Full Paper

Dracorhodin Perchlorate Induces Apoptosis via Activation of Caspases and Generation of Reactive Oxygen Species

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Abstract. Dracorhodin perchlorate inhibited proliferation of several tumor cell lines. The drug induced oligonucleosomal fragmentation of DNA in HeLa cells and increased caspase-3, -8, -9 activities followed by the degradation of caspase-3 substrates, inhibitor of caspase-dependent DNase, and poly-(ADP-ribose) polymerase. It also increased caspase-1 activity and a caspase-1 inhibitor, Ac-YVAD-cmk, and a caspase-10 inhibitor z-AEVD-fmk, also reduced dracorhodin-perchlorate-induced HeLa cell death. Dracorhodin perchlorate decreased the expression of anti-apoptotic mitochondrial protein, Bcl-X_L, but not Bcl-2; and it increased the expression of pro-apoptotic protein, Bax. Dracorhodin perchlorate induced a sustained generation of reactive oxygen species (ROS) in HeLa cells; caspase-1 inhibitor, Ac-YVAD-cmk, and caspase-3 inhibitor, z-DEVD-fmk, attenuated the generation of ROS. Taken together, our results indicate that dracorhodin perchlorate alters the intracellular redox status, changed the balance of Bcl-X_L and Bax protein expression, and induces apoptosis through caspase pathways in HeLa cells.

Keywords: dracorhodin perchlorate, Bax, Bcl-X_L, caspase, reactive oxygen species

Introduction

The red pigment, dracorhodin, is an anthocyanin isolated in the exudates of the fruit of the plant *Daemonorops draco* (1), known as “Dragon’s Blood”, which has been often used in traditional Chinese medicine (2) where it is known as *Kirin-kakketsu* in Japanese for the treatment of injuries, to control bleeding. Biological effects of dracorhodin have not been investigated except for its antimicrobial effects (3). Dracorhodin perchlorate is a synthetic analog of dracorhodin (4), the pharmacological and toxicological effects of which have not previously been reported to our knowledge. Recently, there is increasing interest in the pharmaceutical function of anthocyanins (5). Several anthocyanins have been reported to have apoptosis-inducing activities in human tumor cells (6, 7); therefore, the cytotoxic effect of dracorhodin perchlorate

is investigated in the present study.

Apoptosis or programmed cell death is an evolutionarily conserved process that removes damaged or unwanted cells. Recent studies have shown that a large number of anticancer drugs exert their therapeutic action by inducing apoptosis in malignant cells (8).

There are two major signaling pathways that induce apoptosis. One is initiated by death receptors such as Fas and tumor necrosis factor (TNF) α receptor, and another is activated by molecules released from mitochondria, the energy generators of cells. Both pathways eventually converge, leading to activation of the central effectors of apoptosis: a group of cysteine proteases, called caspases (9, 10). Activated death receptors in turn recruit and activate caspase-8 and -10 that initiate a cascade of terminal or “executioner” caspases, including caspase-3 and others. Unleashing of executioner caspases results in cleavage of a number of caspase substrates responsible for apoptotic demise of the cell, including protein kinases, cytoskeleton associated

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proteins, transcription factors, and proteins involved in DNA repair and chromatin structure such as poly-(ADP-ribose) polymerase (PARP).

The Bcl-2 protein family is a large family of apoptosis-regulating proteins that modulate the mitochondrial pathway. It includes both antiapoptotic members such as Bcl-2 and Bcl-X_L and proapoptotic members such as Bax and Bak (11). These proteins govern mitochondrial membrane permeability, either promoting or suppressing release of apoptogenic proteins from these organelles. Among the mitochondrial proteins released into the cytosol during apoptosis is cytochrome *c*, which binds and activates Apaf-1, an oligomeric protein that activates the cell death protease, pro-caspase-9. This event triggers a cascade of proteolytic events, especially executioner caspases, and initiates terminal events that overlap with those initiated by death receptors (12).

Several studies suggest that reactive oxygen species (ROS) affects the intrinsic apoptotic pathway, with mitochondria being the major source and the primary target of these ROS. ROS may also change the mitochondrial potential, oxidize mitochondrial pores, induce the release of cytochrome-*c*, triggering the subsequent apoptotic events. ROS generation may also change the redox status of cells with subsequent effect on specific kinases, phosphatases and transcription factors that alter the cell's sensitivity to apoptotic stimuli (13–17).

