JPP Journal of Pharmacy and Pharmacology

JPP 2002, 54: 257–262

Received March 13, 2001
Accepted August 15, 2001
ISSN 0022-3573

Potent apoptotic effects of saponins from Liliaceae plants in L1210 cells

E. Candra, K. Matsunaga, H. Fujiwara, Y. Mimaki, M. Kuroda, Y. Sashida and Y. Ohizumi

Abstract

We isolated eight saponins, a hexacyclic lanosterol tetraglycoside (**1**), a 27-norlanosterol tetraglycoside (**2**) and six spirostanol oligoglycosides (**3**–**8**), from the plants of the family Liliaceae. In murine leukaemic L1210 cells, saponins **5** and **7** at a concentration of 1 μM showed potent cytotoxic activity and the activities were in the following decreasing order: **5**, **7**, **1**, **3**, **2**, **8**, **4**, **6**. At a concentration of 10 μM, not only **5** and **7** but also **3** and **8** markedly caused cell death. The flow cytometric analysis indicated that **7** and **8** caused a concentration- and time-dependent apoptosis of L1210 cells (EC50 value = $\sim$ 5 μM). The morphological observation using a light microscope revealed that both **7** and **8** induced shrinkage in cell soma and chromatin condensation, suggesting apoptotic cell death. Moreover, in agarose gel electrophoretic analysis, a typical apoptotic DNA ladder pattern was observed after treatment with both **7** and **8**. These results suggest that **7** and **8** caused the death of L1210 cells through the apoptotic process. These compounds may become powerful pharmacological tools for studying the molecular mechanism of apoptosis.

Department of Pharmaceutical Molecular Biology, Graduate School of Pharmaceutical Sciences, Tohoku University, Aoba, Aramaki, Aoba-ku, Sendai 980-8578, Japan

E. Candra, K. Matsunaga, H. Fujiwara, Y. Ohizumi

School of Pharmacy, Tokyo University of Pharmacy and Life Science, Hachioji, Tokyo 192-0392, Japan

Y. Mimaki, M. Kuroda, Y. Sashida

Correspondence: Y. Ohizumi, Department of Pharmaceutical Molecular Biology, Graduate School of Pharmaceutical Sciences, Tohoku University, Aoba, Aramaki, Aoba-ku, Sendai 980-8578, Japan

Acknowledgments and Funding: The authors thank Dr Tohru Yamakuni of the Graduate School of Pharmaceutical Sciences, Tohoku University, for useful advice and helpful discussion. We also thank Professor Kazuo Sugamura and Dr Naoto Ishii of Tohoku University Graduate School of Medicine for encouragement. This work was partially supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

Introduction

A complex interplay of intracellular signals and metabolic process is involved in the regulation of cell death (Orrenius 1995). Two principal patterns of cell death have been described: necrosis and apoptosis. Necrosis is associated with inflammation, whereas apoptosis is a selective process of physiological cell deletion that plays an important role in the balance between cell proliferation and death. Apoptosis is characterized by cell shrinkage, membrane blebbing, chromatin condensation and formation of a DNA ladder of multiples of 180 base pairs caused by internucleosomal DNA cleavage (Kerr et al 1972; Steller 1995). Since it has recently been reported that cancer chemotherapeutics exert part of their pharmacological effect by triggering apoptotic cell death, apoptosis-inducing compounds in tumour cells have become useful as leading compounds for the development of anticancer drugs.

Numerous natural products have been used as pharmacological tools for pharmacological, physiological and biochemical studies (Ohizumi 1997). In our screening programme for pharmacologically active substances from natural resources, much attention has been given to compounds inducing apoptosis because of their important roles in the development of new types of anticancer drugs and in the basic study of signal transduction (Kugawa et al 1998). Liliaceae plants are known as a rich source of saponins (Mahato et al 1982). It has been reported

that saponins possess diverse biological and pharmacological activities, such as antitumour activity and inhibitory activities of cyclic AMP phosphodiesterase and Na^+/K^+ ATPase (Nagumo et al 1991; Kuroda et al 1995; Mimaki et al 1994, 1995, 1999, 2000). Recently, we isolated eight saponins, a hexacyclic lanosterol tetraglycoside (**1**), a 27-norlanosterol tetraglycoside (**2**) and six spirostanol oligoglycosides (**3**–**8**), from Liliaceae plants (Figure 1). Ginsenoside saponins have been shown to inhibit tumour cell proliferation, invasion, metastasis and tumour growth (Iishi et al 1997; Nakata et al 1998) and induce differentiation and apoptosis (Kim et al 1999; Lee et al 2000). However, apart from ginsenosides there have been few reports on saponins having apoptotic activity. In this study we present the first paper on the evaluation of apoptotic effects of saponins from Liliaceae plants. Saponins **7** and **8**, which possess potent apoptotic activity, may become not only leading compounds for anticancer drugs but also useful pharmacological tools for studying the molecular mechanism of apoptosis.

