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# Induction of apoptosis of RAW 264.7 cells by the cytostatic macrolide apicularen A

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## Abstract

In RAW 264.7 cells, a mouse leukaemic monocyte cell line, apicularen A decreased cell growth and survival as assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay in a concentration-dependent manner at 10–1000 nM. Apicularen B, an *N*-acetyl-glucosamine glycoside of apicularen A, was 10–100-fold less effective than apicularen A. Apicularen A induced a DNA ladder, an increase in the percentage of sub-$G_1$ cells and annexin V-binding cells, and promoted the activation of caspase as revealed by the cleavage of poly(ADP-ribose) polymerase, indicating that apicularen A induced apoptosis in RAW 264.7 cells. In addition, apicularen A phosphorylated p44/42 mitogen-activated protein kinase (MAPK) and p38 MAPK. The p44/42 MAPK inhibitor PD98059 rescued the cells from apicularen-induced decrease in cell growth and survival as determined by the MTT assay, while the p38 MAPK inhibitor SB203580 augmented the effect of apicularen A. This suggested the activation of p44/42 MAPK to be pro-apoptotic and the activation of p38 MAPK anti-apoptotic in apicularen A-treated RAW 264.7 cells.

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## Introduction

The novel highly cytostatic macrolide apicularen A was isolated from the myxobacterial genus *Chondromyces* (Kunze et al 1998). The chemical structure of apicularen A is characterized by a salicylic acid residue as part of a 10-membered lactone bearing an acylenamine side chain. Apicularen A is reported to be highly cytostatic in several types of human and animal cells, such as the ovarian carcinoma cell line SK-OV-3, kidney carcinoma cell line A-498, and cervix carcinoma cell line KB-3-1. The $IC_{50}$ values of apicularen A range between 0.1 and 10 ng $mL^{-1}$ (0.227 and 22.7 nM, respectively) (Kunze et al 1998). Apicularen B, an *N*-acetyl-glucosamine glycoside of apicularen A, is distinctly less cytostatic than apicularen A, with $IC_{50}$ values ranging between 0.2 and 1.2 $\mu$g $mL^{-1}$ (0.317 and 1.87 $\mu$M, respectively) (Kunze et al 1998). Due to its strong cytostatic activity, apicularen A might be a potent lead compound for anti-cancer drugs. Previously, we had found that apicularen A had strong cytotoxic activity in RAW 264.7 cells, a mouse leukaemia monocyte cell line. Therefore, we have analysed the mechanism of action of apicularen A from the viewpoint of apoptosis.

The term apoptosis describes a tightly regulated process of cell death characterized by marked changes in cellular morphology, chromatin condensation, and intranucleosomal DNA fragmentation (Wyllie et al 1980; Leist & Nicotera 1997). During the process of apoptosis, several target proteins including poly(ADP-ribose) polymerase (PARP), lamin A, lamin B, actin and protein kinase C (PKC) $\delta$ (Porter et al 1997) are cleaved by various caspases. Apoptosis is regulated by intracellular signalling molecules including tyrosine kinase, serine/threonine kinase and phosphatases. The roles of p38 mitogen-activated protein kinase (MAPK) (Roulston et al 1998; Gálan et al 2000), p44/42 MAPK (Chin et al 1999), PKC (Jun et al 1999) and phosphatidylinositol 3-kinase (PI3K) (Koh et al 1998) in apoptosis have progressively been clarified although there are several inconsistencies.

In this study, we have examined the effects of apicularen A on cell growth and survival, cell morphology, and DNA fragmentation in RAW 264.7 cells. The effects on

the percentage of sub-$G_1$ cells and annexin V-binding cells, the activation of caspase and phosphorylation of p44/42 MAPK and p38 MAPK were examined to show that apicularen A induced apoptosis in RAW 264.7 cells. Finally, the roles of p38 MAPK and p44/42 MAPK in apicularen A-induced decrease in cell growth and survival in RAW 264.7 cells were analysed.

## Materials and Methods

### Reagents

Propidium iodide (Wako Pure Chemical Inc., Osaka, Japan), the mitogen-activated protein kinase kinase (MEK) inhibitor PD98059 (Dudley et al 1995) (New England Biolabs Inc., Beverly, MA), the p38 MAPK inhibitor SB203580 (Cuenda et al 1995) (Calbiochem Novabiochem Japan, Tokyo, Japan), apicularen A (2,4-heptadienamide, *N*-[(1E)-3-[(3S,5R,7R,9S)-3,4,5,6,7,8,9,10-octahydro-7,14 dihydroxy-1-oxo-5,9-epoxy-1H-2-benzoxacyclododecin-3-yl]-1 propenyl]-, (2Z,4Z)- (9CI)) and apicularen B (2,4-heptadienamide, *N*-[(1E)-3-[(3S,5R,7R,9S)-7-[[2-(acetylamino)-2-deoxy-$\beta$-D-glucopyranosyl]oxy]-3,4,5,6,7,8,9,10-octahydro 14-hydroxy-1-oxo-5,9-epoxy-1H-2-benzoxacyclododecin-3-yl]-1 propenyl]-, (2Z,4Z)- (9CI)) were dissolved in dimethyl sulfoxide (DMSO). A sample of each solution was added to the medium, and the final concentration of DMSO in the medium was adjusted to 0.1% (v/v). The control medium contained the same amount of the vehicle. Apicularens A and B were purified from culture medium of *Chondromyces apiculatus* JW 184 according to the method described by Jansen et al (2000). The compounds isolated were identified by a comparison of spectral data (NMR, IR, UV) using authentic compounds, and the purity of each isolated compound was confirmed to be more than 99%. Chemical structures of apicularens A and B are shown in Figure 1.

