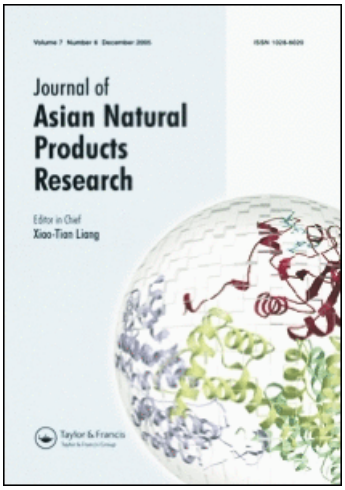

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Dracorhodin perchlorate induces apoptosis in HL-60 cells

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Dracorhodin perchlorate, an anthocyanin red pigment, induces human premyelocytic leukemia HL-60 cell death through apoptotic pathway. Caspase -1, -3, -8, -9, and -10 inhibitors partially reversed the cell death induced by dracorhodin perchlorate. Caspase-3 and -8 were activated followed to the degradation of caspase-3 substrates, inhibitor of caspase-activated DNase (ICAD) and poly-(ADP-ribose) polymerase (PARP). Dracorhodin perchlorate up-regulated the expression ratio of mitochondrial proteins, Bax/Bcl-X_L. The cell death was accompanied with phosphorylation of ERK, JNK and p38 MAPK and partially reduced by MEK inhibitor (PD98059), JNK MAPK inhibitor (SP600125) and p38 MAPK inhibitor (SB 203580). Taken together, dracorhodin perchlorate-induced apoptosis in HL-60 cells via up-regulation of Bax, activation of caspases and ERK/p38/JNK MAPKs.

Keywords: Dracorhodin perchlorate; Human premyelocytic leukemia HL-60 cell; Apoptosis

1. Introduction

Dracorhodin perchlorate is a synthetic analogue of the antimicrobial anthocyanin red pigment dracorhodin (figure 1) which is isolated from the exudates of the fruit of *Daemonorops draco* [1–3], known as "Dragon's Blood" in Traditional Chinese Medicine (TCM) [4] for the treatment in injury and bleeding. Many flavonoids have been reported to have apoptosis-inducing activities in human tumour cells [5–7], therefore the cytotoxic action of dracorhodin perchlorate on human premyelocytic leukemia HL-60 cell is examined in this study. We found that dracorhodin perchlorate induces apoptosis in human premyelocytic leukemia HL-60 cells via up-regulating Bax level, activation of caspases and MAPKs.

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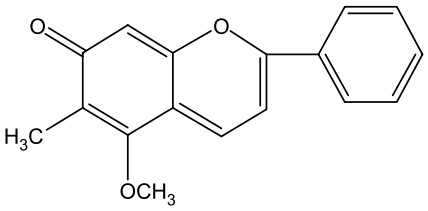
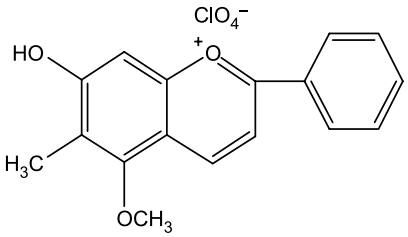


Dracorhodin



Dracorhodin perchlorate

Figure 1. Chemical structures of dracorhodin and dracorhodin perchlorate.

2. Results and discussion

2.1 Cytotoxic effects of dracorhodin perchlorate on HL-60 cells

As shown in figure 2, dracorhodin perchlorate inhibited HL-60 cell growth in a dose- and time-dependent manner.

2.2 Dracorhodin perchlorate induced apoptosis in HL-60 cells

In order to examine the characteristics of dracorhodin perchlorate-induced HL-60 cell death, the morphologic changes and DNA fragmentation were observed. In HL-60 cells, exposure to 20 μmol L^{-1} dracorhodin perchlorate for 6 h resulted in morphologic alterations