Materials and Methods

Materials

Saponin **3** was isolated from the rhizomes of *Anemarrhena asphodeloides* purchased from a wholesale firm in Uchida-Wakanyaku (Tokyo, Japan). The rhizomes of *A. asphodeloides* (dry weight 250 g) were extracted with hot methanol (1 L) twice. The extract was concentrated under reduced pressure and the residue (74 g) was passed through a Diaion HP-20 (Mitsubishi-Kasei, Tokyo, Japan) column eluted with stepwise gradient mixtures of water/methanol (7:3; 1:1; 3:7), methanol, ethanol and finally ethyl acetate (each 1 L). The methanol eluate fraction (12 g), which contained steroidal saponins in abundance, was chromatographed over silica gel (Fiji-Silysia Chemicals, Aichi, Japan) eluted with trichloromethane/methanol/water (40:10:1) to give five fractions (I–V). Fraction II (3.4 g) was subjected to column chromatography over octadecylsilanized silica gel (Nacalai Tesque, Kyoto, Japan) with methanol/water (4:1) to yield **3** (459 mg) (Nagumo et al 1991). The structure of **3** was identified as that shown in Figure 1 by the comparison of its physical and nuclear magnetic resonance (NMR) spectral data with those reported in a previous paper (Nagumo et al 1991). The compounds **1**, **2**, **4**, **5**, **6**, **7** and **8** were isolated from *Scilla peruviana*, *Eucomis bicolor*, *Allium chinense*, *Allium jesdianum*, *Paris polyphylla* var. *chinensis* and *Tritelia lactea*, respectively, and were identified as previously reported (Mimaki et al 1994, 1995, 1999, 2000; Kuroda et al 1995). The purity of these compounds was confirmed by NMR analysis. The following reagents and serum were used: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Dojindo, Kumamoto, Japan), calcium ionophore A23187 (Sigma, St Louis, MO), proteinase K (Takara, Tokyo, Japan), RNase A (Sigma, St Louis, MO), fetal calf serum (FCS) (Cell Culture Laboratory, Cleveland, OH) and RPMI 1640 (Nissui Pharmaceuticals, Tokyo, Japan). All other reagents or drugs were of analytical grade.

Cell culture

L1210 and C6 cells were maintained in RPMI 1640 supplemented with 10% (v/v) FCS, 4 mM L-glutamine, 50 units mL^{-1} penicillin and 50 μg mL^{-1} streptomycin at 37°C in a 5% CO_2 and 95% air-humidified incubator.

Cell viability assay

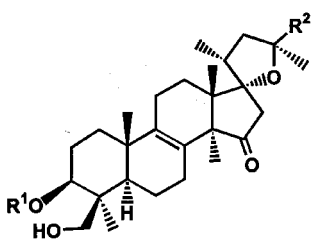
L1210 and C6 cells were seeded in 96-well plates at a density of 1×10^4 cells per well. After 24 h, cells were treated with saponins for 24 h, then 0.5 mg mL^{-1} MTT was added to each well and the cells were incubated at 37°C for 4 h. The plates were centrifuged at 170 *g* for 5 min and the medium was replaced with dimethylsulfoxide. After 15 min, the plates were read on the microplate reader (Model 450, Bio-Rad) at a test wavelength of 595 nm.

Flow cytometric analysis

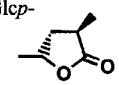
Apoptotic cells were detected according to the method of Darzynkiewicz et al (1992) with slight modifications. L1210 cells were seeded in 6-well plates at a density of 2×10^5 cells per well. After 24 h, the cells were treated with saponins at different concentrations and time periods as described below. Cells were washed with phosphate-buffered saline (PBS) and fixed with 70% ethanol at –20°C for 3 h. Cells were rinsed with PBS, incubated with 50 μg mL^{-1} RNase A at 37°C for 15 min and stained with 50 μg mL^{-1} propidium iodide for 10 min. Apoptotic analysis was performed using a flow cytometer (FACSCan and FACSCalibur, Becton Dickinson Immunocytometry Systems, Franklin Lakes, NJ).