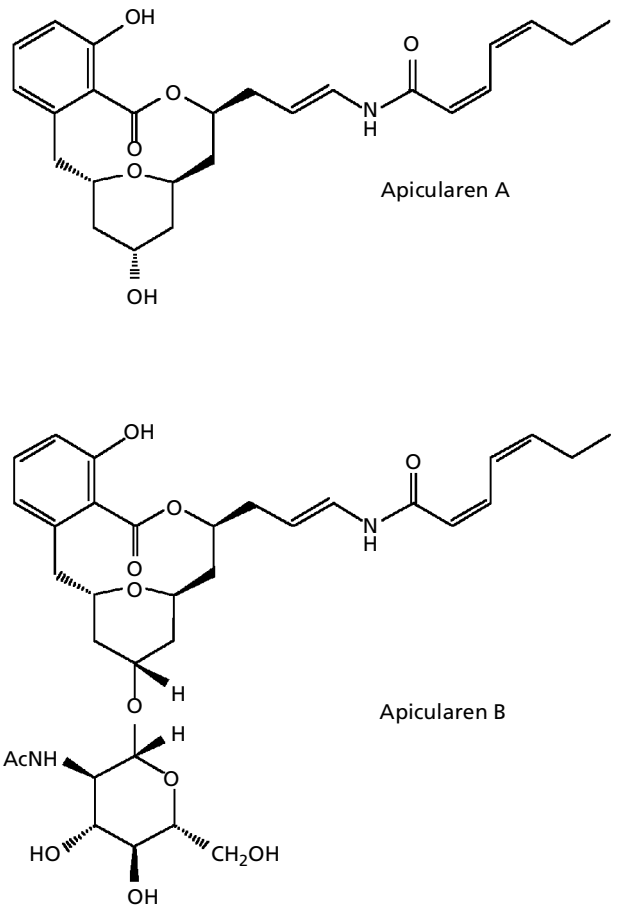


**Figure 1** Chemical structures of apicularens A and B.

### Cell culture

RAW 264.7 cells (RIKEN Gene Bank, Tsukuba, Japan) were cultured in Eagle's minimal essential medium (EMEM) (Nissui Seiyaku, Tokyo, Japan) containing 10% (v/v) foetal bovine serum (FBS, Dainippon Pharmaceutical, Osaka, Japan) and 1% (v/v) non-essential amino acid solution (Sigma Chemical Co., St Louis, MO). The cells were incubated at 37 °C under 5% $CO_2$–95% air.

### Cell growth and survival assay

The cell growth and survival were assessed by a procedure using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma), which is based on the ability of glycolytic pathway enzyme to cleave MTT to the blue compound formazan (Berridge & Tan 1993; Liu et al 1997). RAW 264.7 cells ($1 \times 10^4$ cells) were incubated at 37 °C for specified periods in 100 $\mu$L medium containing drugs in each well of a 96-well plate (Nalge Nunc International, Roskilde, Denmark). After three washes with phosphate-buffered saline (PBS, pH 7.4), 10 $\mu$L MTT solution in PBS (5 mg mL$^{-1}$) was added to each well, and cells were incubated at 37 °C for a further 4 h. After 100 $\mu$L 0.04 M HCl solution in isopropanol was added, the cells were dissolved by pipetting and the resultant coloured product was read on a Microplate Reader (Bio-Rad, Richmond, CA) at 570 nm.

### Detection of DNA fragmentation by electrophoresis

RAW 264.7 cells ($5.0 \times 10^6$ cells) were incubated at 37 °C for 24 h in 10 mL medium in 100-mm dishes (Corning Coster Japan, Tokyo, Japan) in the presence or absence of drugs. After the incubation, cells were washed twice with PBS, 0.5 mL DNA extraction buffer (10 mM EDTA-2Na, 50 mM Tris-HCl, 1% (w/v) sodium dodecylsulfate and 100 $\mu$g mL$^{-1}$ proteinase K (Sigma), pH 8.0) was added, and the cells were incubated at 55 °C for 16 h. DNA was extracted twice with phenol/chloroform/isoamylalcohol (25:24:1, v/v), and precipitated with a 0.1 vol 3 M sodium acetate and 2 vol ethanol at −20 °C. Precipitated DNA was dissolved in TE buffer (1 mM Tris-HCl and 0.1 mM EDTA-2Na, pH 7.5) containing

$20\,\mu g\,mL^{-1}$ ribonuclease I (Sigma), and incubated at 37 °C for 16 h. Electrophoresis of DNA was performed in a 1.4% (w/v) agarose gel at 50 V for 3 h. After the electrophoresis, DNA was visualized by ethidium bromide staining and photographed.

