

ORIGINAL ARTICLE

Allantopyrone A, an α -pyrone metabolite from an endophytic fungus, inhibits the tumor necrosis factor α -induced nuclear factor κ B signaling pathway

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Tumor necrosis factor α (TNF- α) induces the activation of transcription factor nuclear factor κ B (NF- κ B), which upregulates a variety of genes, including the gene encoding intercellular adhesion molecule-1 (ICAM-1). Allantopyrone A, a recently identified α -pyrone metabolite from an endophytic fungus, was found to inhibit the TNF- α -induced expression of ICAM-1 in human lung carcinoma A549 cells. Allantopyrone A also inhibited the TNF- α -induced luciferase expression of an NF- κ B-responsive reporter. In the NF- κ B signaling pathway, allantopyrone A inhibited the nuclear translocation of NF- κ B subunits as well as the phosphorylation and subsequent degradation of the inhibitor of NF- κ B (I κ B) α proteins. By contrast, allantopyrone A did not directly affect the catalytic activity of active I κ B kinase β . These findings indicate that allantopyrone A inhibits the NF- κ B signaling pathway at a step upstream of I κ B α phosphorylation.

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INTRODUCTION

Proinflammatory cytokines, such as tumor necrosis factor α (TNF- α), induce intracellular signaling pathways, one of which leads to the activation of transcription factor nuclear factor κ B (NF- κ B).¹ NF- κ B plays a role in the expression of many genes essential for inflammation and tumor promotion.² In response to TNF- α stimulation, TNF receptor 1 recruits the TNF receptor-associated death domain (TRADD), followed by the recruitment of other adaptor proteins, including receptor-interacting protein 1 (RIP1).^{3,4} RIP1 is then ubiquitinated and thereby recruits the TGF- β -activated kinase 1 (TAK1) complex and the inhibitor of the NF- κ B (I κ B) kinase complex in its polyubiquitin chains.⁵ The I κ B kinase complex is subsequently activated and the activated complex phosphorylates N-terminal serine residues of I κ B, which promotes I κ B polyubiquitination and proteolysis by proteasomes.^{6,7} NF- κ B heterodimers, such as p65/p50, are associated with I κ B in the cytoplasm, and following I κ B degradation they are released and translocate to the nucleus.⁸ NF- κ B heterodimers activate the transcription of target genes, including the gene encoding intercellular adhesion molecule-1 (ICAM-1).⁹

We have recently isolated a new α -pyrone metabolite designated allantopyrone A (Figure 1) from an endophytic fungus, *Allantophomopsis lycopodina* KS-97.¹⁰ Allantopyrone A was found to have high cytotoxicity and induce DNA fragmentation in human leukemia HL-60 cells.¹⁰ Thus far, the biological activities of allantopyrone A

have been poorly understood, except that allantopyrone A possesses an α,β -unsaturated carbonyl moiety, which reacts with cysteinyl thiol groups. It has been reported that natural and synthetic compounds possessing an α,β -unsaturated carbonyl moiety inhibit multiple steps of the NF- κ B signaling pathway.¹¹ We have found that the inhibitory effect of allantopyrone A on the TNF- α -induced expression of ICAM-1 is stronger than its cytotoxic effect on human lung carcinoma A549 cells. In this study, we further investigated the molecular mechanism underlying the inhibition of ICAM-1 expression by allantopyrone A.

MATERIALS AND METHODS

Cell culture

Human lung carcinoma A549 cells (JCRB0076) were provided by the National Institute of Biomedical Innovation JCRB Cell Bank (Osaka, Japan). A549 cells were maintained in RPMI 1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% heat-inactivated fetal calf serum (Nichirei Biosciences, Tokyo, Japan) and a penicillin-streptomycin mixed solution (Nacalai Tesque, Kyoto, Japan). Human embryonic kidney (HEK) 293C8L cells were maintained in Dulbecco's modified Eagle Medium supplemented with 10% heat-inactivated fetal calf serum and the penicillin-streptomycin mixed solution.¹²

Reagents

Allantopyrone A was prepared as described previously.¹⁰ 15-deoxy- $\Delta^{12,14}$ -prostaglandin J₂ (15d- $\Delta^{12,14}$ -PGJ₂) was obtained from Cayman Chemical (Ann Arbor, MI, USA).