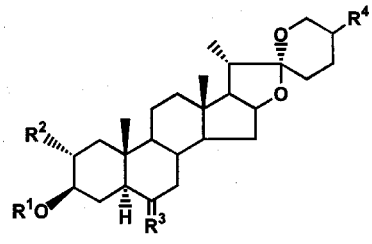
Morphological analysis

L1210 cells were seeded in 24-well plates at a density of 2.5×10^4 cells per well. After 24 h, the cells were treated with saponins for 24 h. The cells were fixed with 1% glutaraldehyde at 37°C for 15 min and rinsed with PBS.

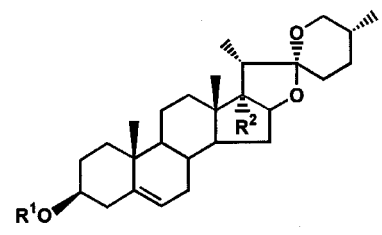


1: R^1 = α-L-Rha*p*-(1→2)-*O*-β-D-Glc*p*-(1→2)-*O*-α-L-Ara*p*-(1→6)-β-D-Glc*p*-
R^2 =



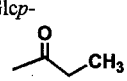


3: R^1 = β-D-Glc*p*-(1→2)-β-D-Gal*p*-
R^2 = H
R^3 = H_2
R^4 = β-CH_3



6: R^1 = α-L-Ara*f*-(1→4)-β-D-Glc*p*-
R^2 = OH

2: R^1 = α-L-Rha*p*-(1→2)-*O*-β-D-Glc*p*-(1→2)-*O*-α-L-Ara*p*-(1→6)-β-D-Glc*p*-
R^2 =



4: R^1 = β-D-Xyl*p*-(1→4)-*O*-[α-L-Ara*p*-(1→6)]-β-D-Glc*p*-
R^2 = H
R^3 = O
R^4 = α-CH_3

7: R^1 = α-L-Rha*p*-(1→2)-*O*-[β-D-Gal*p*-(1→3)]-β-D-Glc*p*-
R^2 = H

5: R^1 = β-D-Glc*p*-(1→2)-*O*-[β-D-Xyl*p*-(1→3)]-*O*-β-D-Glc*p*-(1→4)-β-D-Gal*p*-
R^2 = OH
R^3 = H_2
R^4 = α-CH_3

8: R^1 = α-L-Rha*p*-(1→2)-*O*-[β-D-Gal*p*-(1→3)]-β-D-Glc*p*-
R^2 = OH

Figure 1 Chemical structures of saponins.

The morphology of the cells was examined using a phase-contrast microscope (IMT-2, Olympus, Tokyo, Japan).

DNA fragmentation analysis

DNA analysis was carried out as previously reported (Fujiwara et al 2000). Briefly, L1210 cells were seeded in 6-well plates at a density of 2×10^5 cells per well and were treated with saponins for 24 h. Cells were washed with PBS and resuspended in cell lysis buffer at 50°C for 2 h. DNA was precipitated in ethanol, rinsed with 70% ethanol and dissolved in tris-EDTA buffer. The DNA preparation was incubated with RNase A. Electrophoresis was carried out in a 2% (w/v) agarose gel at 50 V for 90 min in tris-borate-EDTA buffer. The gel was stained with ethidium bromide and visualized under UV light.

Statistical analysis of the data

The data are expressed as means ± s.e.m. Statistical comparisons were made by using Student's *t*-test. $P < 0.05$ was considered significant.

Results and Discussion

Apoptosis is a mechanistically driven form of cell death that is either developmentally regulated or activated in response to specific stimuli or various forms of cell injury. There are three criteria for judging apoptosis: morphology of cells, detection of DNA fragmentation and subdiploid DNA content measured by flow cytometry. Apoptosis can be induced by various intracellular signals, including growth factor deprivation (Araki et al 1990) and activation of cytokine receptor (Laster et al 1988; Nagata & Golstein 1995). Several compounds have been reported to cause apoptosis by enhancing or suppressing these signals (Muthukkumar et al 1995; Yao & Cooper 1995; Stefanis et al 1999).

