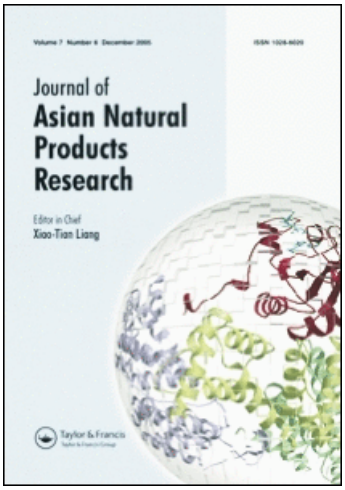

Journal of Asian Natural Products Research
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713454007

Atypical apoptosis in L929 cells induced by evodiamine isolated from *Evodia rutaecarpa*

Ying Zhang[a]; Qi-Hui Zhang[b]; Li-Jun Wu[b]; Shin-Ichi Tashiro[c]; Satoshi Onodera[c]; Takashi Ikejima[a]
[a] China-Japan Research Institute of Medical and Pharmaceutical Sciences, Shenyang Pharmaceutical University, China [b] Department of Phytochemistry, Shenyang Pharmaceutical University, Shenyang, China [c] Department of Clinical and Biomedical Sciences, Showa Pharmaceutical University, Tokyo, Japan

To cite this Article Zhang, Ying , Zhang, Qi-Hui , Wu, Li-Jun , Tashiro, Shin-Ichi , Onodera, Satoshi and Ikejima, Takashi(2004) 'Atypical apoptosis in L929 cells induced by evodiamine isolated from *Evodia rutaecarpa*', Journal of Asian Natural Products Research, 6: 1, 19 — 27
To link to this Article: DOI: 10.1080/1028602031000119772
URL: http://dx.doi.org/10.1080/1028602031000119772

Journal of Asian Natural Products Research, March 2004, VOL. 6 (1), pp. 19–27

ATYPICAL APOPTOSIS IN L929 CELLS INDUCED BY EVODIAMINE ISOLATED FROM *EVODIA RUTAECARPA*

YING ZHANG[a], QI-HUI ZHANG[b], LI-JUN WU[b], SHIN-ICHI TASHIRO[c], SATOSHI ONODERA[c] and TAKASHI IKEJIMA[a,*]

[a]*China–Japan Research Institute of Medical and Pharmaceutical Sciences, Shenyang Pharmaceutical University, Shenyang 110016, China;* [b]*Department of Phytochemistry, Shenyang Pharmaceutical University, Shenyang, 110016, China;* [c]*Department of Clinical and Biomedical Sciences, Showa Pharmaceutical University, Tokyo 194-8543, Japan*

(Received 2 January 2003; Revised 7 March 2003; In final form 11 March 2003)

Two alkaloids, evodiamine and rutaecarpine, isolated from the dried fruits of *Evodia rutaecarpa* Bentham were evaluated *in vitro* for antiproliferation activity on tumor cells versus human peripheral blood mononuclear cells (PBMC). Evodiamine had more potent cytotoxic effects on five tumor cell lines (human malignant melanoma A375-S2, human cervical cancer HeLa, human breast adenocarcinoma MCF7, human acute monocytic leukemia THP-1, murine fibrosarcoma L929) than rutaecarpine. Moreover, evodiamine did not affect PBMC viability for a 36 h culture period. Although apoptotic bodies were observed in evodiamine-treated L929 cells stained with Hoechst 33258, DNA fragmentation as a hallmark of apoptosis was not found. Caspases were involved in the protection of L929 cells against cell death. Evodiamine initiated atypical apoptosis in L929 cells by cycle arrest at the G0/G1 phase.

Keywords: Evodiamine; Rutaecarpine; Apoptosis; Caspases

INTRODUCTION

Evodiamine and rutaecarpine (Fig. 1) are major quinazoline alkaloids of the dried, unripe fruits of *Evodia rutaecarpa* Bentham. They are reported to have a wide variety of biological activities, such as antinociceptive, vasorelaxant and catecholamine-secretory effects [1]. Recently, Ogasawara and his colleagues have reported that evodiamine also had a marked inhibitory activity on tumor cell migration *in vitro* [2]. However, the mechanism of this antitumor effect remains unclear.

