

ORIGINAL ARTICLE

Allantopyrone A activates Keap1–Nrf2 pathway and protects PC12 cells from oxidative stress-induced cell death

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Keap1–Nrf2 system is known as a sensor of electrophilic compounds, and protects cells from oxidative stress through induction of various antioxidant enzymes. We found by proteomic analysis that allantopyrone A, a metabolite isolated from an endophytic fungus, upregulates the expression of proteins that are regulated by the transcription factor Nrf2. Indeed, allantopyrone A increased the antioxidant enzyme heme oxygenase-1 in PC12 cells. Moreover, it induced localization of Nrf2 in the nucleus. Affinity purification of allantopyrone A-binding protein showed that this compound could bind directly to Keap1. Allantopyrone A suppressed intracellular reactive oxygen species level and cell death induced by H₂O₂ in PC12 cells. These results indicate that allantopyrone A protects PC12 cells from oxidative stress-induced cell death through direct binding with Keap1 and activation of the Keap1–Nrf2 pathway.

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INTRODUCTION

Many clinical drugs have been discovered and developed from natural sources. In particular, microbial metabolites contain bioactive compounds that have a huge variety of chemical structures and biological activities.¹ For example, avermectin from *Streptomyces avermitilis* greatly contributed to the prevention and therapy of parasite diseases.² Based on these advantages, we focused on the metabolites of endophytic fungi.³ Since endophytic fungi produce characteristic compounds through the stimulation from host plants, it is suggested that endophytic fungi have a different metabolic pathway from other microorganisms.⁴ Previously, we have found the novel bioactive compounds such as eremoxylin A and B (inhibitors of Ca²⁺ signal transduction),^{5,6} and bispyrrocin (inhibitor of prolyl oligopeptidase),⁷ from metabolites of endophytic fungi.

Allantopyrone A (Figure 1) was isolated from an endophytic fungus *Allantophomopsis lycopodina* KS-97.⁸ We previously reported that this compound shows cytotoxicity in HL60 cells (IC₅₀ = 0.32 μM). Recently, we also found that allantopyrone A inhibits the tumor necrosis factor α-induced nuclear factor κB signaling pathway.⁹ Though α,β-unsaturated carbonyl moiety of allantopyrone A is important, its molecular targets and biological effects are not fully understood.

Keap1–Nrf2 system is known as a sensor of oxidative stress and electrophilic compounds.¹⁰ In this pathway, the expression of many antioxidant and phase-2 detoxification enzymes, including glutathione S-transferase, NADPH quinone oxidoreductase 1, were regulated.

They can induce to protect cells from several environmental stresses such as oxidants and carcinogenesis. Some of the electrophilic compounds, including sulforaphane¹¹ and curcumin,¹² activate this pathway and exhibit protective effect against hepatocytes or neurons through the induction of Nrf2-mediated enzyme expression.

In this study, we investigated the effects of a microbial-derived electrophilic compound, allantopyrone A, on the Keap1–Nrf2 pathway in PC12 cells.

MATERIALS AND METHODS

Isolation of allantopyrone A

Allantopyrone A was obtained from *A. lycopodina* KS-97 isolated from a dead branch collected from Mt. Gassan in Yamagata, Japan. The isolation procedure was described in a previous report.⁸

Cell line and culture

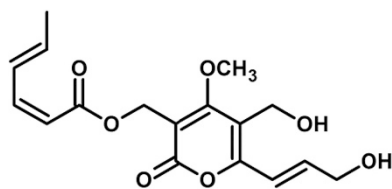
PC12 cells (RCB0009, RIKEN BioResource Center, Tsukuba, Japan) were grown in DMEM medium (Gibco, Invitrogen Corp., Carlsbad, CA, USA) supplemented with 10% heat-inactivated fetal bovine serum (BioWest Co. Ltd, Vancouver, BC, Canada), 10% heat-inactivated horse serum (Gibco, Invitrogen Corp.) and penicillin (50 units per ml)–streptomycin (50 μg ml⁻¹) (Gibco, Invitrogen Corp.) in a humidified atmosphere at 37 °C under 5% CO₂.