There have been few reports on the apoptotic activity of saponins, apart from for ginsenosides. In the present experiment the cytotoxic and apoptotic effects of saponins from Liliaceae plants were examined. The effects of eight saponins from Liliaceae plants on the viability of L1210 cells are summarized in Figure 2A. At a concentration of 1 μM, **5** and **7** showed potent cytotoxicity and the other six saponins weakly induced cell

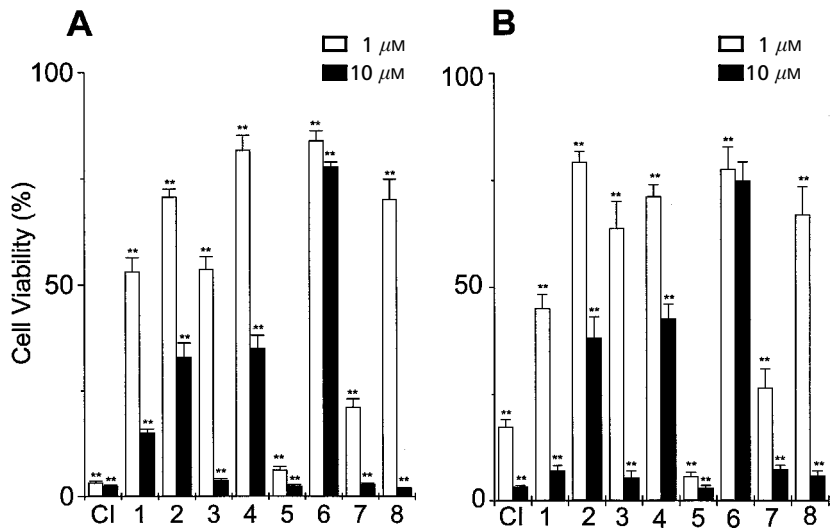


Figure 2 Effect of saponins on the viability of L1210 cells (A) and C6 cells (B). Cells were treated as described in Materials and Methods. L1210 and C6 cells were treated with 1 μM and 10 μM saponins. Cell viability is expressed as percentage against the control viability (100%) in the absence of saponins. Values represent means±s.e.m. from five independent experiments. Calcium ionophore A23187 (CI) was used as a positive control. $*P < 0.05$ and $**P < 0.01$ compared with the control viability.

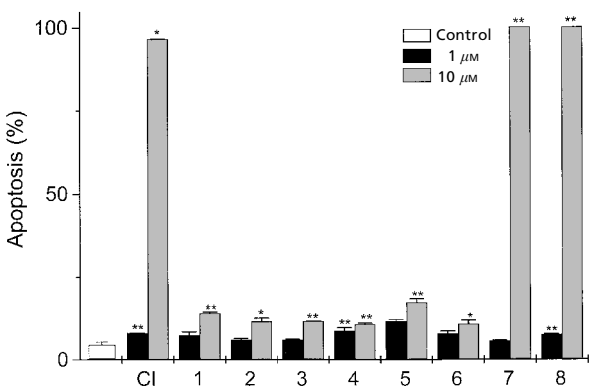


Figure 3 Flow cytometric analysis of the apoptotic effect of saponins. L1210 cells were treated with 1 μM and 10 μM saponins for 24 h. Cells with apoptotic DNA were quantified by propidium iodide staining and flow cytometric analysis. Apoptosis is expressed as the percentage of the number of apoptotic cells to the total number of cells. Values represent means±s.e.m. from three independent experiments. Calcium ionophore A23187 (CI) was used as a positive control. $*P < 0.05$ and $**P < 0.01$ compared with the control.