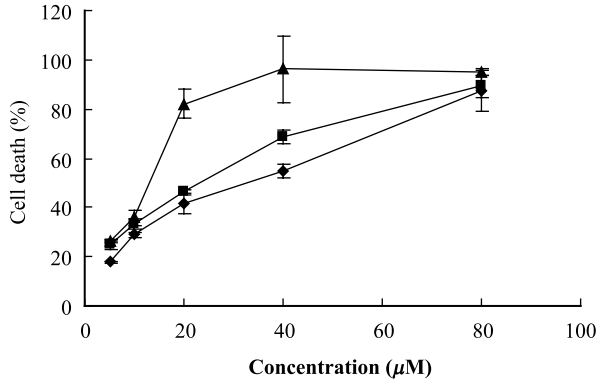


Figure 2. Dose-dependent inhibition effect of dracorhodin perchlorate on HL-60 cells. The cells were cultured for 6 (diamond), 12 (square) and 24 (triangle) h with different concentrations of dracorhodin perchlorate. $n = 3$. Mean ± S.D.

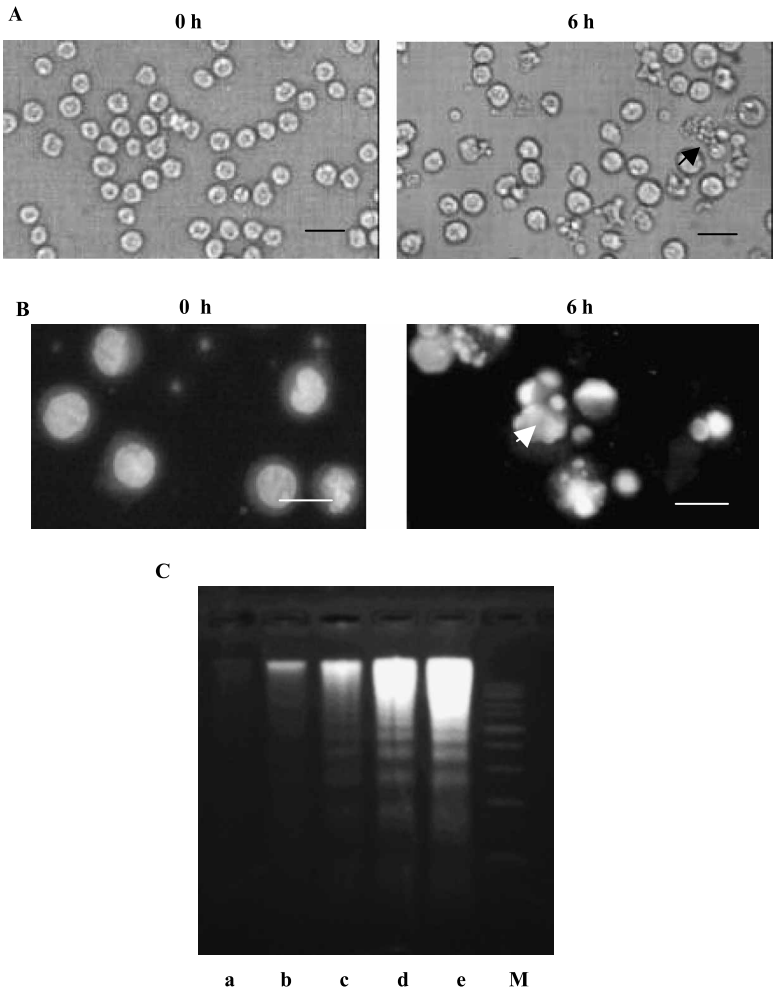


Figure 3. Dracorhodin perchlorate-induced morphologic changes and DNA fragmentation of HL-60 cells. (A) HL-60 cells were incubated in the medium alone for 6 h (a) or the medium containing 20 μmol L^{-1} dracorhodin perchlorate for 6 h (b) (magnification × 200). Arrow indicates multiblebbing cells and apoptotic bodies. Scale bar represents 100 μm. (B) HL-60 cells were incubated in the medium alone for 6 h or the medium containing 20 μmol L^{-1} dracorhodin perchlorate for 6 h (magnification × 400, arrow indicates fragmented nuclei) and stained by Hoechst 33258. Scale bar represents 100 μm. (C) HL-60 cells were cultured with dracorhodin perchlorate 20 μmol L^{-1} for 0 (a), 1 (b), 3 (c), 6 (d) and 9 (e) h. *Hae* III-digested phage ϕx-174 DNA fragments were used as molecular weight markers (M).

characteristic of apoptosis, including membrane blebbing, nuclear condensation and fragmentation (figure 3A and B). In a gel electrophoresis, after 3, 6 or 9 h treatment with 20 μmol L^{-1} dracorhodin perchlorate, a time-dependent typical DNA fragmentation was observed which is another hallmark of apoptosis (figure 3C). Thus, dracorhodin perchlorate induced HL-60 cell death by apoptotic pathways.