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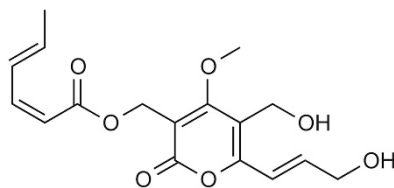


Figure 1 Structure of allantopyrone A.

Cell-enzyme-linked immunosorbent assay

The cell-surface expression level of ICAM-1 was measured by cell-enzyme-linked immunosorbent assay as described previously.^{13,14} The expression level of ICAM-1 (%) was calculated using the following formula: (test samples – background)/(positive control – background) \times 100.

Assay of cell viability

Cell viability was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described previously,^{13,14} except that A549 cells were pulsed with MTT (500 μ g ml⁻¹) for 2 h.

Reporter assay

The NF- κ B-responsive firefly luciferase gene was controlled by two copies of κ B sequences from the I κ B enhancer.¹⁵ A549 cells were transfected with reporter vectors encoding the NF- κ B-responsive firefly luciferase gene and a CMV-promoter-driven *Renilla* luciferase gene using HilyMax transfection reagent (Dojindo Laboratories, Kumamoto, Japan) for 8 h. The transfected A549 cells were seeded in 24-well culture plates and incubated overnight. The reporter assay of the cells was performed as described previously.¹²

Western blotting

Cell lysates were prepared and western blotting was performed as described previously.¹⁶ Antibodies to β -actin (AC-15; Sigma-Aldrich, St Louis, MO, USA), I κ B α (clone 25; BD Biosciences, Franklin Lakes, NJ, USA), I κ B kinase β (10A9B6; BD Biosciences), NF- κ B p50 (H-119; Santa Cruz Biotechnology, Santa Cruz, CA, USA), NF- κ B p65 (C-20; Santa Cruz Biotechnology), phospho-I κ B α (Ser32/36) (5A5; Cell Signaling Technology, Danvers, MA, USA) and poly(ADP-ribose) polymerase (C-2-10; Sigma-Aldrich) were used for western blotting. The protein bands were analyzed using an ImageQuant LAS 4000 Mini (GE Healthcare, Piscataway, NJ, USA).

I κ B kinase assay

I κ B kinase assay was performed as described previously.¹⁷ HEK293C8L cells were transfected with the pCR3 expression vector encoding FLAG-tagged human I κ B kinase β by the calcium phosphate method. Cell lysates were prepared using NP-40 lysis buffer and immunoprecipitated with anti-FLAG antibody-conjugated agarose beads (Sigma-Aldrich). The obtained immunoprecipitates were incubated with glutathione S-transferase (GST)-tagged I κ B α (1–54) (1.5 μ g) at 37 °C for 30 min in the presence or absence of ATP (10 μ M). Reaction mixtures were analyzed by western blotting.

Statistical analysis

Statistical significance was assessed by one-way analysis of variance followed by Tukey's test for multiple comparisons. Differences with $P < 0.05$ were considered to be statistically significant.

RESULTS

Allantopyrone A inhibits TNF- α -induced expression of ICAM-1

TNF- α stimulation markedly induces the cell-surface expression of ICAM-1 in an NF- κ B-dependent manner in A549 cells.¹⁸ A549 cells were preincubated with allantopyrone A for 1 h and then incubated with TNF- α for 6 h in the presence of allantopyrone A. Allantopyrone A at concentrations higher than 32 μ M inhibited the TNF- α -induced ICAM-1 expression almost completely (Figure 2a). For the same incubation period, allantopyrone A at 100 μ M decreased cell viability