Caspases are a family of cysteine proteases, which cleave protein substrates after their Asp residues. Caspases appear to be involved in regulating the activation of apoptotic signal transmission and are divided into two classes based on the lengths of their N-terminal prodomains, including up-stream caspases such as caspase-2, -8, -10 and downstream caspases such as caspase-3, -6, -7 [3]. The activation of caspases is believed to be required

*Corresponding author. Tel.: +86-24-23844463. Fax: +86-24-23844463. E-mail: ikejimat@vip.sina.com

ISSN 1028-6020 print/ISSN 1477-2213 online © 2004 Taylor & Francis Ltd
DOI: 10.1080/1028602031000119772

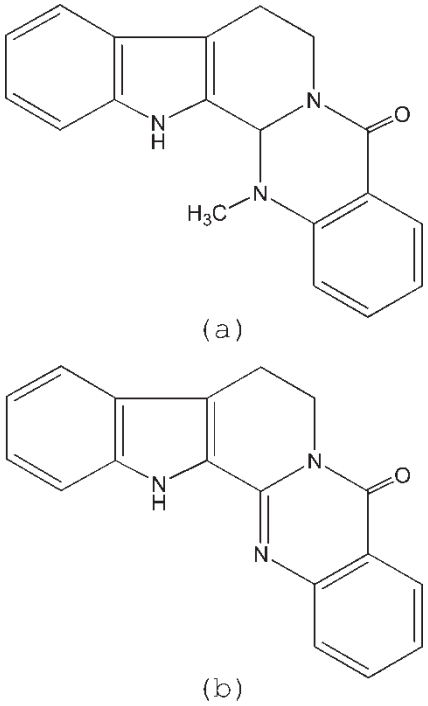

FIGURE 1 Structures of evodiamine (a) and rutaecarpine (b).

for apoptosis. However, some workers have reported that caspases were involved in the protection against TNFα (tumor necrosis factor) or Fas-induced L929 cell death [4,5], suggesting that the effects of caspases on cell death are not limited to initiation of apoptosis.

In this study we have demonstrated that evodiamine has potent anti-tumor effects and induced atypical apoptosis in L929 cells without DNA fragmentation. Moreover, caspases protected L929 cells against apoptosis induced by evodiamine.

RESULTS AND DISCUSSION

Comparison of Antitumor Activities of Evodiamine and Rutaecarpine

Cytotoxic effects of evodiamine and rutaecarpine were detected in A375-S2, HeLa, MCF-7, THP-1, L929 cells and PBMC. The IC_{50} values of evodiamine against five tumor cell lines range from 10 to 22 μM with 24 h drug treatment (Table I) and evodiamine (2–40 μM) had no cytotoxic effects on human PBMC (Fig. 2a). The results suggested that despite a lack of specificity for the five tumor cells, evodiamine had potential anti-tumor effects. Moreover, evodiamine induced tumor cell death, but had no inhibitory effect on human PBMC viability, suggesting that if evodiamine were applied to cancer treatment, it would, unlike other chemotherapeutic drugs, have less cytotoxic effects on human normal cells. Compared to evodiamine, the antiproliferative effects of rutaecarpine were less potent. The IC_{50}s of rutaecarpine against the five tumor cells were all above 150 μM, at which rutaecarpine induced 70% PBMC cell death (Fig. 2b).

TABLE I Antiproliferative effects of evodiamine and rutaecarpine from *Evodia rutaecarpa* on L929, THP-1, MCF7, HeLa, and A375-S2 cells; activity was evaluated by MTT assay after 24 h treatment with drugs; data are expressed as the mean ± SD of three independent experiments

Cell lines	*Evodiamine* (IC_{50} μM)	*Rutaecarpine* (IC_{50} μM)
L929	20.3 ± 3.5	239.1 ± 10.3
THP-1	18.1 ± 1.1	218.6 ± 9.7
MCF7	22.7 ± 1.3	160.2 ± 10.4
HeLa	15.4 ± 0.7	199.8 ± 7.8
A375-S2	10.1 ± 1.9	150.4 ± 6.4

Atypical Apoptosis in L929 Cells Induced by Evodiamine

Although these two alkaloids have very similar structures (Fig. 1) and some biological activities, their anti-tumor effects were remarkably different. This could be due to the methyl group on N-14 of evodiamine, which is absent in rutaecarpine.