2.3 Caspases were activated during dracorhodin perchlorate induced-HL-60 cell death

Caspases are a family of cysteine proteases that mediate apoptosis. Caspase-8 and -10 can be activated by the extrinsic pathway triggered by the binding of death ligands such as FasL

to their cognate receptors. Cellular stresses such as UV irradiation result in engagement of the intrinsic cell death pathway leading to procaspase-9 and -2 activation. Both pathways converge on a cascade of executioner caspases (-3, -6, and -7) which results in cleavage of a number of caspase substrates responsible for apoptotic demise of the cell by cleaving intracellular proteins, altering or negating protein functions [8–11]. In the present study, pan-caspase inhibitor (z-VAD-fmk), caspase-1 inhibitor (Ac-YVAD-cmk), caspase-3 inhibitor (z-DEVD-fmk), caspase-8 inhibitor (z-IETD-fmk), caspase-9 inhibitor (z-LEHD-fmk), and caspase-10 inhibitor (z-AEVD-fmk) 40 $\mu mol\,L^{-1}$ effectively reduced 20 $\mu mol\,L^{-1}$ dracorhodin perchlorate-induced HL-60 cell death (figure 4A). Western blot analysis was applied to further confirm the participation of caspase-3 and -8. The 54 kDa band of procaspase-8 and the 32 kDa band of procaspase-3 were degraded after 1 h treatment with dracorhodin perchlorate 20 $\mu mol\,L^{-1}$, indicating the activation of these two caspases (figure 4B). Two caspase-3 substrates, inhibitor of caspase dependent DNase (ICAD) and poly-ADP-ribose polymerase (PARP), were also examined to ascertain the participation of caspase-3. After exposure to 20 $\mu mol\,L^{-1}$ dracorhodin perchlorate for 3 h, ICAD was down-regulated and the amount of the 116 kDa protein of PARP declined and the 85 kDa degraded product increased in amount (figure 4C). ICAD is cleaved to be inactivated and allow CAD to execute the characteristic fragmentation of DNA [12]. PARP cleavage leads to its inactivation, thus preventing futile DNA repair cycles [13]. Taken together, these results suggest that the activation of caspase cascade occurred in HL-60 cells after exposure to 20 $\mu mol\,L^{-1}$ dracorhodin perchlorate. The protection from caspase-8 and -10 inhibitors indicate the death receptor signalling pathway may be involved in this process.

2.4 Involvement of Bax in dracorhodin perchlorate-induced apoptosis

The Bcl-2 family members are critical regulators of mitochondrial pathway that induces activation of caspases intrinsically; it includes both anti-apoptotic members, such as Bcl-2 and Bcl-X_L, and pro-apoptotic members such as Bax and Bak [14,15]. A balance between members of the Bcl-2 family is thought to determine whether mitochondria remain intact or become permeabilised and release proteins that promote cell death [16]. In this study, after 1 h treatment with dracorhodin perchlorate, Bax began to increase while the expression of Bcl-X_L remained unchanged (figure 5).

Overexpression of Bax increases mitochondrial permeability causes the release of pro-apoptotic factors, including cytochrome *c* [15,17]. Released cytochrome *c* binds to Apaf-1, forms the apoptosome, which proteolytically activates procaspase-9, and in turn activates the effector caspases (caspase-3, -6, and -7). In this study we found that, at 7 h after dracorhodin perchlorate treatment, the expression of the pro-apoptotic protein Bax increased followed by the activation of caspse-3 and the degradation of the caspase-3 substrates. The caspase-9 inhibitor protected cell death induced by dracorhodin perchlorate. Thus, it is suggested that dracorhodin perchlorate induces HL-60 cell apoptosis by up-regulation of Bax, subjecting to the initiation of intrinsic mitochondria-apoptosome pathways.