Proteomic analysis

Proteomic analysis was performed as previously described.^{13,14} Briefly, HeLa cells were treated with 20 μM allantopyrone A for 18 h. Proteome analysis of cell lysates was performed using a 2D-difference gel electrophoresis (DIGE) system

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Allantopyrone A

Figure 1 Chemical structure of allantopyrone A.

(GE Healthcare UK Ltd, Buckinghamshire, UK), and images of the gels were analyzed using Progenesis SameSpots (Nonlinear Dynamics, Newcastle upon Tyne, UK). Out of more than 1000 spots detectable in each 2D-DIGE gel, the 296 spots that were found in common between gels of reference compound-treated cells were selected as described. Next, the volume of each spot was normalized using the average of the corresponding control values from dimethyl sulfoxide-treated HeLa cells. Statistical analysis was performed with a two-tailed unpaired Student's *t*-test. The values are the mean calculated from the experiment in triplicate. Significance was assumed with $*P < 0.03$ and $**P < 0.01$. To determine the protein in the spots, we performed peptide mass fingerprinting with a matrix-assisted laser desorption/ionization time-of-flight mass analysis.

Protein extraction

PC12 cells were treated with allantopyrone A in a 60 mm dish, and the cells were washed with phosphate-buffered saline. Total protein was extracted using RIPA buffer (10 mM Tris-HCl (pH 7.5), 1% NP-40, 0.01% sodium deoxycholate, 0.1% SDS, 150 mM NaCl and 1 mM EDTA) containing protease inhibitor cocktail Complete Mini (Roche Diagnostics, Basel, Switzerland) and phosphatase inhibitor cocktail (Wako Pure Chemical Industries, Osaka, Japan) by incubating at 4 °C for 30 min. After centrifugation (15 000 g for 30 min at 4 °C), supernatant was collected as whole-cell lysate. For the cytosolic and nuclear extracts, the cells were rinsed with ice-cold phosphate-buffered saline, and the pellet was dissolved in 100 μ l of buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM ethylene glycol tetraacetic acid (EGTA) and 1 mM dithiothreitol) containing protease inhibitor cocktail Complete Mini and phosphatase inhibitor cocktail. After 15 min of incubation, 10 μ l of 10% NP-40 was added, and the lysate was vortexed for 15 s. Thereafter, the lysate was repeatedly centrifuged for 10 min (1000 g, 4 °C) to separate the nuclei from the cytosolic fraction. The supernatant (cytosolic fraction) was stored on ice. The pellet was resuspended in 100 μ l of buffer B (20 mM HEPES, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA and 1 mM dithiothreitol) containing protease inhibitor cocktail Complete Mini and phosphatase inhibitor cocktail, and incubated on ice for 15 min with vortexing for every 2 min. After a final centrifugation step for 10 min (20 000 g, 4 °C), the supernatant (nuclear extract) was collected. The protein concentrations were determined using a BCA protein assay kit (Pierce, Waltham, MA, USA).

Western blotting

The prepared samples containing 20 μ g total protein were loaded on SDS-polyacrylamide gels. After electrophoresis, proteins were transferred onto a polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA) and blocking was performed in Tris-buffered saline containing 1% polyvinylpyrrolidone and 0.1% Tween 20. The membranes were then incubated with primary antibodies. After washing membranes with 0.1% Tween 20 in tris-buffered saline-T, each blot was incubated with horseradish peroxidase-conjugated secondary antibodies. The protein bands were detected using ECL (GE Healthcare, Little Chalfont, England) and ImageQuant LAS 4000 (GE Healthcare, Piscataway, NJ, USA). Primary antibodies against heme oxygenase-1 (HO-1) were purchased from Enzo Life Sciences, Inc., Farmingdale, NY, USA, against PARP was purchased from Cell Signaling Technology, Danvers, MA, USA, against Nrf2 and Keap1 were purchased from Santa Cruz Biotechnology, Inc., CA, USA. Secondary antibodies, horseradish

peroxidase-conjugated anti-mouse IgG and anti-rabbit IgG were purchased from Signaling Technology, and horseradish peroxidase-conjugated anti-goat IgG was purchased from Abcam, Cambridge, UK.