We further investigated whether the cytotoxic effect of evodiamine was mediated by an apoptotic mechanism. With evodiamine (20 μM) treatment for 24 h, L929 cells became round, and dead cells were non-adherent at the later stages (Fig. 3). Morphological changes were further confirmed by Hoechst 33258 staining (Fig. 4). In the control group, L929 nuclei were round and stained homogeneously. Evodiamine-treated cells showed marked blebbing nuclei and granular apoptotic bodies.

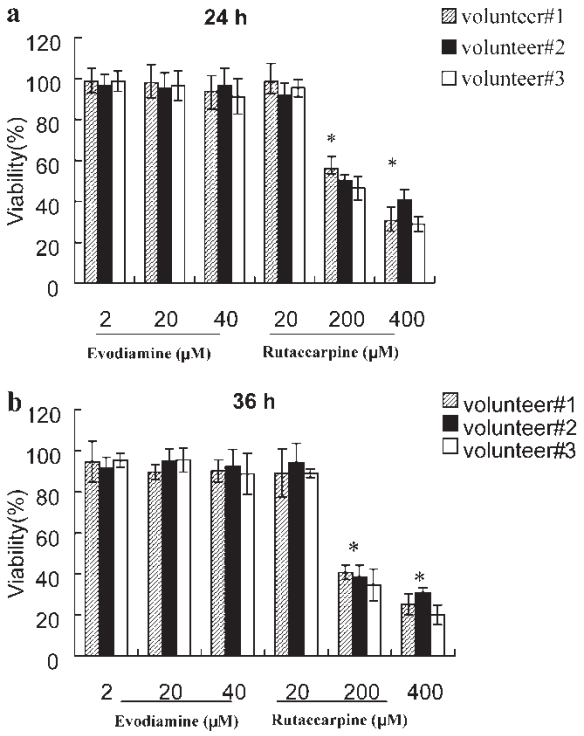

FIGURE 2 Cytotoxic effects of evodiamine and rutaecarpine on human PBMC. Activity was evaluated by MTT assay with drugs treatment for (a) 24 and (b) 36 h. Data are expressed as the mean ± SD of two independent experiments. $^*P < 0.001$ *vs.* negative control.

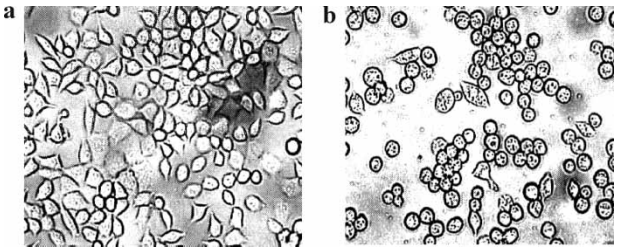

FIGURE 3 Morphological changes of L929 cells treated with evodiamine. Cellular morphology was observed at (a) 0 and (b) 24 h in the presence or absence of evodiamine (20 μM) with × 100 magnification.

To determine whether evodiamine initiated necrosis other than by the apoptosis pathway, the rates of LDH released from viable cells, floating dead cells and the culture medium were measured. In the presence of evodiamine, the number of apoptotic cells increased from 27% to 53% at 36 h; however, that of necrotic cells was still below 20% (Fig. 5). Therefore, evodiamine-induced L929 cell death was mainly due to apoptosis.

DNA fragmentation was assayed by agarose gel electrophoresis. A DNA ladder that is the hallmark of typical apoptosis was not observed in evodiamine-treated L929 cells (data not shown).