Because of the existence of the p53-binding domain in the Bax gene promoter region, Bax is in general thought to be up-regulated by the tumour suppressor p53 and mediates collapse of mitochondria [18,19]. However, HL-60 is an exceptional human tumour cell line that does not express p53 [20], therefore dracorhodin perchlorate up-regulates Bax in a p53 independent pathway.

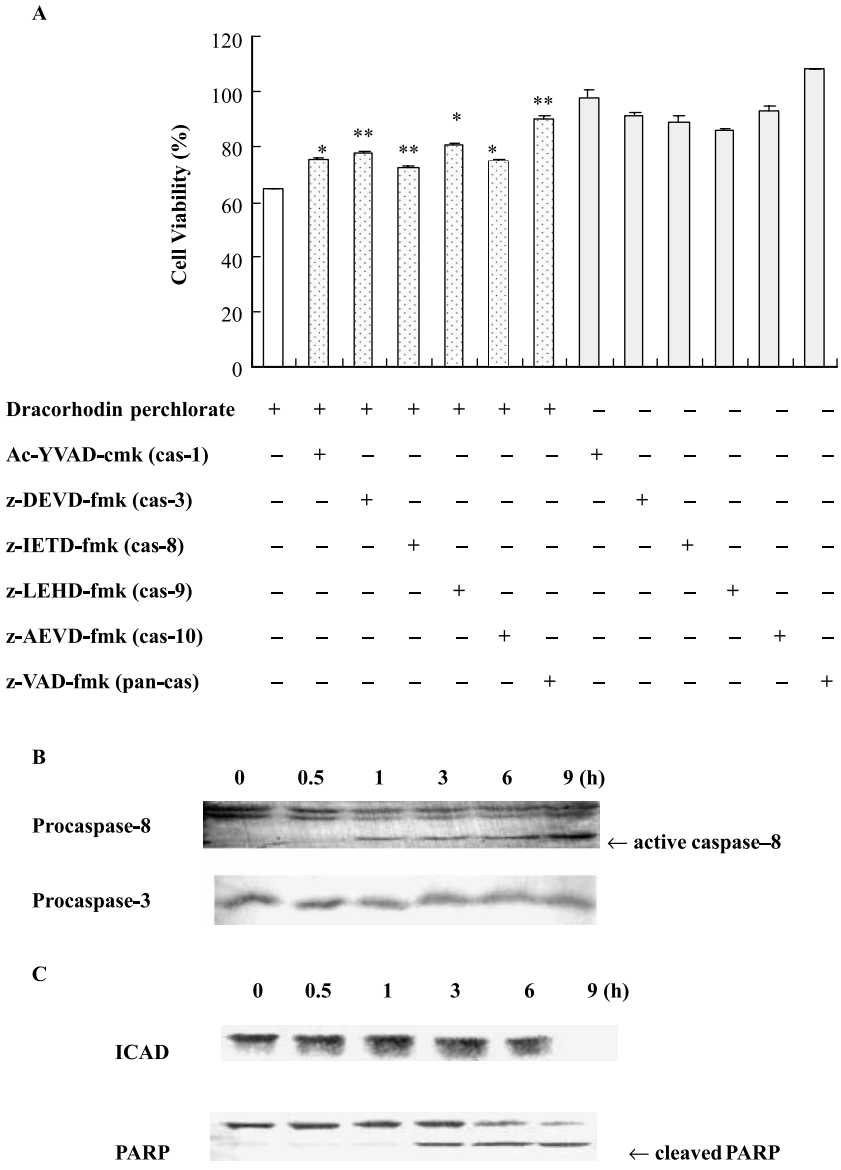


Figure 4. Involvement of caspases in dracorhodin perchlorate-induced apoptosis of HL-60 cells. (A) Effects of caspase inhibitors on 20 μmol L^{-1} dracorhodin perchlorate induced HL-60 cell death. The cells were cultured in the absence or presence of caspase inhibitors (40 μmol L^{-1}) 1 h prior to the addition of dracorhodin perchlorate, then incubated for 6 h. $n = 3$. Mean ± S.D. *$p < 0.05$ and **$p < 0.01$ vs. dracorhodin perchlorate 20 μmol L^{-1} group. (B) Degradation of caspase-3 and -8 in dracorhodin perchlorate-treated HL-60 cells. The protein bands were detected after treatment with dracorhodin perchlorate 20 μmol L^{-1} for indicated times by Western blot analysis. (C) Degradation of ICAD and PARP in dracorhodin perchlorate-treated HL-60 cells. The cells were treated with dracorhodin perchlorate 20 μmol L^{-1} for the indicated times. ICAD and PARP were analysed by Western blotting.

2.5 Dracorhodin perchlorate induced activation of ERK, p38 and JNK MAPKs

Mitogen-activated protein kinases (MAPKs), include extracellular signal-regulated kinase (ERK), c-Jun NH2-terminal protein kinase (JNK), and p38 kinase, also played an important role in the regulation of apoptosis. Dracorhodin perchlorate induced transient

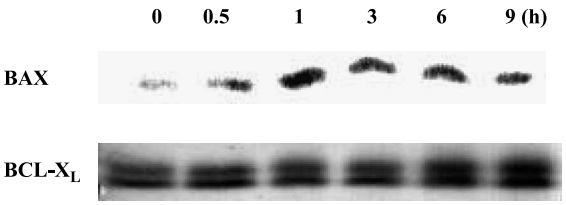


Figure 5. Expression of Bax and Bcl-X_L in dracorhodin perchlorate-trated HL-60 cells. The cells were treated with dracorhodin perchlorate 20 μmol L^{-1} for the indicated times. Bax and Bcl-X_L were detected by Western blot analysis.