Affinity chromatography

Allantopyrone A beads were prepared as previously described.¹⁵ PC12 cells were harvested, washed with phosphate-buffered saline, and then lysed in binding buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA and 2.5 mM EGTA) containing 1 mM dithiothreitol, 0.1 mM MgCl₂, 1% NP-40 and Complete Mini (Roche Diagnostics, Basel, Switzerland). After 30 min, samples were centrifuged (4 °C, 15 000 g, 30 min), and the supernatant was collected as cell lysate. The cell lysate (3 mg of protein) was then incubated with allantopyrone A beads (20 μ l) for 12 h at 4 °C. The reacted beads were washed with binding buffer containing 0.2% NP-40, and the binding proteins were eluted with SDS-PAGE sample buffer (140 mM Tris-HCl (pH 6.8), 22.4% glycerol, 6% SDS, 10% 2-mercaptoethanol, 0.02% bromophenol blue), separated by SDS-PAGE, and detected by western blotting using anti-Keap1 antibody.

Microscopic observation

PC12 cells were pretreated with allantopyrone A for 18 h in a 24-well plate, and then treated with 200 μ M H₂O₂ for 12 h. These cells were stained with Hoechst 33342 (Dojindo Laboratories, Kumamoto, Japan) and propidium iodide (PI) (Wako Pure Chemical Industries) for 15 min at 37 °C. Stained cells were resuspended in medium and observed using confocal laser microscope (C2, Nikon, Tokyo, Japan).

Cell viability

Cell viability was assessed using trypan blue dye exclusion assay. PC12 cells were seeded in 24-well plates and were pretreated with allantopyrone A for 18 h, and then treated with 200 μ M H₂O₂ for 12 h. These cells were then stained with trypan blue. The percentage of viable cells was determined using a hemocytometer. Cell viability (%) denotes the ratio of the number of trypan blue impermeable cells in total cell counts.

Detection of intracellular ROS level

Intracellular reactive oxygen species (ROS) level was detected using 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA). PC12 cells were seeded and were pretreated with allantopyrone A for 18 h in 96-well white plates. Then, 200 μ M H₂O₂ was added to the wells. After 1 h, 5 μ M DCFH-DA (Sigma Aldrich Corp., St Louis, MO, USA) was added to the wells 30 min prior to measurement. Fluorescent intensity (excitation/emission wavelength = 530-/590 nm) was measured using a micro plate reader (Tecan, Männedorf, Switzerland).

RESULTS

Allantopyrone A upregulated the targeting proteins of Nrf2

We performed proteomic analysis using 2D-DIGE to investigate the effect of allantopyrone A on protein expression. HeLa cells were treated with allantopyrone A for 18 h, and the whole-cell lysates were subjected to 2D-DIGE. As a result, 16 proteins were significantly increased and interestingly, 5 proteins that are regulated by transcription factor Nrf2 (aldo-keto reductase family 1 member C2, UDP-glucose 6-dehydrogenase, D-3-phosphoglycerate dehydrogenase, glutathione S-transferase P and peroxiredoxin-6), were upregulated by allantopyrone A treatment (Table 1).