Cell Cycle Arrest in L929 Cells Treated with Evodiamine

The changes in the cell cycle of L929 cells during treatment with evodiamine (20 μM) from 8 to 36 h were monitored for DNA contents (Fig. 6). The results showed that cell numbers at the sub-G1 phase increased progressively over the time period. There was a marked increase in the number of apoptotic cells at 36 h. Furthermore, evodiamine caused apparent cell cycle arrest at the G0/G1 phase, which contributed to evodiamine-induced L929 cell death. Most RNA and protein were synthesized at the G0/G1 phase; thus, G0/G1 arrest induced by evodiamine might block protein synthesis, exchanging the balance between cell survival and apoptosis.

Protectional Effects of Caspases in L929 Cell Death Induced by Evodiamine

As caspases are known to mediate apoptosis we further examined the effects of caspase inhibitors on evodiamine-induced L929 cell death. Unexpectedly, pan-caspase inhibitor (z-VAD-fmk) rendered the cells even more sensitive to evodiamine and an inhibitor of

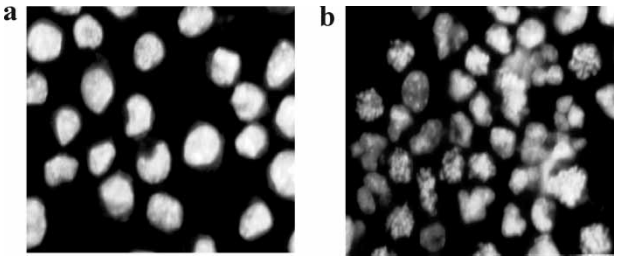

FIGURE 4 Morphological changes of cell nuclei. L929 cells were incubated in the medium alone (a) or the medium containing evodaimine (20 μM) for 24 h (b). The cells were then stained with Hoechst 33258 to identify apoptotic nuclei with × 400 magnification.

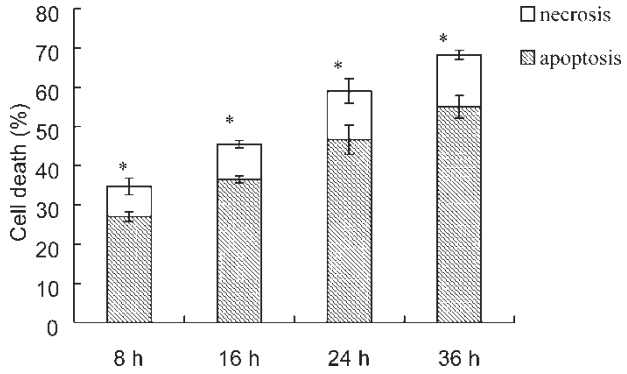

FIGURE 5 Characterization of cell death in L929 cells treated with evodiamine (20 μM). Cell death assessed by LDH activity-based assays was expressed as percentage of apoptosis and necrosis. Data are represented as the mean ± SD of three independent experiments. *$P < 0.001$ *vs.* the percentage of apoptosis.

caspase-3 (z-DEVD-fmk) also weakly augmented the cell death (Fig. 7). Caspase-1 and -8 had no effect on L929 cell death.

At 24 h, z-VAD-fmk increased the cell death rate from 40% (cultured with evodiamine alone) to 72%. z-VAD-fmk has been reported to inhibit caspase-1 [6,7], -8 [8], -3, -4 and -11, and very weakly inhibit caspase-7 [7]. Thus, the inhibitory effect of z-VAD-fmk might be more prominent than z-DEVD-fmk's. However, these inhibitors did not block cell death, but augmented the evodiamine-induced apoptosis. It has also been reported that caspase inhibitors stimulated TNFα-induced L929 necrosis. This sensitization may be correlated with a higher production of reactive oxygen radicals [9]. However, z-VAD-fmk blocked the apoptosis in Fas-treated L929 cells [10]. In our studies, we demonstrated the protection effect of caspase against atypical apoptosis in evodiamine-treated L929 cells. It still remains to be elucidated whether the protection effects of caspases are specific to L929 cells or common to the other cell death pathways.