phosphorylation of p38 and JNK46 and sustained phosphorylation ERK42/44 MAPK (figure 6A). The specific inhibitor SB203580 for p38, SP600125 for JNK and PD98059 for MEK/ERK signalling pathways attenuated dracorhodin perchlorate 20 μmol L^{-1}-induced apoptosis (figure 6B). The activation of JNK is associated with release of cytochrome *c* and Smac (second mitochondria-derived activator of caspases) from mitochondria to cytosol in apoptosis. Sustained p38 MAPK activation contributes to the UVB-induced apoptosis by mediating the release of mitochondrial cytochrome *c* into the cytosol [21,22]. Although more often associated with survival, a pro-apoptotic function for the ERK pathway has also been suggested in several apoptotic model systems. For example, in cisplatin-induced apoptosis of HeLa cells, ERK activation functions upstream of caspase activation to initiate the apoptotic signal and in quercetin treated A549 lung cancer cells, high and sustained activation of ERK is necessary for cleavage of PARP and apoptosis [23,24].

In dracorhodin perchlorate-induced HL-60 cell apoptosis, activation of p38, ERK and JNK MAPKs was detected which is associated with the inhibitory effect from p38 specific inhibitor SB 203580, MEK inhibitor PD98059 and JNK specific inhibitor SP 600125, indicating an active role of p38, ERK and JNK in mediating dracorhodin perchlorate-induced HL-60 cell apoptosis related to mitochondria-mediated caspase activation.

3. Experimental

3.1 Chemical reagents

Dracorhodin perchlorate was purchased from the Beijing Institute of Biological Products (Beijing, China). The purity of dracorhodin perchlorate was measured by HPLC and determined to be about 98%. Dracorhodin perchlorate was dissolved in dimethyl sulfoxide (Me_2SO) and Me_2SO concentration in all cell cultures was kept below 0.1%, which had no detectable effect on cell growth or apoptosis.

Pan-caspase inhibitor (z-VAD-fmk), caspase-3 (z-DEVD-fmk), capase-8 (z-IETD-fmk), caspase-9 (z-LEHD-fmk), and caspase-10 (z-AEVD-fmk) inhibitors were purchased from Enzyme Systems (Livermore, CA, USA). Caspase-1 inhibitor (Ac-YVAD-cmk) was obtained from Balchem (Bubendorf, Switzerland). SB203580 and PD98059 were from Calbiochem (La Jolla, CA, USA), SP600125 was purchased Alexis (Lausanne, Switzerland).