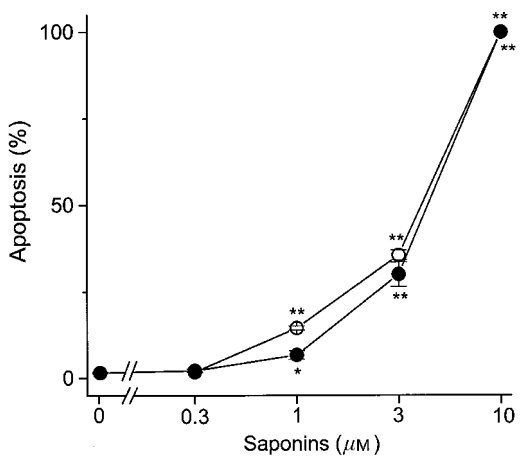


Figure 4 Concentration-dependent increase in apoptosis induced by **7** and **8** in L1210 cells. Cells were treated with several concentrations of **7** (○) and **8** (●) for 24 h. Apoptosis is expressed as the percentage of the number of apoptotic cells to the total number of cells. Values represent means±s.e.m. from three independent experiments. $*P < 0.05$ and $**P < 0.01$ compared with the control.

death. At a higher concentration (10 μM), potent cytotoxic effects were observed in four saponins: **3**, **5**, **7** and **8**. The cytotoxic potency of these saponins in C6 cells was also similar to that obtained from L1210 cells (Figure 2B). Examination of the cytotoxic effect of the saponins on normal cells is under way. The marked influence of saponins on the viability of L1210 cells led us to examine whether cell death by these compounds was a result of apoptosis. In L1210 cells the flow cytometric analysis showed that **7** and **8** (10 μM) caused marked apoptotic cell death (Figure 3). **7** or **8** caused concentration-dependent apoptosis in the range 0.3 to 10 μM with an EC50 value around 5 μM (Figure 4). Apoptosis was induced by **7** and **8** within 6 h and the apoptotic cells reached around 90 and 70% of the total cell number after 48 h in L1210 cells treated with **7** and

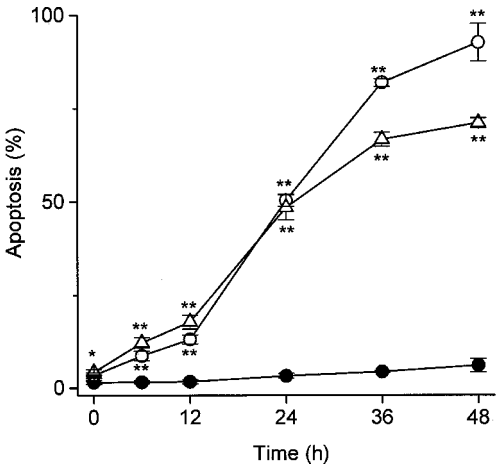


Figure 5 Time-dependent increase in apoptosis in the presence or absence of 5 μM of **7** and **8** in L1210 cells (●, control; ○, **7**; △, **8**). Apoptosis is expressed as the percentage of the number of apoptotic cells to the total number of cells. Values represent means±s.e.m. from three independent experiments. $*P < 0.05$ and $**P < 0.01$ compared with the control.

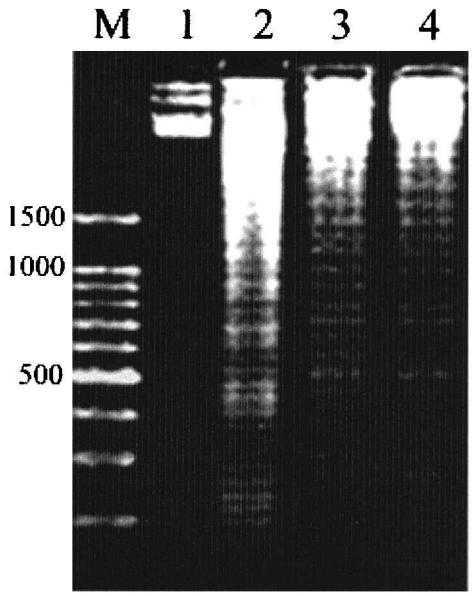


Figure 7 DNA fragmentation of L1210 cells. The 180 bp band can be seen on the agarose gel electrophoresis (M, marker; lane 2, 10 μM calcium ionophore A23187; lane 3, 10 μM **7**; lane 4, 10 μM **8**) while DNA from untreated cells remains intact (lane 1).

8, respectively (Figure 5). The apoptotic effects of both the compounds were almost equal, suggesting that a hydroxyl group at C-17 in the spirostanol moiety of **7** and **8** is not important for exerting apoptotic effect. Furthermore, **6**, which has a different structure to that of **8** in the triglycoside moiety, did not induce apoptosis, indicating that the triglycoside moiety in saponins that have a spirostanol part, such as **7** and **8**, plays an important role in apoptotic activity.