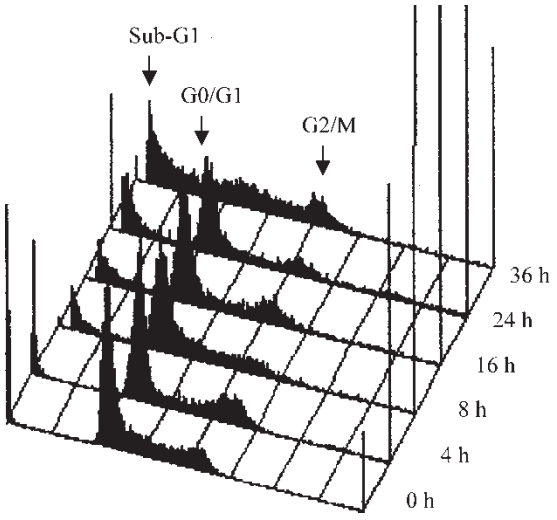

FIGURE 6 Flow cytometric analysis of the cell cycle distribution of L929 cells treated with evodiamine (20 μM) for 0, 4, 8, 16, 24 and 36 h.

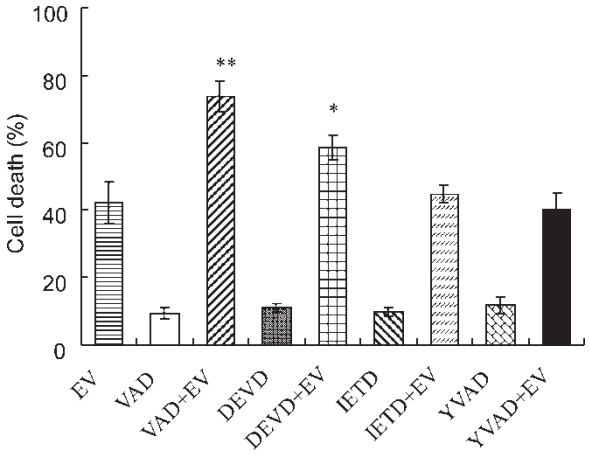

FIGURE 7 Effects of caspase inhibitors on evodiamine-induced cell death. L929 cells were cultured in the presence or the absence of caspase inhibitors, z-VAD-fmk (20 μM), z-DEVD-fmk (20 μM), z-IETD-fmk (20 μM) or Ac-YVAD-cmk (20 μM), 1 h prior to the administration of evodiamine (EV) 20 μM, then incubated for 24 h. The mean ± SD are given for three independent experiments. $*P < 0.05$, $**P < 0.01$ *vs.* evodiamine alone.

EXPERIMENTAL

General Experimental Procedures

Thin-layer chromatography (TLC) was performed on silica gel 60 F254 plates or silica gel G plates (0.25 mm thick, Qingdao Haiyang Chemical group Co., China) and the spots were detected under UV light (254 and 365 nm) or by the use of 10% aqueous sulfuric acid reagent and Dragendorff reagent. ^{1}H and ^{13}C NMR spectra were recoded on a Bruker ARX-300 NMR spectrometer, using TMS as an internal standard.

Plant Material

The dried, unripe fruits of *Evodia rutaecarpa* Bentham (Rutaceae) were purchased from Anhui Province (China). The original plant was identified by Professor Q.-S. Sun of Shengyang Pharmaceutical University, China, and a voucher specimen has been deposited at the Department of Phytochemistry, Shenyang Pharmaceutical University. All the chemicals used were analytical grade reagents.

Isolation and Purification of Active Compounds

The dried fruits of *Evodia rutaecarpa* Bentham (2 kg) were defatted with light petroleum refluxed in MeOH (3 ×), as described in previous reports [11]. The methanol extract was concentrated, then acidified with 2% HCl. The acidic extract solution was submitted to an ion-exchange resin, eluted with NH_4OH, and extracted with Et_2O. The Et_2O extract (2 g) was then chromatographed on silica gel (60 g) with gradient light petroleum–EtOAc (9:1 → 1:1) to give fractions 1–10. Fraction 2 and 4 were submitted to repeated column chromatography on silica gel to afford evodiamine (80 mg) and rutaecarpine (43 mg). The purity of both compounds was over 99%, as assessed by NMR techniques.