3.2 Cell culture

The human premyelocytic leukemia HL-60 cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA) and were cultured in RPMI-1640 medium (Gibco,

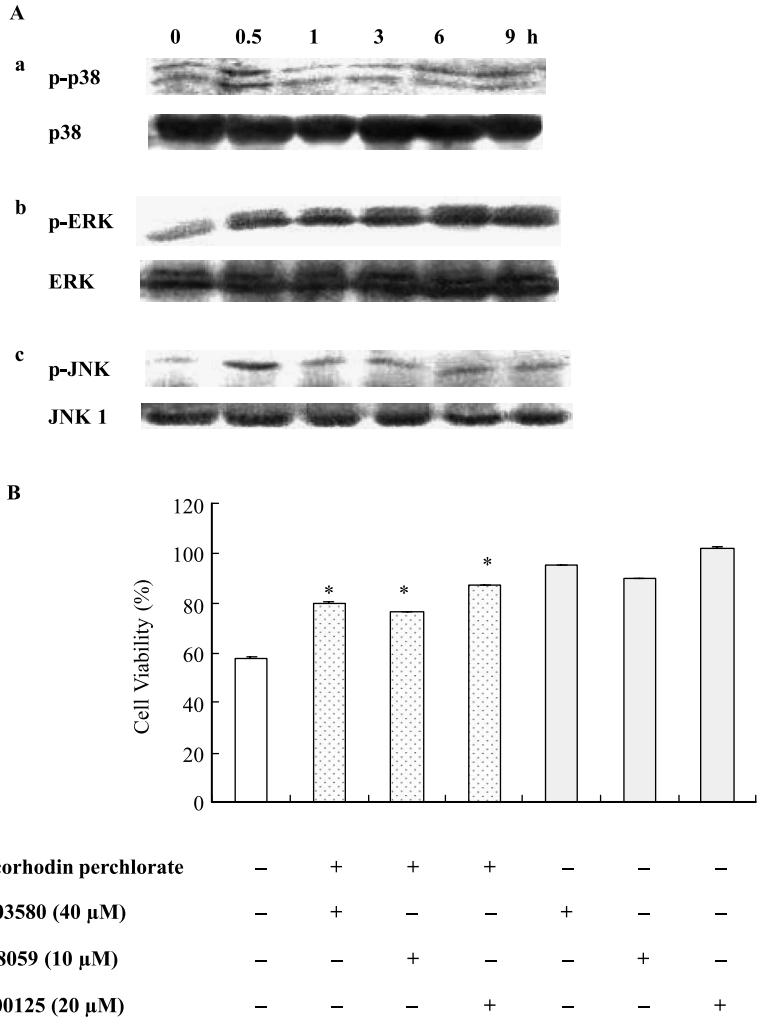


Figure 6. MAPKs are activated in dracorhodin perchlorate induced HL-60 cell death. (A) The expression of (a) p-p38 and p38; (b) p-ERK and ERK; (c) p-JNK and JNK in dracorhodin perchlorate-treated HL-60 cells. The cells were treated with dracorhodin perchlorate 20 μmol L^{-1} for the indicated times. p-p38, p38, p-JNK, JNK, p-ERK and ERK were analysed by Western blotting. (B) Effects of MAPK inhibitors on 20 μmol L^{-1} dracorhodin perchlorate induced HL-60 cell death. The cells were cultured in the absence or presence of different MAPK inhibitors, 1 h prior to the addition of dracorhodin perchlorate, then incubated for 6 h. $n = 3$. Mean ± S.D.*$p < 0.05$ vs. dracorhodin perchlorate 20 μmol L^{-1} group.

Grand Island, NY, USA) containing 10% foetal bovine serum (FBS) (Yuanhengshengma Biological Reagent Institute, Beijing, China) and 0.03% L-glutamine (Gibco) in 5% CO_2 at 37°C. Cells in the exponential phase of growth were used in the experiments.