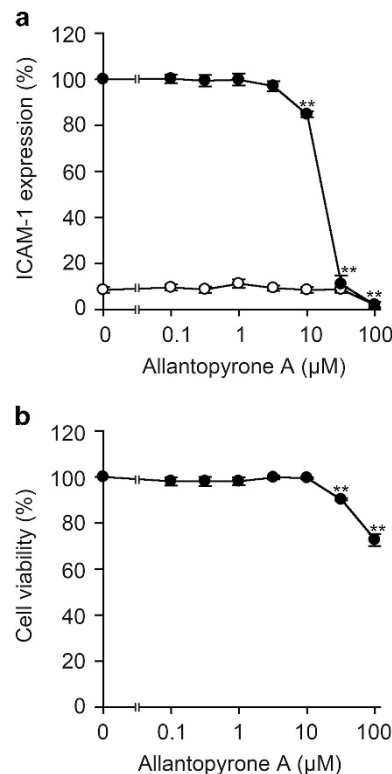


Figure 2 Allantopyrone A inhibits TNF- α -induced ICAM-1 expression. (a) A549 cells were preincubated with allantopyrone A at various concentrations for 1 h and then incubated with (filled circles) or without (open circles) TNF- α (2.5 ng ml⁻¹) for 6 h in the presence of allantopyrone A. ICAM-1 expression (%) is shown as the mean \pm s.e. of three independent experiments. ** $P < 0.01$, compared with control. (b) A549 cells were incubated with allantopyrone A at various concentrations for 7 h. Cell viability (%) is shown as the mean \pm s.e. of three independent experiments. ** $P < 0.01$, compared with control.

by \sim 30% (Figure 2b), indicating that allantopyrone A exerts a moderately strong cytotoxic effect within 7 h on A549 cells.

Allantopyrone A inhibits TNF- α -induced NF- κ B-dependent luciferase activity

To minimize the cytotoxic effect of allantopyrone A, A549 cells were exposed to allantopyrone A for a shorter period in further experiments. A549 cells were preincubated with allantopyrone A for 1 h and then incubated with TNF- α for 2.5 h in the presence of allantopyrone A. TNF- α induced an approximately eightfold increase in NF- κ B-dependent luciferase reporter activity (Figure 3a). Allantopyrone A at concentrations higher than 32 μ M markedly decreased the TNF- α -induced NF- κ B luciferase reporter activity (Figure 3a). By contrast, allantopyrone A did not affect cell viability at these concentrations up to 3.5 h (Figure 3b).

Allantopyrone A inhibits TNF- α -induced nuclear translocation of NF- κ B subunits

The NF- κ B p65/p50 heterodimers are localized in the cytoplasm and translocate to the nucleus upon TNF- α stimulation.^{16,18} A549 cells were preincubated with allantopyrone A for 1 h and then incubated with TNF- α for 30 min in the presence of allantopyrone A. TNF- α augmented the amounts of p65 and p50 in the nuclear fraction within 30 min (Figure 4). Allantopyrone A at concentrations higher than 32 μ M significantly inhibited the accumulation of nuclear p65 and p50 (Figure 4).