Biological Materials

Cell Lines

Human malignant melanoma A375-S2; human cervical cancer HeLa; human breast adenocarcinoma MCF7; human acute monocytic leukemia THP-1; and murine fibrosarcoma L929 were purchased from American Type Culture Collection (ATCC, VA, USA). Human PBMC were obtained from healthy adult volunteers.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), ribonuclease (RNase), proteinase K, propidium iodide (PI) and Hoechst 33258 were purchased from the Sigma-Aldrich Chemical Company (MO, USA). z-Val-Ala-Asp-fmk (z-VAD-fmk), z-Asp-Glu-Val-Asp-fmk (z-DEVD-fmk) and z-Ile-Glu-Asp-fmk (z-IETD-fmk) were purchased from Enzyme Systems (CA, USA). Ac-Tyr-Val-Ala-Asp-chloromethyl-ketone (Ac-YVAD-cmk) was obtained from Bachem (Bubendorf, Switzerland).

In Vitro Evaluation of Antiproliferation Effects

Drug Solutions

Evodiamine and rutaecarpine were dissolved in DMSO to make stock solutions, then diluted in cell culture medium at different concentrations and immediately used. In all assays, the final concentration of DMSO in the culture medium was below 0.01%.

Cell Culture

The cells were cultured in RPMI-1640 medium (GIBCO, NY, USA) supplemented with 10% fetal bovine serum (FBS) (Dalian Biological Reagent Factory, Dalian, China) and 0.03% L-glutamine (GIBCO, NY, USA) at 37°C in 5% CO_2.

MTT Assays

Five tumor cell lines were seeded at 7×10^3 cells well^{-1} in 96-well plates (NUNC, Denmark). The cells were cultured with evodiamine and rutaecarpine for 24 h. Cell growth was measured by MTT as previously described [12] using a plate reader (Bio-Rad, USA). The percentage of cell viability and cell death was calculated as follows: cell viability (%) = $[A_{570}(\text{evodiamine})]/A_{570}(\text{control}) \times 100\%$; cell death (%) = $[A_{570}(\text{control}) - A_{570}(\text{evodiamine})]/A_{570}(\text{control}) \times 100\%$.

Separation of Human PBMC

Blood was obtained from three healthy adult volunteers. PBMC were separated by density centrifugation in the cell separation solution (Shanghai Biological Reagent Factory, China) and suspended in RPMI-1640 medium containing 2% heat-inactivated human AB serum. PBMC, 1×10^4 cells well^{-1}, were seeded in the 96-well culture plates. Cell viability was measured by MTT assay.

Observation of Morphological Changes

L929 cells in RPMI-1640 containing 10% FBS were seeded into 6-well culture plates (NUNC, Denmark) and cultured overnight. Evodiamine (20 μM) was added to the cell culture and the cellular morphology was observed using phase contrast microscopy (LEICA, Germany) after 24 h.

Nuclear Damage Observed by Hoechst 33258 Staining

Apoptotic nuclear morphology was assessed using Hoechst 33258 as described previously [13]. Cells were fixed with 3.7% paraformaldehyde for 2 h at room temperature, then washed and stained with 167 μM Hoechst 33258 for 10 min at 37°C. At the end of incubation, the cells were washed and resuspended in PBS for observation of nuclear morphology using a fluorescence microscope (Nikon, Japan).

LDH Activity-based Cytotoxicity Assays [14]

Lactate dehydrogenase (LDH) activity was assessed using a standardized kinetic determination kit (Zhong Sheng High-Tech Bioengineering Company, Beijing, China). The cells were cultured with evodiamine for 8, 12, 24 and 36 h. Floating dead cells were collected from the culture medium by centrifugation (240*g* for 10 min at 4°C), and the LDH content from the pellets was used as an index of apoptotic cell death (LDHp). The LDH released in the culture medium (extracellular LDH or LDHe) was used as an index of necrotic cell death. The LDH present in the adherent viable cells was referred to as intracellular LDH (LDHi). The percentage of apoptotic and necrotic cell death was calculated as follows: % apoptosis = LDHp/(LDHp + LDHe + LDHi) × 100; % necrosis = LDHe/(LDHp + LDHe + LDHi) × 100.