To characterize the mode of cell death more clearly, morphological change was observed by a light microscope and the apoptotic effects of these compounds on DNA were examined using agarose gel electrophoresis. **7**- and **8**-treated cells expressed the morphological characteristics of apoptosis, including chromatin condensation and cell shrinkage (Figures 6B and 6C) in contrast to the untreated L1210 cells (Figure 6A). An apoptotic DNA ladder was observed in the cells treated with 10 μM of **7** and **8** (Figure 7). We can confirm that compounds **7** and **8** apparently induced apoptosis using flow cytometry and morphological and DNA ladder experiments.

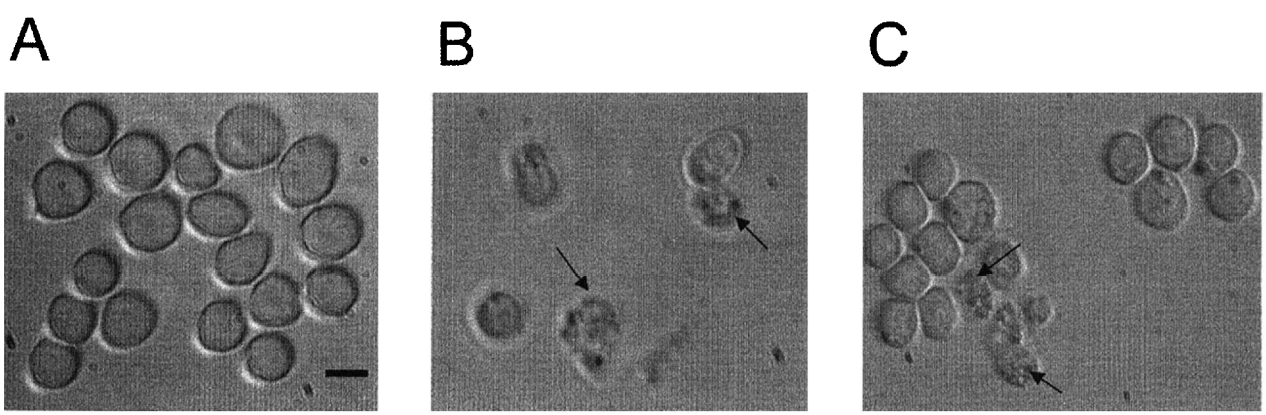


Figure 6 Morphological change. Phase-contrast views are shown in the absence (A) or presence of 10 μM of **7** (B) and **8** (C). Arrows indicate condensed and fragmented nuclei. Scale bar = 50 μm.

In conclusion, **7** and **8** caused the death of L1210 cells through an apoptotic mechanism. Both the compounds may provide valuable leading compounds for anticancer drugs, as well as being pharmacological tools for clarifying the apoptotic molecular mechanism.

References

Araki, S., Shimada, Y., Kaji, K., Hayashi, H. (1990) Apoptosis of vascular endothelial cells by fibroblast growth factor deprivation. *Biochem. Biophys. Res. Commun.* **168**: 1194–1200

Darzynkiewicz, Z., Bruno, S., Del bino, G., Gorczyca, W., Hotz, M. A., Lassota, P., Traganos, F. (1992) Features of apoptotic cells measured by flow cytometry. *Cytometry* **13**: 795–808

Fujiwara, H., Matsunaga, K., Kumagai, H., Ishizuka, M., Ohizumi, Y. (2000) Ophiobolin A, a novel apoptosis-inducing agent from fungus strain f-7438. *Pharm. Pharmacol. Commun.* **6**: 427–431

Iishi, H., Tatsuta, M., Baba, M., Uehara, H., Nakaizumi, A., Shinkai, K., Akedo, H., Funai, H., Ishiguro, S., Kitagawa, I. (1997) Inhibition by ginsenoside Rg3 of bombesin-enhanced peritoneal metastasis of intestinal adenocarcinomas induced by azoxymethane in Wistar rats. *Clin. Exp. Metas.* **15**: 603–611

Kerr, J. F. R., Wyllie, A. H., Currie, A. R. (1972) Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br. J. Cancer* **26**: 239–257