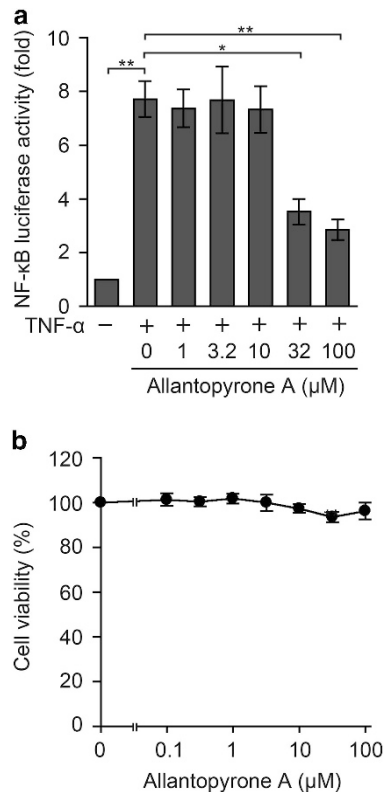


Figure 3 Allantopyrone A inhibits TNF- α -induced NF- κ B-responsive reporter activity. **(a)** A549 cells were transfected with reporter vectors encoding an NF- κ B-responsive luciferase gene and a CMV promoter-driven *Renilla* luciferase gene. A549 cells were preincubated with allantopyrone A at various concentrations for 1 h and then incubated with (+) or without (-) TNF- α (2.5 ng ml⁻¹) for 2.5 h in the presence of allantopyrone A. NF- κ B activation (fold) is shown as means \pm s.e. of three independent experiments. * P <0.05 and ** P <0.01, compared with TNF- α (+). **(b)** A549 cells were incubated with various concentrations of allantopyrone A for 3.5 h. Cell viability (%) is shown as the mean \pm s.e. of three independent experiments. ** P <0.01, compared with control.

Allantopyrone A inhibits TNF- α -induced I κ B α phosphorylation and degradation

TNF- α stimulation induces I κ B α phosphorylation within 5 min and the subsequent I κ B α proteolysis by the ubiquitin-proteasome system within 15 min in A549 cells.¹⁹ A549 cells were preincubated with allantopyrone A for 1 h and then incubated with TNF- α for 15 min in the presence of allantopyrone A. The amount of I κ B α markedly decreased 15 min after TNF- α stimulation (Figure 5a). Allantopyrone A at concentrations higher than 32 μ M inhibited the TNF- α -induced I κ B α degradation (Figure 5a).

The amount of the phosphorylated form of I κ B α transiently increased 5 min following TNF- α stimulation (Figure 5b). Allantopyrone A at 100 μ M inhibited the phosphorylation and subsequent degradation of I κ B α almost completely (Figure 5b). These findings suggest that allantopyrone A blocks the NF- κ B signaling pathway at a step upstream of I κ B α phosphorylation.

Allantopyrone A does not directly inhibit the catalytic activity of I κ B kinase β

FLAG-tagged I κ B kinase β was transiently overexpressed in HEK293C8L cells and immunoprecipitated with anti-FLAG-antibody-conjugated beads. The immunoprecipitates containing I κ B