3.3 Cytotoxicity assay

The cells were cultured at 1×10^4 cells per well in 96-well plates (Nunc, Roskilde, Denmark). After pre-incubation with caspase inhibitors, z-VAD-fmk, z-DEVD-fmk, z-IETD-fmk, z-LEHD-fmk, z-AEVD-fmk and Ac-YVAD-cmk at given concentrations for 1 h, the cells were incubated with or without dracorhodin perchlorate for 6 h. Cell growth was

measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay using a plate reader (Tecan, Austria) as previously described [25].

3.4 Observation of morphologic changes

HL-60 cells in RPMI-1640 containing 10% FBS were seeded into 25-mL culture bottles and incubated overnight. Dracorhodin perchlorate 20 μmol L^{-1} was added to the cell culture and the cellular morphology was observed using phase contrast microscopy (Leica, Germany) at 0 and 6 h.

3.5 Nuclear damage observed by Hoechst 33258 staining

HL-60 cells in RPMI-1640 containing 10% FBS were seeded into a 6-well plate and cultured overnight. Dracorhodin perchlorate 20 μmol L^{-1} was added to the cell culture and incubated for 0 and 6 h. The cells were fixed with 3.7% paraformaldehyde at room temperature for 2 h, then washed twice with Ca^{2+}- and Mg^{2+}-free phosphate buffered saline (PBS) and stained with Hoechst 33258 (Sigma, St. Louis, MO, USA) 167 μmol L^{-1} at 37°C for 15 min. At the end of incubation, nuclear morphology of the cells were observed by using a fluorescence microscope (Leica) [26].

3.6 DNA extraction and detection of DNA fragments

The cells (1×10^6 cells) were harvested and centrifuged at 1000 × *g* for 10 min, and washed once in PBS. Cell pellets were lysed in 100 μl cell lysis buffer (10 mmol L^{-1} Tris–HCl pH 7.4, 10 mmol L^{-1} EDTA pH 8.0, 0.5% Triton X-100) at 4°C for 15 min, and centrifuged at 15,000 × *g* for 20 min. The supernatants were incubated with 40 μg mL^{-1} proteinase K (Merck, Darmstadt, Germany) and 40 μg mL^{-1} RNase A (Sigma) at 37°C for 2 h. The lysate was extracted with 0.5 mol L^{-1} NaCl and 50% 2-propanol and incubated at −20°C overnight, and centrifuged at 15,000 × *g* for 20 min. The pellets were suspended in TE buffer (10 mmol L^{-1} Tris–HCl pH 7.4 and 1 mmol L^{-1} EDTA pH 8.0). DNA was separated by 2% agarose gel electrophoresis at 100 V for 40 min and stained with 0.1 μg mL^{-1} ethidium bromide [27].

3.7 Western blot analysis

Immunoblotting of cell lysates was performed as previously described [28]. The cells were harvested, washed twice with ice-cold PBS, then lysed in lysis buffer (50 mmol L^{-1} Hepes pH 7.4, 1% Triton X-100, 2 mmol L^{-1} sodium orthovanadate, 100 mmol L^{-1} sodium fluoride, 1 mmol L^{-1} EDTA, 1 mmol L^{-1} EGTA, 1 mmol L^{-1} PMSF, 10 μg mL^{-1} aprotinin and 10 μg mL^{-1} leupeptin) at 4°C for 60 min. The lysate was centrifuged at 15,000 × *g* for 10 min and the supernatant was used for Western blot analysis. Equivalent amounts of protein were separated by SDS-PAGE and wet-electrotransferred onto nitrocellulose membranes, and equivalent loading was confirmed by Bio-Rad protein assay. Proteins were detected with antibodies against Bax, ICAD, Bcl-X_L, caspase-3 and caspase-8 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and PARP (Upstate Biotechnology, Lake Placid, NY, USA) followed by horseradish peroxidase (HRP)-conjugated secondary antibody (Santa Cruz Biotechnology) and visualised by using 3,3-diaminobenzidine tetrahydrochloride as the HRP substrate.

3.8 Statistical analysis

Statistical analysis was conducted using Student's *t*-test for analysis of significance between the different values. Values were expressed as the mean ± S.D. and they were considered significant at a p of less than 0.05. All ps are two-tailed.