Kim, S. E., Lee, Y. H., Park, J. H., Lee, S. K. (1999) Ginsenoside-Rs4, a new type of ginseng saponin concurrently induces apoptosis and selectively elevates protein levels of p53 and p21WAF1 in human hepatoma SK-HEP-1 cells. *Eur. J. Cancer* **35**: 507–511

Kugawa, F., Arae, K., Ueno, A., Aoki, M. (1998) Buprenorphine hydrochloride induces apoptosis in NG 108-15 nerve cells. *Eur. J. Pharmacol.* **347**: 105–112

Kuroda, M., Mimaki, Y., Kameyama, A., Sashida, Y., Nikaido, T. (1995) Steroidal saponins from *Allium chinense* and their inhibitory activities on cyclic AMP phosphodiesterase and Na^+/K^+ ATPase. *Phytochemistry* **40**: 1071–1076

Laster, S. M., Wood, J. G., Gooding, L. R. (1988) Tumor necrosis factor can induce both apoptotic and necrosis forms of cell lysis. *J. Immunol.* **141**: 2629–2634

Lee, S. J., Ko, W. G., Kim, J. H., Sung, J. H., Moon, C. K., Lee, B. H. (2000) Induction of apoptosis by a novel intestinal metabolite of ginseng saponin via cytochrome c-mediated activation of caspase-3 protease. *Biochem. Pharmacol.* **60**: 677–685

Mahato, S. B., Ganguly, A. N., Sahu, N. P. (1982) Steroid saponins. *Phytochemistry* **21**: 959–978

Mimaki, Y., Nishino, H., Ori, K., Kuroda, M., Matsui, T., Sashida, Y. (1994) Lanosterol oligosaccharides from the plants of the subfamily Scilloideae and their tumor-promoter activity. *Chem. Pharm. Bull.* **42**: 327–332

Mimaki, Y., Nakamura, O., Sashida, Y., Nikaido, T., Ohmoto, T. (1995) Steroidal saponins from the bulbs of *Triteleia lactea* and their inhibitory activities on cyclic AMP phosphodiesterase. *Phytochemistry* **38**: 1279–1286

Mimaki, Y., Kuroda, M., Fukasawa, T., Sashida, Y. (1999) Steroidal glycosides from the bulbs of *Allium jesdianum*. *J. Nat. Prod.* **62**: 194–197

Mimaki, Y., Kuroda, M., Obata, Y., Sashida, Y., Kitahara, M., Yasuda, A., Naoi, N., Xu, Z. W., Li, M. R., Rao, A. N. (2000) Steroidal saponins from the rhizomes of *Paris polyphylla* var *chinensis* and their cytotoxic activity. *Nat. Prod. Lett.* **14**: 357–364

Muthukkumar, S., Nair, P., Sells, S. F., Maddiwar, N. G., Jacob, R. J., Rangnekar, V. M. (1995) Role of EGR-1 thapsigargin-inducible apoptosis in the melanoma cell line A375-C6. *Mol. Cell Biol.* **15**: 6262–6272

Nagata, S., Golstein, P. (1995) The Fas death factor. *Science* **267**: 1449–1456

Nagumo, S., Kishi, S., Inoue, T., Nagai, M. (1991) Saponins of Anemarrhenae rhizoma. *Yakugaku Zasshi* **111**: 306–310

Nakata, H., Kikuchi, Y., Tode, T., Hirata, J., Kita, T., Ishii, K., Kudoh, K., Nagata, I., Shinomiya, N. (1998) Inhibitory effects of ginsenoside Rh2 on tumor growth in nude mice bearing human ovarian cancer cells. *Jpn J. Cancer Res.* **89**: 733–740

Ohizumi, Y. (1997) Application of physiologically active substances isolated from natural resources to pharmacological studies. *Jpn J. Pharmacol.* **73**: 263–289

Orrenius, S. (1995) Apoptosis: molecular mechanisms and implications for human disease. *J. Intern. Med.* **237**: 529–536

Stefanis, L., Park, D. S., Friedman, W. J., Greene, L. A. (1999) Caspase-dependent and independent death of camptothecin-treated embryonic cortical neurons. *J. Neurosci.* **19**: 6235–6247

Steller, H. (1995) Mechanism and genes of cellular suicide. *Science* **267**: 1445–1449

Yao, R., Cooper, G. M. (1995) Requirement for phosphatidylinositol-3 kinase in the prevention of apoptosis by nerve growth factor. *Science* **267**: 2003–2006