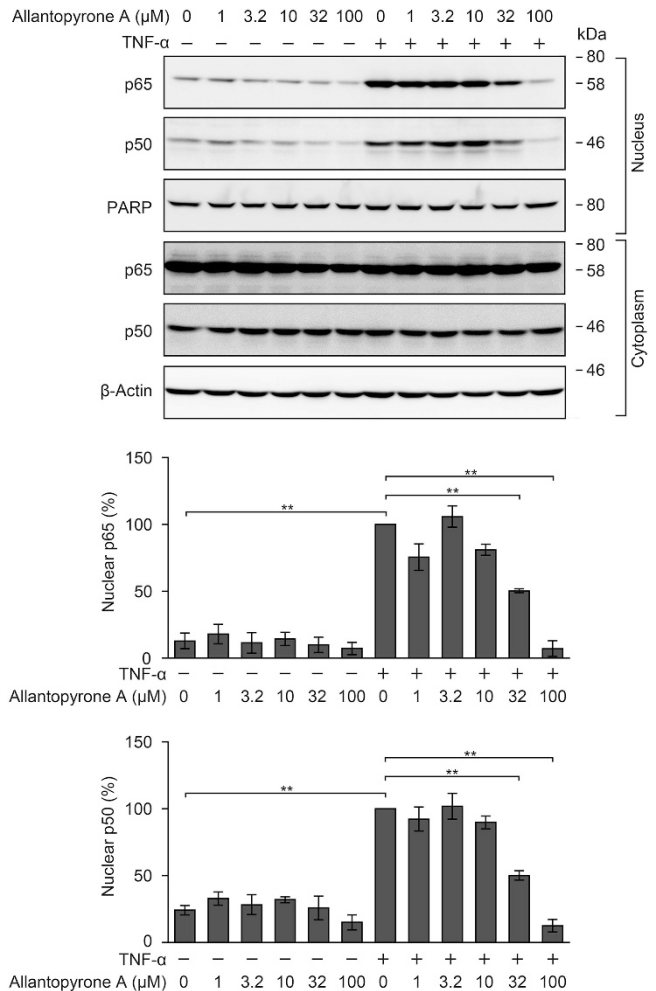


Figure 4 Allantopyrone A inhibits TNF- α -induced nuclear translocation of NF- κ B subunits. A549 cells were preincubated with allantopyrone A at various concentrations for 1 h and then incubated with (+) or without (-) TNF- α (2.5 ng ml⁻¹) for 30 min in the presence of allantopyrone A. Nuclear and cytoplasmic fractions were analyzed by western blotting. The amounts of nuclear p65 and p50 were normalized with that of PARP. The amounts of nuclear p65 and p50 (%) are shown as the mean \pm s.e. of three independent experiments. ** P <0.01, compared with TNF- α (+).

kinase β were capable of phosphorylating GST-tagged I κ B α (1-54) in the presence of ATP. As reported previously,^{20,21} 15d- Δ ^{12,14}-PGJ₂ markedly inhibited the phosphorylation of I κ B α (1-54) (Figure 6). The MW of I κ B kinase β treated with 15d- Δ ^{12,14}-PGJ₂ slightly increased (Figure 6), suggesting that 15d- Δ ^{12,14}-PGJ₂ binds covalently to I κ B kinase β . By contrast, allantopyrone A at concentrations up to 100 μ M did not inhibit the phosphorylation of I κ B α (1-54) (Figure 6). These findings suggest that allantopyrone A does not directly inhibit the catalytic activity of active I κ B kinase β .

DISCUSSION

We have recently isolated allantopyrone A from *A. lycopodina* KS-97.¹⁰ Allantopyrone A has been shown to have high cytotoxicity and induce DNA fragmentation in HL-60 cells.¹⁰ Thus far, the biological activities of allantopyrone A have been poorly characterized. In line with our previous finding,¹⁰ allantopyrone A decreased the viability of A549 cells by \sim 30% following the 7-h incubation. More importantly, we found the novel biological activity