References

[1] H. Brockmann, H. Junge. *Ber. Dtsch. Chem. Ges*, **76**, 751 (1943).
[2] G.S.R. Rao, M.A. Gehart, R.T. Lee III, L.A. Mitscher, S. Drake. *J. Nat. Prod.*, **45**, 646 (1982).
[3] W.F. Gao, H. Zheng, Y.S. Wang, Z.H. Zhang, J.C. Lu, S.F. Zhang. *Chin. J. Pharmacol. (Zhongguo. Yiyao. Zazhi)*, **20**, 247 (1989).
[4] S.C. Li. *Pen-tsao Kang Mu*, p. 1959, People's Sanitation Press, Shanghai (2000).
[5] I.K. Wang, S.Y. Lin-Shiau, J.K. Lin. *Eur. J. Cancer*, **35**, 1517 (1999).
[6] M. Russo, R. Palumbo, A. Mupo, M. Tosto, G. Iacomino, A. Scognamiglio, I. Tedesco, G. Galano, G.L. Russo. *Oncogene*, **22**, 3330 (2003).
[7] S. Kuntz, U. Wenzel, H. Daniel. *Eur. J. Nutr.*, **38**, 133 (1999).
[8] N.A. Thornberry, Y. Lazebnik. *Science*, **281**, 1312 (1998).
[9] A. Ashkenazi. *Nat. Rev. Cancer*, **2**, 420 (2002).
[10] F.H. Igney, P.H. Krammer. *Nat. Rev. Cancer*, **2**, 277 (2002).
[11] M.O. Hengartner. *Nature*, **407**, 770 (2000).
[12] H. Sakahira, M. Enari, S. Nagara. *Nature*, **391**, 96 (1998).
[13] Y.A. Lazebnik, S.H. Kaufmann, S. Desnoyers, G.G. Poirier, W.C. Earnshaw. *Nature*, **371**, 346 (1994).
[14] J.M. Adams, S. Cory. *Science*, **281**, 1322 (1998).
[15] A. Hunt, G. Evan. *Science*, **293**, 1784 (2001).
[16] D.C. Huang, A. Strasser. *Cell*, **103**, 839 (2000).
[17] X. Li, M. Marani, J. Yu, B. Nan, J.A. Roth, S. Kagawa, B. Fang, L. Denner, M. Marcelli. *Cancer Res.*, **61**, 186 (2001).
[18] T. Miyashita, J.C. Reed. *Cell*, **80**, 293 (1995).
[19] M. Schuler, E. Bossy-Wetzel, J.C. Goldstein, P. Fitzgerald, D.R. Green. *J. Biol. Chem.*, **275**, 7337 (2000).
[20] D. Wolf, V. Rotter. *Proc. Natl Acad. Sci. USA*, **82**, 790 (1985).
[21] Z. Assefa, A. Vantieghem, M. Garmyn, W. Declercqi, P. Vandenabeelei, J.R. Vandenheede, R. Bouillon, W. Merlevede, P. Agostinis. *J. Biol. Chem.*, **275**, 21416 (2000).
[22] D. Chauhan, G. Li, T. Hideshima, K. Podar, C. Mitsiades, N. Mitsiades, N. Munshi, S. Kharbanda, K.C. Anderson. *J. Biol. Chem.*, **278**, 17593 (2003).
[23] X. Wang, J.L. Martindale, N.J. Holbrook. *J. Biol. Chem.*, **275**, 39435 (2000).
[24] T.T.T. Nguyen, E. Tran, T.H. Nguyen, P.T. Do, T.H. Huynh, H. Huynh. *Carcinogenesis*, **25**, 647 (2004).
[25] F. Takano, T. Tanaka, W. Tsukamoto, N. Yahagi, S. Fushiya. *Planta. Med.*, **69**, 321 (2003).
[26] Y. Zhang, Q.H. Zhang, L.J. Wu, S. Tashiro, S. Onodera, T. Ikejima. *J. Asian. Nat. Prod. Res*, **6**, 19 (2004).
[27] Y. Zhang, L.J. Wu, S. Tashiro, S. Onodera, T. Ikejima. *Biol. Pharm. Bull.*, **26**, 1543 (2003).