### Measurement of DNA fragmentation by flow-cytometry

RAW 264.7 cells ($1 \times 10^6$ cells) in each well of the 12-well plate (Corning Coster Japan) were incubated at 37 °C for specified periods with or without the drugs. After the incubation, the cells were washed three times with PBS and detached from the plate using 0.02% (w/v) EDTA in PBS. The cells were then fixed with 70% (v/v) ethanol at −20 °C for 3 h, and incubated in PBS containing $50\,\mu g\,mL^{-1}$ propidium iodide (Wako Pure Chemical Inc.) and $20\,\mu g\,mL^{-1}$ ribonuclease I (Sigma) at 37 °C for 5 min. Subsequently, the fluorescence intensity of propidium iodide in 10 000 cells was analysed by flow-cytometry using FACScan (Becton Dickinson, San Jose, CA). The percentage of sub-$G_1$ cells was calculated using CELLQuest Software (Becton Dickinson).

### Measurement of annexin V binding by flow-cytometry

For measurement of annexin V binding, RAW 264.7 cells ($1 \times 10^6$ cells per 1.5 mL tube (Corning Coster Japan)) were incubated at 37 °C for 3.25 h in medium in the presence or absence of drugs. After incubation, the cells were washed three times with PBS and stained by propidium iodide and Annexin-V-FLUOS according to a manufacturer's protocol using Annexin-V-FLUOS Staining Kit (Roche Diagnostics GmbH, Mannheim, Germany). Subsequently, the fluorescence intensities of propidium iodide and Annexin-V-FLUOS in 10 000 cells were analysed by flow-cytometry using FACScan (Becton Dickinson).

### Western blotting analysis of poly(ADP-ribose) polymerase (PARP) and mitogen-activated protein kinases

RAW 264.7 cells ($4 \times 10^5$ cells) in 60-mm dishes (Corning Coaster Japan) were incubated at 37 °C for specified periods with or without the drugs. After incubation, the cells were washed three times with PBS, lysed in ice-cold lysis buffer (20 mM HEPES, 1% (v/v) Triton X-100, 1 mM EDTA, 50 mM NaF, 2.5 mM *p*-nitrophenyl phosphate, 1 mM $Na_3VO_4$, $10\,\mu g\,mL^{-1}$ leupeptin and 10% (v/v) glycerol, pH 7.3), and sonicated using a Handy Sonic Disrupter (Tomy Seiko Co., Tokyo, Japan). After centrifugation at 15 000 *g* and 4 °C for 5 min, the protein content of the supernatant fraction was determined as described by Bradford (1976). The same amount of protein was loaded in each well of a SDS-polyacrylamide gel, which underwent electrophoresis and was then transferred onto a nitrocellulose membrane (Schleicher & Schuell GmbH, Dassel, Germany). Immunoblotting was carried out by using antibodies to PARP (Santa Cruz Biotechnology Inc., Santa Cruz, CA), phosphorylated-p44/42 MAPK (New England Biolabs Inc., Beverly, MA), rat MAP kinase R2 (erk1-CT) (Upstate Biotechnology Inc., Lake Placid, NY), and phospho-p38 (New England Biolabs Inc.) and p38 (Santa Cruz).

### Statistical analysis

The results are expressed as the means ± s.e.m. from at least four samples. Statistical analysis was performed by Kruskal-Wallis test and Nemenyi's test.

## Results

### Effects of apicularens A and B on cell growth and survival

RAW 264.7 cells were incubated with apicularen A at concentrations of 0.1–1000 nM for various periods, and the effect on the cell growth and survival was determined by the MTT assay. As shown in Figure 2A, at 18 h and thereafter apicularen A at 10 nM and above significantly decreased cell growth and survival. At 24 h, the effect of apicularen A was concentration-dependent from 10 to 1000 nM (Figure 2B), and the $IC_{50}$ value was calculated to be 600 nM. The effect of apicularen A was 10–100-times that of apicularen B (Figure 2B). Changes in the number of adherent cells 24 h after incubation almost paralleled those of the values obtained by the MTT assay (data not shown).

### Induction of apoptosis by apicularen A

To analyse that the decrease in cell growth and survival by apicularen A determined by the MTT assay was due to the induction of apoptosis, DNA fragmentation was examined by flow-cytometric analysis. Consistent with the result of the MTT assay, apicularen A (100 nM) increased the percentage of sub-$G_1$ cells from 18 h (Figure 3A), indicating that apicularen A (100 nM) induced DNA fragmentation in RAW 264.7 cells. The percentage of sub-$G_1$ cells at 24 h was increased in a concentration-dependent manner at 10–1000 nM apicularen A (Figure 3B). DNA fragmentation was also detected by electrophoresis after 24-h incubation with 100 nM apicularen A (Figure 3C). In addition, double staining of the cells with Annexin-V-FLUOS and propidium iodide after 12-h incubation revealed that apicularen A (100 nM) increased annexin-V-FLUOS-positive but propidium iodide-negative cells (LR quadrant in Figure 3D), which were in the early stage of apoptosis.