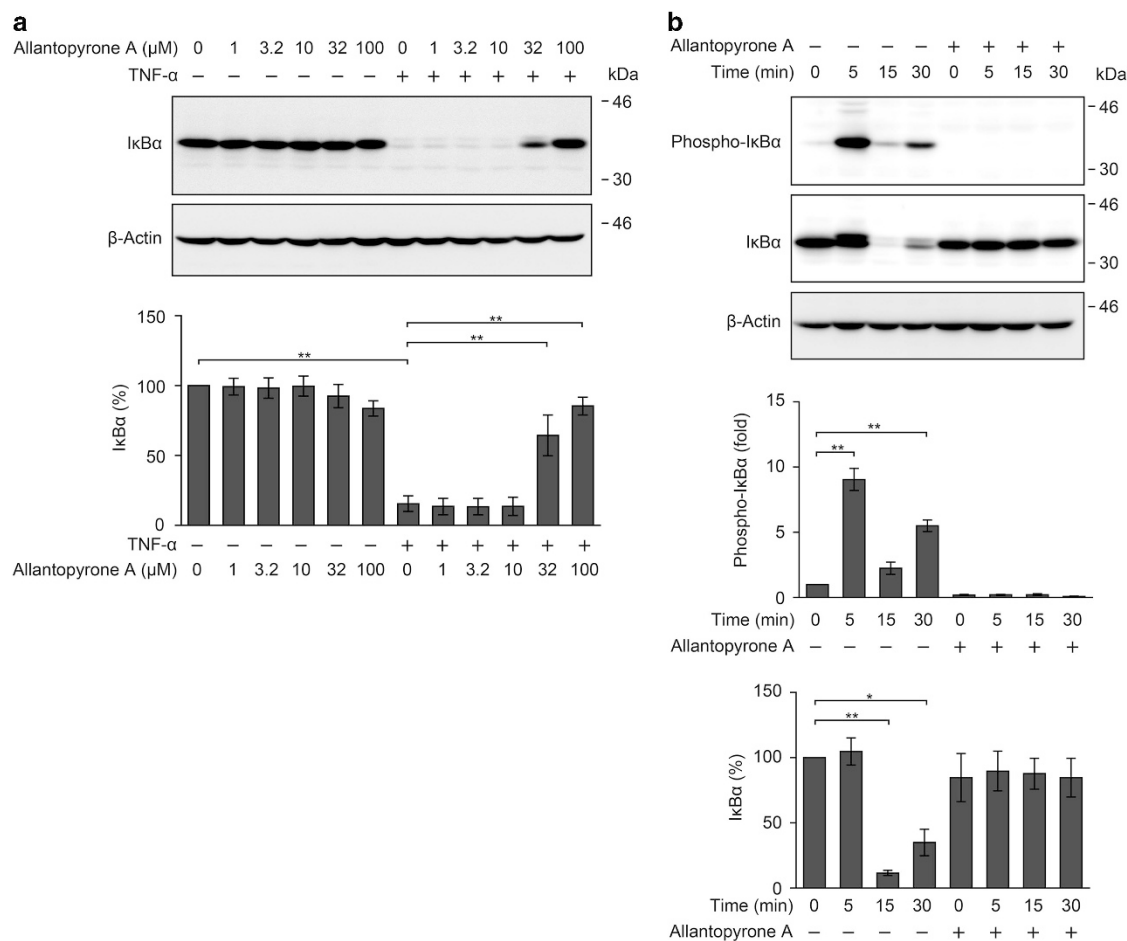


Figure 5 Allantopyrone A inhibits TNF- α -induced phosphorylation and degradation of I κ B α . **(a)** A549 cells were preincubated with allantopyrone A at various concentrations for 1 h and then incubated with (+) or without (-) TNF- α (2.5 ng ml $^{-1}$) for 15 min in the presence of allantopyrone A. Cell lysates were analyzed by western blotting. The amount of I κ B α was normalized with that of β -actin. I κ B α (%) is shown as the mean \pm s.e. of four independent experiments. ** P < 0.01, compared with TNF- α (+). **(b)** A549 cells were preincubated with (+) or without (-) allantopyrone A (100 μ M) for 1 h and then incubated with TNF- α (2.5 ng ml $^{-1}$) for indicated times in the presence of allantopyrone A. Cell lysates were analyzed by western blotting. The amounts of phospho-I κ B α and total I κ B α were normalized with that of β -actin. Phospho-I κ B α (fold) and I κ B α (%) are shown as the mean \pm s.e. of three independent experiments. * P < 0.05 and ** P < 0.01, compared with control.

that allantopyrone A selectively inhibits the NF- κ B signaling pathway in A549 cells under conditions that do not affect cell viability.

As compounds structurally related to allantopyrone A, islandic acid-I and islandic acid-II as well as their methyl esters were previously isolated from *Penicillium islandicum* Sopp.^{22,23} The islandic acid-I and islandic acid-I methyl esters showed a 100% inhibitory activity against the growth of Yoshida sarcoma cells (rat tumor cells) at concentrations of 100 μ g ml $^{-1}$ (286 μ M) and 1 μ g ml $^{-1}$ (2.7 μ M), respectively.²³ We previously showed that allantopyrone A and islandic acid-II methyl ester exert the cytotoxic effect on HL-60 cells at IC $_{50}$ values of 0.32 and 6.55 μ M, respectively.¹⁰ Thus, it seems that allantopyrone A is one of most potent compounds with antitumor activity among the α -pyrone metabolites.