Allantopyrone A increased HO-1 and nuclear localization of Nrf2 in PC12 cells

Proteomic analysis suggested that allantopyrone A activates the Keap1-Nrf2 pathway. This pathway enhances the expression of many antioxidant and detoxification enzymes such as HO-1 and γ -glutamylcysteine synthetase. We examined the protein levels of HO-1 to clarify the effect of allantopyrone A on the Keap1-Nrf2

pathway. As a result, allantopyrone A increased the protein levels of HO-1 in dose- and time-dependent manners (Figure 2a). In the course of activation of Keap1–Nrf2 pathway, Nrf2 is released from Keap1 and localized to the nucleus where it promotes expression of target proteins as a transcriptional factor. Nrf2 was accumulated in the nuclear fraction by allantopyrone A (Figure 2b). These results indicate that allantopyrone A activates the Keap1–Nrf2 pathway.

The thiol-containing compounds attenuated the effects of allantopyrone A

Allantopyrone A has an α,β -unsaturated carbonyl moiety, and it is known that this structure is involved in the activation of the Keap1–Nrf2 pathway through the direct binding with the thiol group of Keap1. Pretreatment of PC12 cells by *N*-acetyl-L-cysteine (NAC) or glutathione for 1 h clearly attenuated the increase in the protein expression of HO-1 (Figure 3a). The nuclear localization of Nrf2 is also suppressed by NAC or glutathione (Figure 3b).

Table 1 Expression data of significant increased spot volumes in HeLa cells treated with allantopyrone A

Spot no	Fold ratio	UniProt Ac	Protein name
1757	1.74*	P04792	Heat-shock protein beta-1
949	1.56*	P08107	Heat-shock 70 kDa protein 1A/1B
934	1.55*	P08107	Heat-shock 70 kDa protein 1A/1B
1499	1.49**	P52895	Aldo-keto reductase family 1 member C2
1975	1.42*	P08107	Heat-shock 70 kDa protein 1A/1B
2164	1.38*	P41250	Glycyl-tRNA synthetase
1113	1.35*	O60701	UDP-glucose 6-dehydrogenase
1754	1.35**	P04792	Heat-shock protein beta-1
1491	1.32*	P04083	Annexin A1
2168	1.31**	Q8WUM4	Programmed cell death 6-interacting protein
1138	1.21*	O43175	D-3-phosphoglycerate dehydrogenase
1579	1.18**	Q15181	Inorganic pyrophosphatase
1756	1.16*	P04632	Calpain small subunit 1
2242	1.16**	P09211	Glutathione S-transferase P
1274	1.14*	P35998	26S protease regulatory subunit 7
1779	1.10*	P30041	Peroxiredoxin-6

Abbreviation: tRNA, transfer RNA.
The fold ratio between control and allantopyrone A was calculated. A two-tailed unpaired Student's *t*-test was performed. Asterisks indicate significant differences from the control (* $P < 0.03$, ** $P < 0.01$).

Allantopyrone A directly bound with Keap1

It was suggested that allantopyrone A bound to Keap1 through an α,β -unsaturated carbonyl moiety. Most of the activators of Keap1–Nrf2 pathway bind covalently with Keap1. Previously, it was reported that 6-(methylsulfinyl)hexyl isothiocyanate (6-MSITC) activates the Keap1–Nrf2 pathway through covalent binding with Keap1.¹⁶ They showed that a band of 6-MSITC-modified Keap1, which is greater than 150 kDa, in the lysate of cells treated with 6-MSITC, was detected by western blotting using anti-Keap1 antibody. To evaluate the covalent binding of allantopyrone A with Keap1, we performed the same experiment. As a result, a protein band (>150 kDa), detected with anti-Keap1 antibody, was observed in an allantopyrone A dose-dependent manner (Figure 4a). Thus, it was suggested that allantopyrone A bound covalently with Keap1.