### Induction of caspase activation by apicularen A

Western blotting analysis revealed that apicularen A (100 nM) induced the cleavage of 115 kDa poly (ADP-ribose)

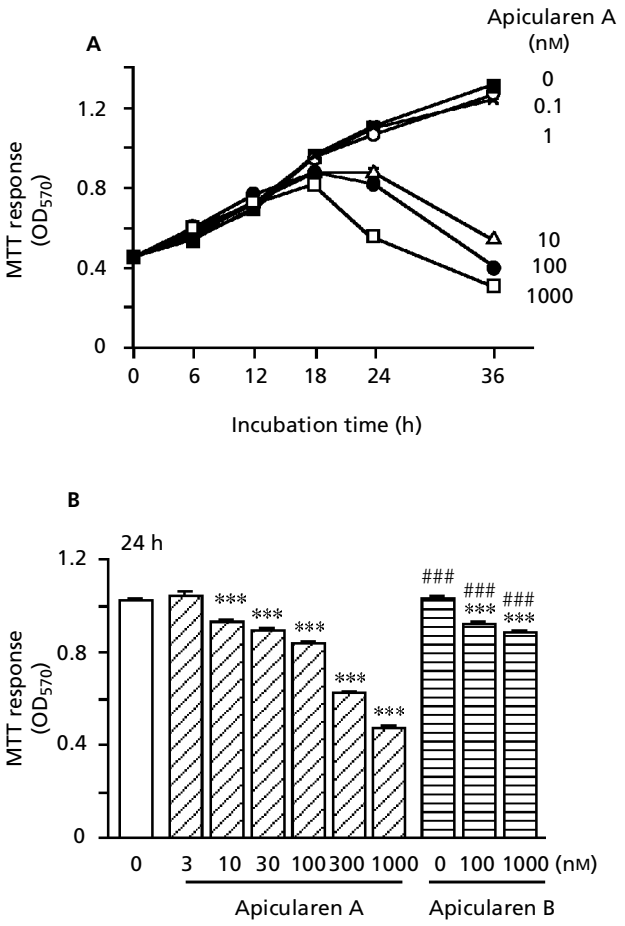


**Figure 2** Effects of apicularens A and B on cell growth and survival in RAW 264.7 cells. A. RAW 264.7 cells ($1 \times 10^4$ cells) were incubated at 37 °C for the periods indicated in the presence of the indicated concentrations of apicularen A. B. RAW 264.7 cells ($1 \times 10^4$ cells) were incubated at 37 °C for 24 h in the presence of the indicated concentrations of apicularen A or B. The cell growth and survival were determined by the MTT assay. The value at 0 h for the control group in B was $0.42 \pm 0.02$. Values are the means from four wells, with s.e.m. shown by vertical bars. In A s.e.m. bars are within the symbols. The results were confirmed by repeating two additional independent sets of experiments. $^{***}P < 0.001$ compared with control; $^{###}P < 0.001$ compared with corresponding concentrations of apicularen A.

polymerase (p115 PARP) and generated the 25 kDa protein (p25 PARP), a cleaved form of p115 PARP, at 18 h and thereafter (Figure 4).

### Effects of apicularen A on phosphorylation of p44/42 MAPK and p38 MAPK

On treatment with apicularen A (100 nM), significantly higher phosphorylation of p44/42 MAPK was induced at 3–24 h (Figure 5). Significantly higher phosphorylation of p38 MAPK was detected at 3–24 h (Figure 5).

### Effects of MAPK inhibitors on the apicularen A-induced decrease in cell growth and survival

Effects of the p44/42 MAPK inhibitor PD98059 and the p38 MAPK inhibitor SB203580 were examined to analyse the role of p44/42 MAPK and p38 MAPK in apicularen A-induced decrease in cell growth and survival. To obtain clear effects of these inhibitors, the concentration of apicularen A was set to 1000 nM. As shown in Figure 6, the effect of apicularen A (1000 nM) at 16 h was ameliorated by PD98059 in a concentration-dependent manner, with an almost complete recovery at 30 $\mu$M. Western blot analysis demonstrated that PD98059 at 30 $\mu$M almost completely suppressed the apicularen A-induced phosphorylation of p44/42 MAPK (data not shown). The effect of PD98059 weakened with time from 20 to 24 h (Figure 6). PD98059 by itself decreased cell growth and survival in a concentration-dependent manner at 20 and 24 h (Figure 6). In contrast, the apicularen A (1000 nM)-induced decrease in cell growth and survival was enhanced by SB203580 at 16, 20 and 24 h in a concentration-dependent manner, at which concentrations SB203580 by itself showed no effect (Figure 7). Western blot analysis demonstrated that 10 $\mu$M SB203580 almost completely suppressed the apicularen A-induced phosphorylation of 38 MAPK (data not shown).

## Discussion

In RAW 264.7 cells, the growth and survival as assessed by the MTT assay were decreased by apicularen A at 24-h incubation (Figure 2A), suggesting that apicularen A was cytotoxic to RAW 264.7 cells. The decrease was apparent at 36-h incubation, but during the 12-h incubation period no significant decrease was observed (Figure 2A). Apicularen B, an *N*-acetyl-glucosamine glycoside of apicularen A, was 10–100-fold less cytotoxic than apicularen A (Figure 2B). The difference in the cytostatic activity of apicularens A and B has been shown in human cancer cell lines also (Kunze et al 1998). The relatively weak cytotoxic activity of apicularen B might be explained by the glycosylation of apicularen A. We have reported that the glycosylation of isoflavones reduces the inhibitory effect of isoflavones on 12-*O*-tetradecanoylphorbol 13-acetate-induced production of prostaglandin $E_2$ in rat peritoneal macrophages (Yamaki et al 2002b). The mechanism by which glycosylation affected this biological activity remains to be elucidated.