Allantopyrone A possesses an α,β -unsaturated carbonyl moiety that is reactive to cysteinyl thiol groups by the Michael addition. It has been thus far reported that natural and synthetic compounds possessing an α,β -unsaturated carbonyl moiety inhibit multiple steps in the NF- κ B signaling pathway.¹¹ I κ B kinase β contains a Cys-179 residue within its activation loop in which two serine residues (Ser-177 and Ser-181) are phosphorylated for kinase activation.^{20,24} It has been reported that 15d- $\Delta^{12,14}$ -PGJ $_2$ targets Cys-179 of I κ B kinase β

and inhibits its catalytic activity.^{20,21} In contrast to 15d- $\Delta^{12,14}$ -PGJ $_2$, allantopyrone A did not directly inhibit I κ B kinase β activity. However, allantopyrone A inhibited the TNF- α -induced I κ B α phosphorylation at the cellular level. These findings suggest that allantopyrone A inhibits a step upstream of the activation of I κ B kinase in the NF- κ B signaling pathway.

We previously showed that peperomin E and 2,6-didehydropeperomin B that possess an α -methylene- γ -lactone moiety reactive to thiol residues, but not peperomin A and peperomin B that possess an α -methyl- γ -lactone moiety unreactive to thiol residues, inhibit the TNF- α -induced activation of I κ B kinase in A549 cells.¹⁷ However, similar to allantopyrone A, peperomin E and 2,6-didehydropeperomin B were not capable of inhibiting I κ B kinase β catalytic activity directly.¹⁷ These findings collectively imply that certain protein(s) containing cysteine residues critical for their biological activities are required for the signaling process upstream of the activation of I κ B kinase. TAK1 might be one of the candidate proteins, because it has been reported that epoxyquinol B inhibits NF- κ B signaling by crosslinking TAK1 through cysteine residues.²⁵

In conclusion, we have shown that allantopyrone A inhibits the TNF- α -induced NF- κ B signaling pathway at a step upstream of I κ B α

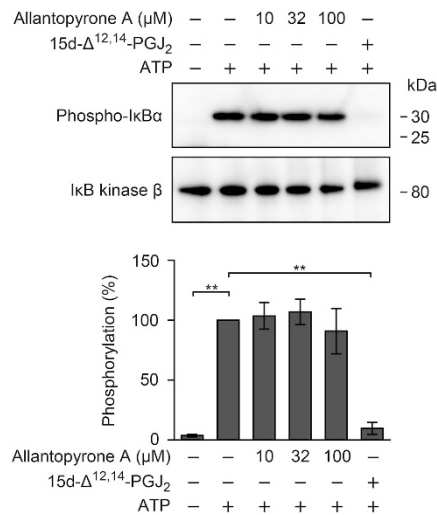


Figure 6 Allantopyrone A does not directly affect I κ B kinase β . HEK293C8L cells were transfected with an expression vector encoding FLAG-tagged I κ B kinase β for 30 h. Cell lysates were immunoprecipitated with anti-FLAG antibody-conjugated agarose beads. Immunoprecipitates were incubated with GST-tagged I κ B α (1–54) in the presence (+) or absence (–) of ATP, allantopyrone A (at indicated concentrations) or 15d- $\Delta^{12,14}$ -PGJ₂ (50 μ M) for 30 min. The amount of phospho-I κ B α (1–54) (GST-tagged) was normalized with that of FLAG-tagged I κ B kinase β . Phosphorylation (%) is shown as the mean \pm s.e. of three independent experiments. ** $P < 0.01$, compared with control.

phosphorylation. NF- κ B targets many genes required for cancer development and progression.²⁶ Thus, agents that block the NF- κ B signaling pathway are expected to have high anticancer activity. It is currently unclear whether the inhibition of NF- κ B activation by allantopyrone A is related to the apoptosis-inducing activity of allantopyrone A, as observed in HL-60 cells. Further experiments will be necessary to clarify the molecular mechanism by which allantopyrone A inhibits the NF- κ B signaling pathway or induces apoptosis.

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