To confirm the direct binding of allantopyrone A to Keap1, we performed affinity purification of binding proteins using allantopyrone A-immobilized beads from the lysate of PC12 cells, and then detected by western blot analysis using anti-Keap1 antibody. Since covalent binding was suggested in Figure 4a, we used a cleavable photoactivatable linker-coated beads.¹⁵ It can detect covalently binding proteins by cleavage of disulfide bond in the linker using reducing agents. As expected, Keap1 was detected as one of the allantopyrone

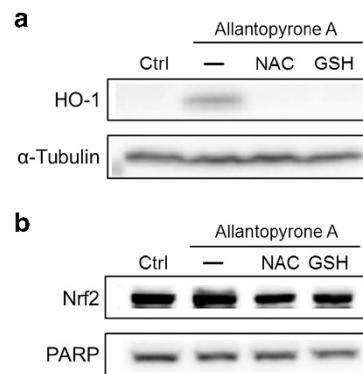


Figure 3 NAC and GSH alleviates the effects induced by allantopyrone A. PC12 cells were pretreated with 5 mM NAC or 5 mM GSH for 1 h and then treated with 1 μ M allantopyrone A for 12 h. Whole-cell lysates (a) or nuclear fraction (b) were subjected to western blot analysis. GSH, glutathione; NAC, *N*-acetyl-L-cysteine.

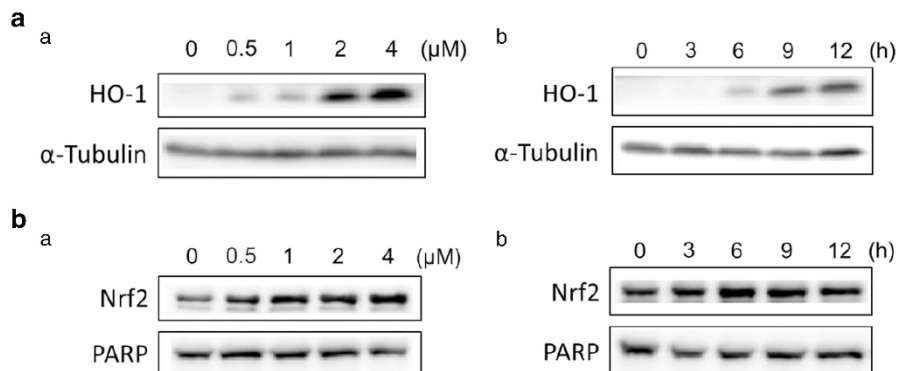


Figure 2 Effects of allantopyrone A on the expression of HO-1 and localization of Nrf2. (a) Effects of allantopyrone A on the protein expression of HO-1. PC12 cells were treated with allantopyrone A for 12 h at indicated doses (a), and 1 μ M allantopyrone A for indicated times (b). Whole-cell lysates were subjected to western blot analysis. α -Tubulin was used as a loading standard. (b) Nuclear localization of Nrf2 induced by allantopyrone A. PC12 cells were treated with allantopyrone A for 12 h at indicated doses (a), and 1 μ M allantopyrone A for indicated times (b), and then fractionated to nuclear and cytosolic fraction. The nuclear fraction was subjected to western blot analysis. PARP was used as a loading standard of nuclear fraction. HO-1, heme oxygenase-1.

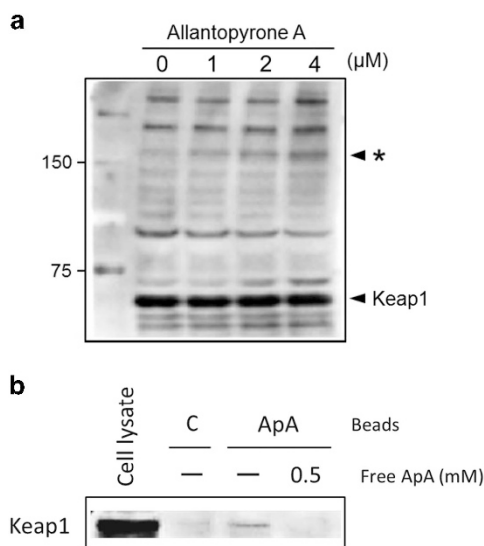


Figure 4 Binding of allantopyrone A with Keap1. (a) Detection of an allantopyrone A-modified Keap1. PC12 cells were treated with allantopyrone A for 1 h, and the whole-cell lysate was prepared. The modified band of Keap1 (asterisk) was detected by western blotting using anti-Keap1 antibody. (b) Detection of direct binding of allantopyrone A to Keap1. Whole-cell lysates of PC12 cells were prepared. Then we performed affinity purification from the PC12 lysates using allantopyrone A (ApA)-immobilized beads. Proteins bound to beads were eluted by SDS sample buffer and boiling, and then subjected to western blot analysis using an anti-Keap1 antibody.