Flow Cytometric Analysis of Cell Cycle [15]

Flow cytometric analysis was conducted as described in previous experiments. In brief, L929 cells, both adherent and floating, were pelleted and washed with PBS. The cells were fixed in 75% ethanol at 4°C overnight. After washing twice with PBS, the cells were stained with propidium iodine (1.0 mL, 50 mg l^{-1}), RNase A (1 g l^{-1}) and 0.1% Triton X-100 in sodium citrate (3.8 mM), followed by incubation on ice for 30 min in the dark. Samples were analyzed by a flow cytometer (Becton Dickinson FACScan, CA, USA) with the Cell Quest software, which was used to determine the percentage of cells in different phases of the cell cycle.

Statistical Analysis

All results were confirmed in at least two separate experiments. IC_{50} values were calculated by regression analysis of the concentration response data. Data of the representatives were analyzed for statistical significance using the Student's *t*-test. *P* values of less than 0.05 were considered to be significant.

References

[1] Chiou, W.F., Chou, C.J. and Shum, A.Y. (1992), *Eur. J. Pharmacol.* **215**, 277–283.
[2] Ogasawara, M., Matsubara, T. and Suzuki, H. (2001), *Biol. Pharm. Bull.* **24**, 917–920.
[3] Porter, A.G., Ng, P. and Jänicke, R.U. (1997), *Bioassays* **19**, 501–507.
[4] Vercammen, D., Beyaert, R., Denecker, G., Goossens, V., Van Loo, G., Declercq, W., Grooten, J., Fiers, W. and Vandenabeele, P. (1998), *J. Exp. Med.* **187**, 1477–1485.
[5] Vercammen, D., Brouckaert, G., Denecker, G., Van de Craen, M., Declercq, W. and Fiers, W. (1998), *J. Exp. Med.* **188**, 919–930.
[6] Sarin, A., Wu, M.L. and Henkart, P.A. (1996), *J. Exp. Med.* **184**, 2445–2450.
[7] Margolin, N., Raybuck, S.A., Wilson, K.P., Chen, W., Fox, T., Gu, Y. and Livingston, D.J. (1997), *J. Biol. Chem.* **272**, 7223–7228.
[8] Muzio, M., Chinnaiyan, A.M., Kischkel, F.C., O'Rourke, K., Shevchenko, A., Scaffidi, J.C., Bretz, M., Zhang, R., Gentz, R., Mann, M., Krammer, P.H., Peter, M.E. and Dixit, V.M. (1996), *Cell* **85**, 817–827.

[9] Medema, J.P., Scaffidi, C., Kischkel, F.C., Shevchenko, A., Mann, M., Krammer, P.H. and Peter, M.E. (1997), *EMBO* **16**, 2794–2804.
[10] Los, M., Mozoluk, M., Ferrari, D., Stepczynska, A., Stroh, C., Renz, A., Herceg, Z., Wang, Z.Q. and Schulze-Osthoff, K. (2002), *Mol. Biol. Cell* **13**, 978–988.
[11] Pang, Y.Q., Feng, X.Z. and Huang, L. (1996), *Acta. Pharm. Sin.* **31**, 151–155 (in Chinese).
[12] Tada, H., Shiho, O. and Kuroshima, K. (1986), *J. Immunol. Methods* **93**, 157–165.
[13] Sarin, A., Hadid, E. and Henkart, P.A. (1998), *J. Immunol.* **161**, 2810–2813.
[14] Kim, Y.M., Talanian, R.V. and Billiar, T.R. (1997), *J. Biol. Chem.* **272**, 31138–31147.
[15] Lee, K.Y., Park, J.A., Chung, E., Lee, Y.H., Kim, S.I. and Lee, S.K. (1996), *Cancer Lett.* **110**, 193–200.