Consistent with the result of the MTT assay, fragmentation of DNA was induced by 100 nM apicularen A at 24 h (Figure 3C). Microscopic observations of RAW 264.7 cells 24 h after incubation revealed that apicularen A 100 nM induced condensation and localization of nuclei with decreased number of cells, but apicularen B 100 nM did not show such morphological changes (figure not shown). As an index of apoptosis (Telford et al 1992), apicularen A increased the percentage of sub-$G_1$ cells (Figure 3A, B) and the population of Annexin-V-FLUOS-stained and propidium iodide-nonstained cells

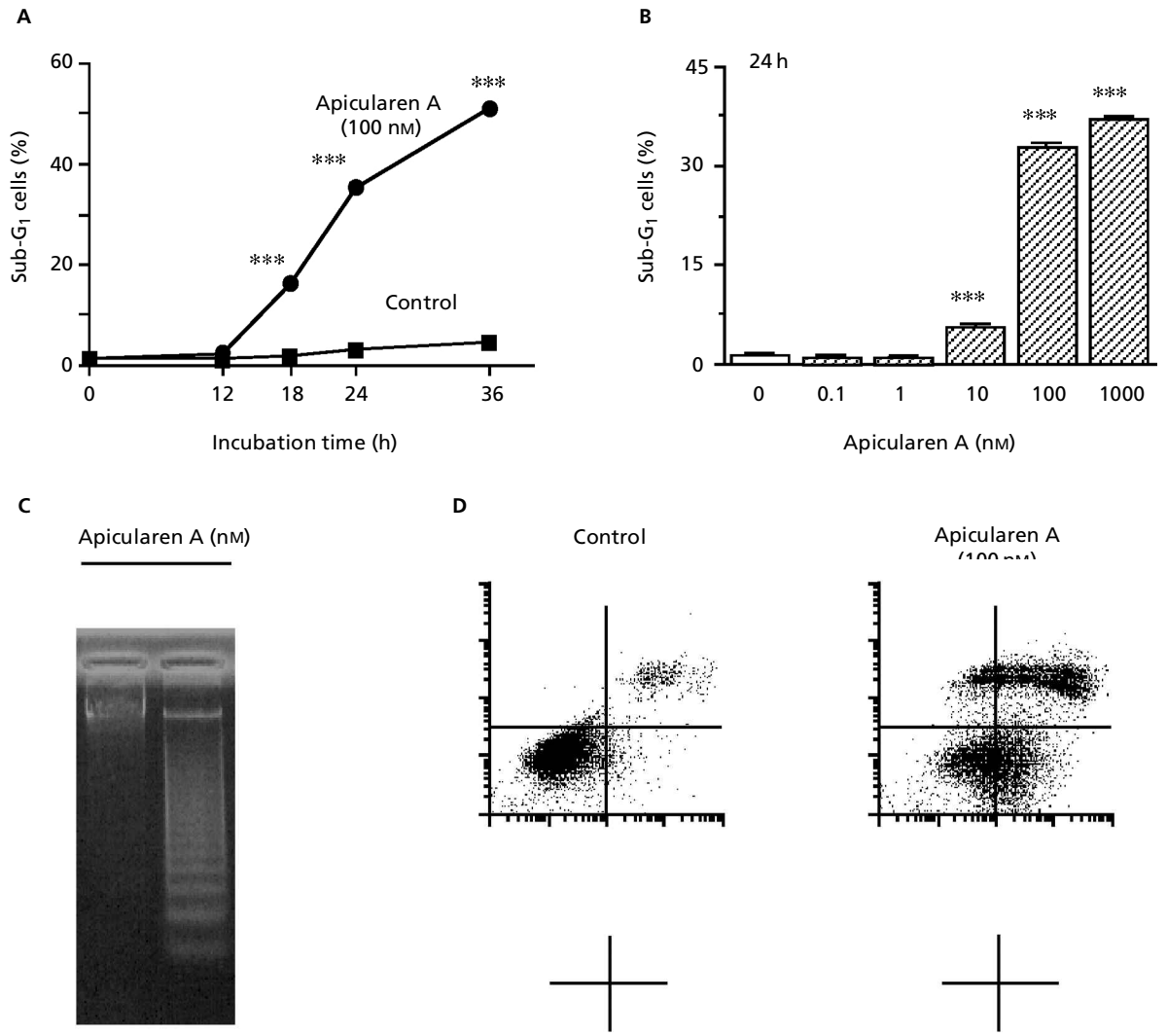


**Figure 3** Effects of apicularen A on the percentage of sub-$G_1$ cells and the percentage of cells stained with Annexin-V-FLUOS and propidium iodide. A. RAW 264.7 cells ($2 \times 10^5$ cells) were incubated at 37 °C for the periods indicated in the presence or absence of apicularen A (100 nM). B. RAW 264.7 cells ($2 \times 10^5$ cells) were incubated at 37 °C for 24 h in the presence of the indicated concentrations of apicularen A. DNA fluorescence was analysed by flow-cytometry, and the percentage of sub-$G_1$ cells was calculated using LISYS II software. Values are the means from four experiments with s.e.m. shown by vertical bars. In A, s.e.m. bars are within the symbols. ***$P < 0.001$ compared with control. C. RAW 264.7 cells ($1 \times 10^6$ cells) were incubated at 37 °C for 24 h in the presence or absence of apicularen A (100 nM). DNA was then extracted, underwent electrophoresis and was visualized by ethidium bromide staining. The experiment was repeated three times with similar results. D. RAW 264.7 cells ($1 \times 10^6$ cells) were incubated at 37 °C for 3.25 h in the presence or absence of apicularen A (100 nM). After incubation, cells were stained with Annexin-V-FLUOS and propidium iodide for 15 min and the fluorescence of the cells was analysed by FACScan. The percentage of the cells in quadrants was calculated by CELLQuest Software. The experiment was repeated three times with similar results. UL, upper left quadrant (not cells); UR, upper right quadrant (late apoptotic and necrotic cells); LL, lower left quadrant (viable cells); LR, lower right quadrant (early apoptotic cells).

(Figure 3D). These findings indicated further that apicularen A induced apoptosis in RAW 264.7 cells. It has been reported that the structurally-related macrolides erythromycin and azithromycin induced apoptosis of neutrophils (Aoshiba et al 1997; Koch et al 2000).