A-binding proteins and it was competed by the addition of free allantopyrone A (Figure 4b), indicating that allantopyrone A directly binds with Keap1.

Allantopyrone A suppressed oxidative stress-induced cell death

Keap1–Nrf2 pathway has a key role in the endogenous antioxidant system. Some of the Keap1–Nrf2 activators protect cells from oxidative stress-induced cell death. We examined whether allantopyrone A affects intracellular ROS levels using DCFH-DA. Pretreatment with allantopyrone A for 18 h suppressed the elevation of intracellular ROS level induced by H_2O_2 in a dose-dependent manner (Figure 5a). In addition, we detected cell death using Hoechst 33342/propidium iodide double staining. Pretreatment with allantopyrone A for 18 h potently suppressed H_2O_2 -induced cell death (Figure 5b). This protective effect was also confirmed by trypan blue dye exclusion assay (Figure 5c). In addition, we examined whether allantopyrone A suppresses oxidative stress-induced cell death through its direct antioxidant effect or not. Co-treatment with NAC, an antioxidant, with H_2O_2 suppressed H_2O_2 -induced cell death. On the other hand, co-treatment of allantopyrone A with H_2O_2 could not suppress it (Figure 5d). Therefore, these results suggest that allantopyrone A suppresses oxidative stress-induced cell death, not through a direct antioxidant effect but by activation of indirect antioxidant systems such as Keap1–Nrf2 pathway.

DISCUSSION

Allantopyrone A has been isolated from endophytic fungi *A. lycopodina*.⁸ We have recently reported that this compound has potent cytotoxicity against HL60 cells and an inhibitory effect on nuclear factor κB signaling.⁹ Allantopyrone A possesses an α,β -unsaturated carbonyl group and this moiety is well known as being highly reactive with several intracellular proteins. Therefore, we

performed proteomic analysis to investigate the effect of allantopyrone A toward mammalian cells. As a result, allantopyrone A upregulated the expression of heat-shock proteins and proteins, which are controlled by the transcription factor Nrf2. Nrf2 regulates protein expression of ~250 antioxidant and detoxification enzymes, and has a protective effect on cells from various stresses such as oxidative stress and carcinogenesis.¹⁷ Thus, the Keap1–Nrf2 pathway is interpreted as a protective system against oxidative stress-related diseases. The upregulation of heat-shock proteins was also shown by allantopyrone A. It is known that heat-shock proteins are frequently increased by various stresses. Moreover, several compounds that have an α,β -unsaturated carbonyl moiety induce the expression of heat-shock proteins through non-specific binding to intracellular proteins.¹⁸ Thus, we focused on the Keap1–Nrf2 system in this study. Allantopyrone A increased HO-1, and induced nuclear localization of Nrf2. These results suggested that allantopyrone A activates the Keap1–Nrf2 pathway. Keap1 has reactive thiol groups and is considered as a sensor of oxidants or electrophilic compounds. Some of the electrophilic compounds, for example, sulforaphane,¹¹ curcumin,¹² zerumbone,^{19,20} piperlongumine,²¹ bardoxolone methyl²² and dimethyl fumarate,²³ activate the Keap1–Nrf2 pathway. They bind directly with the thiol group of Keap1 and causes conformational changes to Keap1. Since allantopyrone A also has an electrophilic α,β -unsaturated carbonyl group, we examined the involvement of this moiety in this effect using thiol-containing compounds NAC and glutathione. NAC and glutathione clearly suppressed the HO-1 upregulation, thus, it was suggested that allantopyrone A interacted with Keap1 through direct binding to its thiol groups. To elucidate the direct binding, we performed affinity purification using allantopyrone A-immobilized beads from a lysate of PC12 cells. As expected, Keap1 was detected as one of the binding proteins of allantopyrone A. Some of the important thiol groups for Keap1 function include Cys151, Cys273 and Cys288, and each compound that directly binds to Keap1 target different combinations of thiol groups.¹⁸ For example, sulforaphane and dimethyl fumarate are Cys151-preferring inducers and 15-deoxy- $\Delta^{12,14}$ -prostaglandin J_2 is a Cys273/288-preferring inducer. Further studies are needed to elucidate which thiol groups of Keap1 are the direct target of allantopyrone A. These results indicate that allantopyrone A activates Keap1–Nrf2 pathway through the direct binding to Keap1.

