsequently transferred to low-serum medium (DMEM supplemented with 0.2% FBS) for 1 d before treatment with 200 μM $H_2O_2$ in low-serum medium for 24 h (control for PC12 death). To test the effects of AP compounds, PC12 cells were re-plated in low-serum medium for 1 d before the addition of 200 μM $H_2O_2$ with either AP1 or AP2 for 24 h. To test whether AP2 inhibited $H_2O_2$-induced death when astrocytic factors were present, the cells were treated as stated above and were cultured in astrocyte-conditioned medium (ACM) or TNF-α-stimulated astrocyte-conditioned medium (SACM) with AP compounds and/or $H_2O_2$ for 24 h. The cell survival and cytotoxicity rates were determined by measuring mitochondria activity (The Cell Growth Determination Kit, MTT-based, Sigma) and LDH release into the medium (LDH Cytotoxicity Detection Kit, Clontech Laboratories, Inc.).

### *Astrocyte culture*

Purified astrocyte cultures were generated from postnatal day 1–3 (P1–P3) Sprague Dawley rat cortices using a modified procedure (McCarthy and de Vellis, 1980). Cerebral cortices were isolated, separated from the meninges, and dissociated in calcium- and magnesium-free Hanks' balanced salt solution with 0.1% trypsin and 0.020% EDTA for 30 min at 37 °C with the addition of 100 ml of 2 mg/ml DNase after 15 min. DMEM-F12 medium (Life Technologies, Gaithersburg, MD) with 10% fetal calf serum (FCS; Sigma, St. Louis, USA) was added, and the tissue was triturated through a fire-polished glass pipette. Next, the cells were cultured in DMEM-F12 medium with 10% fetal calf serum for 12–15 d, and astrocytes were enriched by vigorously shaking the Petri dish to remove non-adherent microglial cells. The percentage of astrocytes and microglial cells were determined by immunostaining for GFAP (an astrocytic marker) and ED-1 (a macrophage marker) along with DAPI (a nuclear marker). After another passage in DMEM-F12 medium with 10% fetal calf serum for 24 h, the cells were cultured in serum-free G5 medium for 24 h before treatment with TNF-α or other compounds.

### *Astrocyte-conditioned medium*

Astrocytes were cultured from dissected neonatal cortices and enriched by culturing for 12–15 days before shaking off microglial cells. Astrocytes were passaged again in DMEM-F12 medium with 10% fetal calf serum for 24 h and were transferred to serum-free G5 medium for 24 h. Then, half of the plates were treated with 10 ng/ml TNF-α. After 24 h, ACM and SACM were collected to be used in later experiments.

In a separate set of cultures, AP1 was added to the astrocytes for 24 h before ACM was collected ($ACM_{AP}$) and used to treat PC12 cells additionally treated with 200 μM $H_2O_2$. This group served as a control for PC12 cells treated with ACM + $H_2O_2$ + AP1.

### *Induction of pro-inflammatory cytokines by TNF-α stimulation in astrocytes*

Highly enriched astrocytes (95%) were obtained as described above, transferred to a 4-cm petri dish for 24 h and were incubated in serum-free G5 medium for 24 h. Next, the cells were cultured in fresh serum-free G5 medium with 10 ng/ml TNF-α for 24 h before

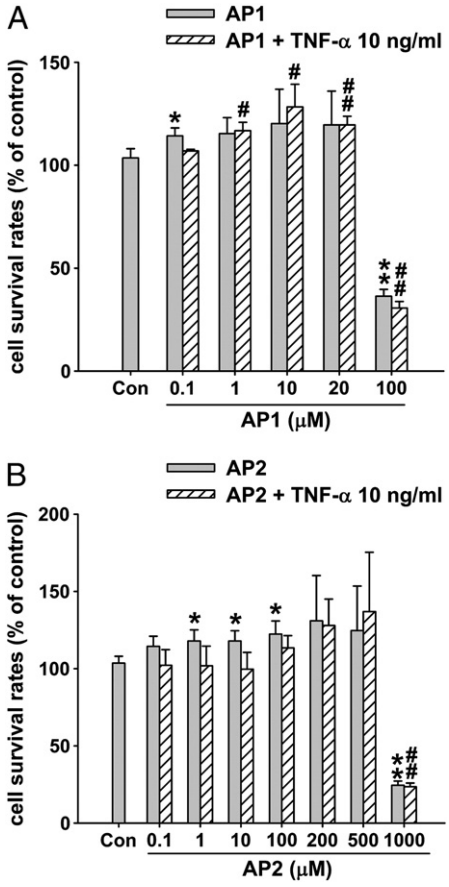


**Fig. 1.** Astrocyte survival rates with AP compounds ± TNF-α. Cell survival of cultured astrocytes as assessed by MTT methods. Primary astrocytes were treated with different doses of AP compounds alone (gray) or in combination with 10 ng/ml TNF-α (hatched lines). Astrocytes treated with 0.6% DMSO (highest concentration in the compounds) were used as controls. * or # indicates significant difference from control ($p<0.05$); ** or ## indicates significant difference from control ($p<0.01$).