To determine whether apicularen A induced apoptosis via the caspase cascade, the activation of caspase was examined by determining the degradation of PARP as a substrate of caspase (Messmer et al 1999). As shown in Figure 4, it was demonstrated that apicularen A induced the cleavage of PARP. These findings supported the view that apicularen A induced apoptosis via the activation of the caspase cascade in RAW 264.7 cells.

As to the role of MAPK in apoptosis, Chin et al (1999) reported that the apoptosis induced by serum deprivation in RAW 264.7 cells was reversed by transforming growth

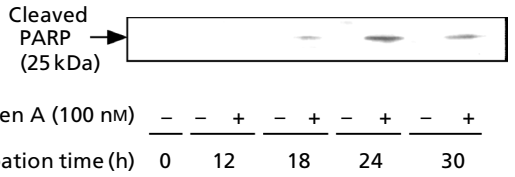


**Figure 4** Effects of apicularen A on cleavage of PARP. RAW 264.7 cells ($4 \times 10^5$ cells) were incubated at 37 °C for the periods indicated in the presence (+) or absence (−) of apicularen A (100 nM). After incubation, total cell lysates underwent electrophoresis and the cleaved form of PARP (25 kDa) was detected by Western blotting. The result shown is representative of three separate experiments.

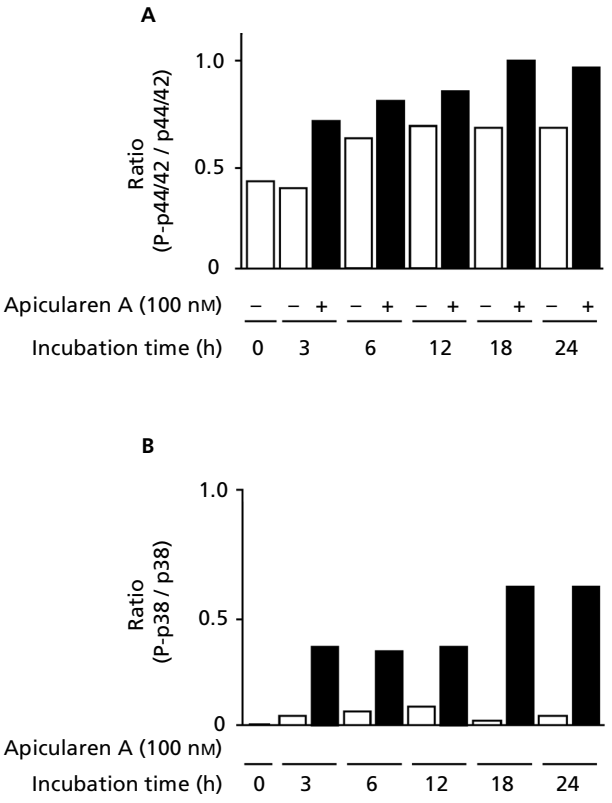


**Figure 5** Effects of apicularen A on phosphorylation of p44/42 MAPK and p38 MAPK. RAW 264.7 cells ($4 \times 10^5$ cells) were incubated at 37 °C for the periods indicated in the presence (+) or absence (−) of apicularen A (100 nM). After incubation, total cell lysates underwent electrophoresis, p44/42 MAPK, phosphorylated p44/42 MAPK (P-p44/42 MAPK), p38 MAPK and phosphorylated p38 MAPK (P-p38 MAPK) were detected by Western blotting, and the density ratios of P-p44/42 MAPK to p44/42 MAPK (A) and P-p38 MAPK to p38 MAPK (B) were calculated. The experiment was repeated three times with similar results, and one representative result is shown. The density ratios at 3 to 24 h in A and B are significantly higher in the presence of apicularen A than in its absence ($P < 0.001$). In A, the density ratios at 6 to 24 h are significantly higher than those at 0 and 3 h ($P < 0.001$).