Oxidative stress is responsible for various diseases such as cancer and neurodegenerative diseases.¹⁸ Indeed, dimethyl fumarate, a Keap1–Nrf2 activator that is used as a clinical drug for multiple sclerosis, acts by suppression of demyelination caused by the oxidative stress. In addition, development of Keap1–Nrf2 activators for diabetic nephropathy, age-related macular degeneration, has been carried out. Based on these cases, we examined the effect of allantopyrone A on oxidative stress-induced cytotoxicity in PC12 cells. Pretreatment with allantopyrone A attenuated the elevation of intracellular ROS levels caused by H_2O_2 . Furthermore, pretreatment of allantopyrone A suppressed H_2O_2 -induced cell death. Although direct antioxidant compounds such as NAC suppress the H_2O_2 -induced toxicity, they can show this effect by co-treatment with H_2O_2 . On the other hand, the protective effects against oxidative stress by indirect antioxidant compounds, such as activators of the Keap1–Nrf2 pathway, are a necessary pretreatment for induction of antioxidant enzymes. Indeed, pretreatment with allantopyrone A protected cells from H_2O_2 -induced cell death, however, co-treatment did not show this effect. Therefore, it suggested that allantopyrone A protects PC12 cells from oxidative stress through the activation of indirect antioxidant systems.