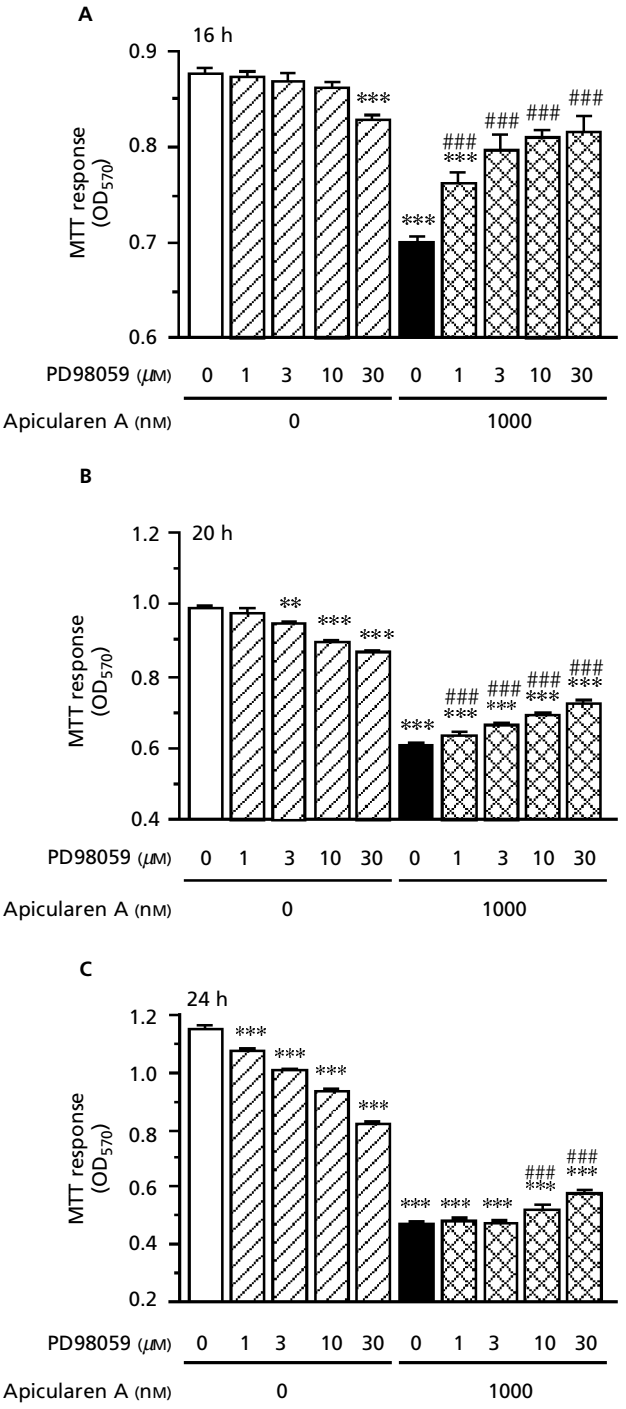


**Figure 6** Effects of PD98059, a MEK inhibitor, on cell growth and survival in RAW 264.7 cells. RAW 264.7 cells ($1 \times 10^4$ cells) were incubated at 37 °C for 16 h (A), 20 h (B) and 24 h (C) in the presence or absence of apicularen A (1000 nM) and the indicated concentrations of PD98059. The cell growth and survival were determined by the MTT assay. Values are the means from four wells with s.e.m. shown by vertical bars. The results were confirmed by repeating two additional independent sets of experiments. $^{**}P < 0.01$, $^{***}P < 0.001$ compared with control; $^{\#\#\#}P < 0.001$ compared with apicularen A (1000 nM) alone.

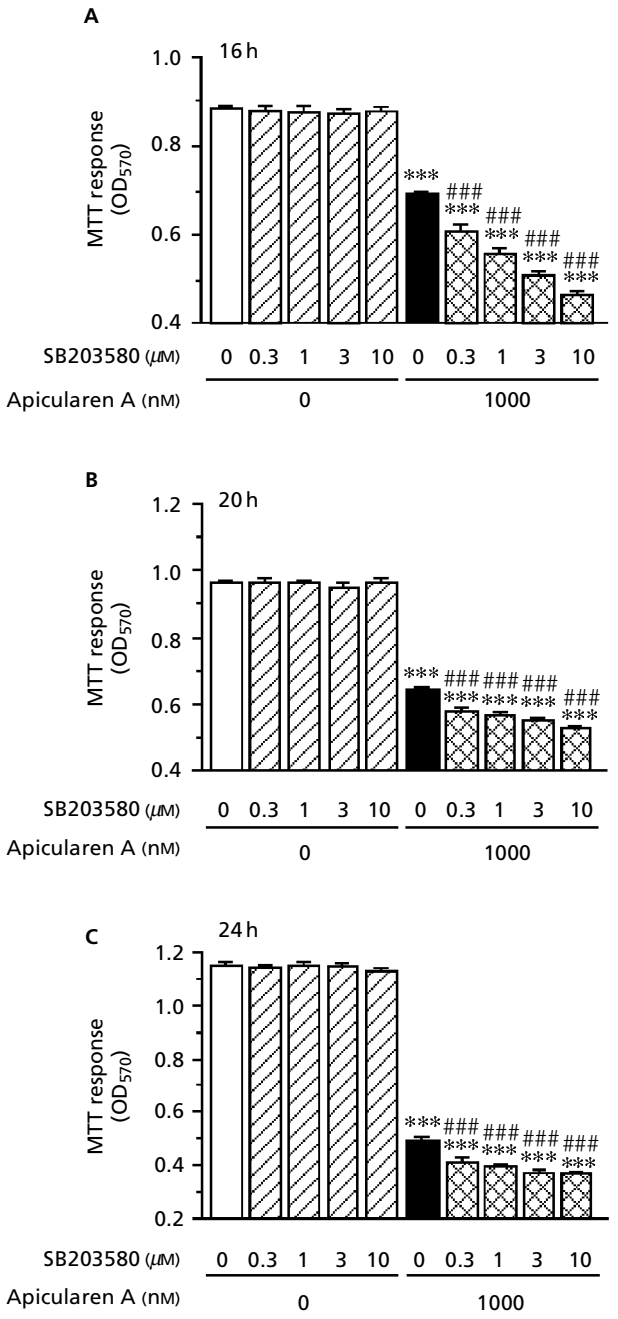


**Figure 7** Effects of SB203580, a p38 MAPK inhibitor, on cell growth and survival in RAW 264.7 cells. RAW 264.7 cells ($1 \times 10^4$ cells) were incubated at 37 °C for 16 h (A), 20 h (B) and 24 h (C) in the presence or absence of apicularen A (1000 nM) and the indicated concentrations of SB203580. The cell growth and survival were determined by the MTT assay. Values are the means from four wells with s.e.m. shown by vertical bars. The results were confirmed by repeating two additional independent sets of experiments. $^{***}P < 0.001$ compared with control; $^{###}P < 0.001$ compared with apicularen A (1000 nM) alone.

factor β1 (TGF-β1) through activation of p44/42 MAPK. Overexpression of a constitutively activated MEK in Rat-1 fibroblast cell lines inhibited the release of cytochrome C from mitochondria, stopped the activation of caspase, and prevented apoptosis after growth factor deprivation (Erhardt et al 1999). In addition, activation of MEK, upstream of p44/42 MAPK, inactivated the pro-apoptotic Bcl-2 family protein Bad in haemopoietic cells (Scheid & Duronio 1998). These reports indicated that p44/42 MAPK activated by survival enhancing factors including serum and TGF-β1 was anti-apoptotic. It was reported that activation of p44/42 MAPK was antagonistic to trichothecene-induced apoptosis in RAW 264.7 cells (Yang et al 2000). On the other hand, p44/42 MAPK activated directly by apoptosis-inducing agents such as nitric oxide (Mohr et al 1998) and TNF-α (Chin et al 1998) and by hyperoxia (Petrache et al 1999) had pro-apoptotic activity in RAW 264.7 cells.

In this study, the phosphorylation of p44/42 MAPK in the absence of apicularen A in RAW 264.7 cells (Figure 5) might have been caused by serum stimulation as reported by Flamigni et al (2001) in human ECV304 cells. This serum-induced phosphorylation of p44/42 MAPK was promoted by apicularen A (Figure 5), indicating that apicularen A activated p44/42 MAPK in RAW 264.7 cells. Because the apicularen A-induced decrease in cell growth and survival determined by the MTT assay was attenuated by the MEK inhibitor PD98059 (Figure 6), the activation of the p44/42 MAPK pathway was suggested to positively regulate the apoptosis. The attenuation of the apicularen A-induced decrease in cell growth and survival by PD98059 was most prominent at 16-h incubation (Figure 6). This suggested that the influence of the activation of p44/42 MAPK on the apicularen A-induced apoptosis was limited to a certain period after the exposure to apicularen A.

In contrast to the possible pro-apoptotic function of the activation of p44/42 MAPK in apicularen A-induced apoptosis in RAW 264.7 cells, the activation of p38 MAPK by apicularen A (Figure 5) was suggested to have an anti-apoptotic effect, because the p38 MAPK inhibitor SB203580 enhanced the apicularen A-induced decrease in cell growth and survival (Figure 7). An anti-apoptotic function of activated p38 MAPK was observed in staurosporine-induced apoptosis in RAW 264.7 cells (Yamaki et al 2002a). It was reported that TNF-α-induced apoptosis in NIH3T3 cells was augmented by SB203580 (Roulston et al 1998). However, in RAW 264.7 cells, it was reported that the inhibition of p38 MAPK by SB203580 partially reduced sodium nitroprusside-induced apoptosis (Jun et al 1999), but had no effect on *S*-nitrosoglutathione-induced apoptosis (Callsen & Brune 1999). In U-937 promonocytic cells, SB203580 inhibited cadmium-induced apoptosis (Gálan et al 2000). Thus, the role of p38 MAPK in apoptosis seems to vary with the type of inducer and cell. It has been reported that the apoptotic process is regulated by PKC (Jun et al 1999), phosphatidylinositol 3-kinase (PI3K) (Koh et al 1998) and c-Jun *N*-terminal kinase (JNK) (Xia et al 1995). Therefore, the contribution of PKC, PI3K and JNK in apicularen A-induced apoptosis in RAW 264.7 cells remains to be elucidated.

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