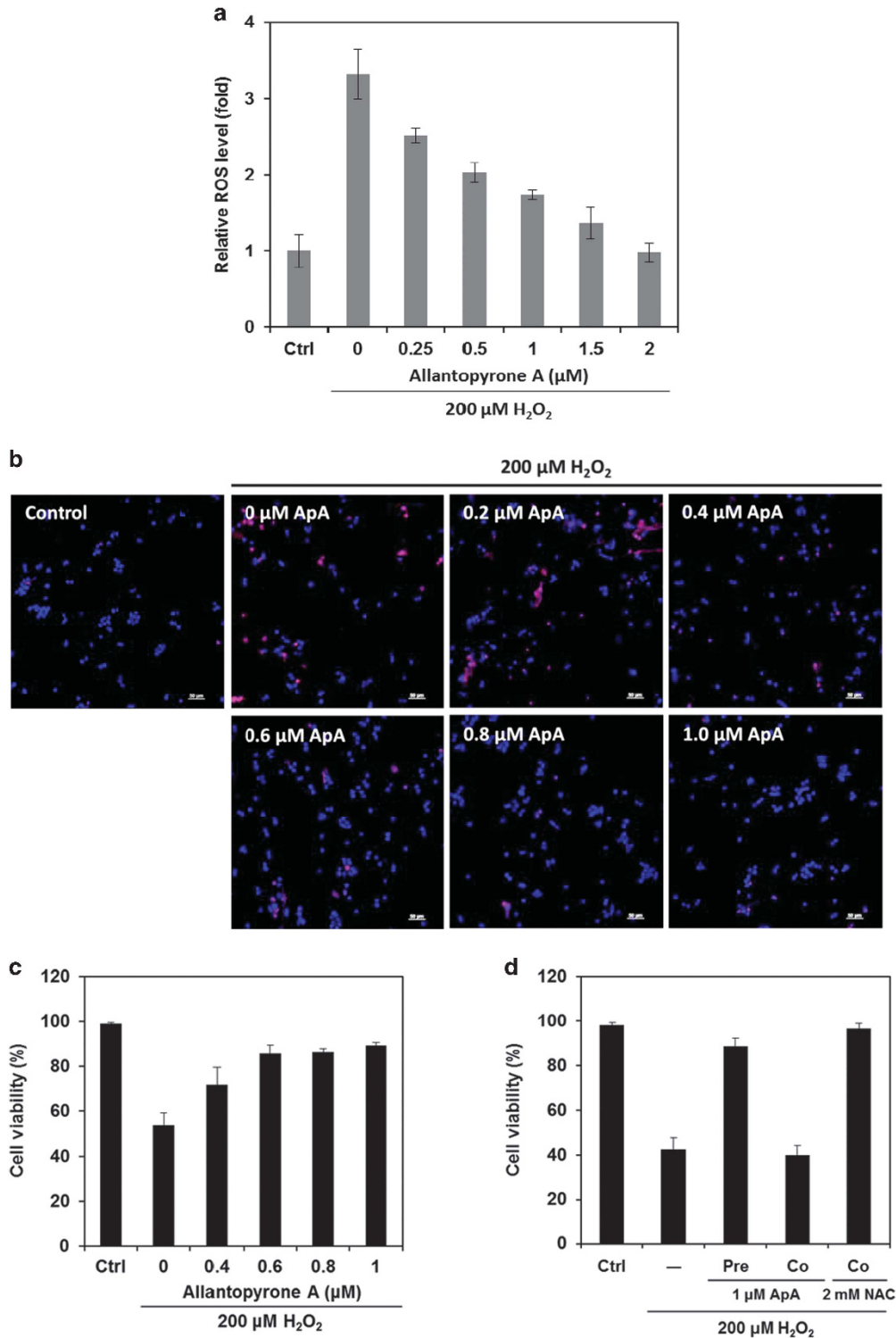


Figure 5 Allantopyrone A (ApA) protects PC12 cells from H_2O_2 -induced cell death. (a) Intracellular reactive oxygen species (ROS) level. PC12 cells were seeded and were pretreated with ApA for 18 h in a 96-well white plate. Then, 200 μM H_2O_2 was added to wells. After 1 h, 5 μM DCFH-DA was added to wells 30 min prior to measurement. Fluorescent intensity (excitation/emission wavelength = 530/590 nm) was measured using a micro plate reader. (b) Detection of cell death by PI staining. PC12 cells were pretreated with ApA for 18 h in a 24-well plate, and then treated with 200 μM H_2O_2 for 12 h. These cells were stained with Hoechst 33342 and PI. Stained cells were observed using a confocal laser microscope. (c) The effect of pretreatment with ApA on H_2O_2 -induced cell death. PC12 cells were seeded in a 24-well plate and were pretreated with ApA for 18 h, and then treated with 200 μM H_2O_2 for 12 h. These cells were stained with trypan blue. The percentage of viable cells was determined using a hemocytometer. (d) Co-treatment with ApA and H_2O_2 did not suppress the cell death induced by H_2O_2 . PC12 cells were seeded in 24-well plates and pretreated with 1 μM ApA for 18 h before the treatment with 200 μM H_2O_2 for 12 h, or co-treated with 1 μM ApA or 2 mM *N*-acetyl-L-cysteine (NAC) with 200 μM H_2O_2 for 12 h.

In conclusion, we have demonstrated that allantopyrone A activates Keap1-Nrf2 pathway through direct binding with Keap1. Furthermore, this compound enhanced antioxidant system and protected PC12 cells from oxidative stress-induced cell death. Although further studies are needed about the effect of allantopyrone A *in vivo*, it may have the potential to be developed as a therapeutic agent for oxidative stress-related diseases including neurodegenerative diseases.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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