We for the first time demonstrate that dracorhodin perchlorate has a cytotoxic effect on tumor cells and induced HeLa cell death through activation of caspases, alteration of Bcl-X_L/Bax balance, and the changes of intracellular redox status.

Materials and Methods

Chemical reagents

Dracorhodin perchlorate (Fig. 1) was purchased from Beijing Institute of Biological Product (Beijing, China). 5-Fluorouracil (5-FU) was purchased from Sigma-Aldrich (St. Louis, MO, USA). The purity of dracorhodin perchlorate was measured by HPLC and determined to be about 98%. Dracorhodin perchlorate was dissolved in dimethyl sulfoxide (Me₂SO) and Me₂SO 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), caspase-8 (z-IETD-fmk), caspase-9 (z-LEHD-fmk), and caspase-10 (z-AEVD-fmk) inhibitors were purchased from Enzyme Systems (Livermore, CA, USA); and caspase-1 inhibitor (Ac-YVAD-cmk) was obtained from Bachem (Bubendorf, Switzerland).

Dracorhodin

Dracorhodin perchlorate

Fig. 1. Chemical structures of dracorhodin and dracorhodin perchlorate.

Cell culture

The human cervical cancer HeLa cells, human malignant melanoma A375-S2, human breast cancer MCF-7, human histiocytic lymphoma U937 cells, and human embryonic lung (HEL 299) cells were obtained from American Type Culture Collection (ATCC, Rockville, MD, USA). The four tumor cell lines were cultured in RPMI-1640 medium (Gibco, Grand Island, NY, USA) and HEL 299 cells were cultured in minimum essential medium (Eagle) (Gibco) containing 10% fetal bovine serum (FBS) (Yuanhengshengma Biological Reagent Institute, Beijing, China) and 0.03% L-glutamine (Gibco) in 5% CO₂ at 37°C. Cells in the exponential phase of growth were used in the experiments.

Cytotoxicity assay

All the cells were cultured at 1×10^4 cells/well in 96-well plates (NUNC, Roskilde, Denmark). After preincubation with caspase inhibitors, z-VAD-fmk, z-DEVD-fmk, z-IETD-fmk, z-LEHD-fmk, z-AEVD-fmk, and Ac-YVAD-cmk at the given concentrations for 1 h, the cells were incubated with or without dracorhodin perchlorate for 12 h. Cell growth was measured with a plate reader at different time points by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) as previously described (Tecan, Grodig, Austria) (18).

Separation of human peripheral blood mononuclear cells (PBMC)

Peripheral blood was obtained from three healthy adult volunteers. PBMC were separated by density centrifugation in cell separation solution (Shanghai Biological Reagent Factory, Shanghai, China) and suspended in RPMI-1640 medium containing 2% human AB type serum. PBMC (5×10^4 cells/well) were seeded into 96-well culture plates. Cell viability was measured by the MTT method.

Observation of morphological changes

HeLa cells in RPMI-1640 containing 10% FBS were seeded into 25-ml culture bottles and cultured overnight. Dracorhodin perchlorate at $60 \mu\text{M}$ was added to the cell culture and the cellular morphology was observed using phase contrast microscopy (Leica, Nussloch, Germany) at 0, 12, 24, 36, and 48 h.

Nuclear damage observed by Hoechst 33258 staining

HeLa cells in RPMI-1640 containing 10% FBS were seeded into a 6-well plate and cultured overnight. Dracorhodin perchlorate at $60 \mu\text{M}$ was added to the cell culture and cultured for 24 and 36 h. Cells were fixed with 3.7% paraformaldehyde at room temperature for 2 h, and then they were washed twice with PBS and stained with Hoechst 33258 (Sigma) at $167 \mu\text{M}$ at 37°C for 15 min. At the end of incubation, nuclear morphology of the cells were observed by using a fluorescence microscope (Leica) (19).

DNA extraction and detection of DNA fragments

Floating and attached HeLa cells (1×10^6 cells) were harvested and centrifuged at $1,000 \times g$ for 10 min and washed once in Ca^{2+} - and Mg^{2+} -free phosphate buffered saline (PBS). Cell pellets were lysed in $100 \mu\text{l}$ cell lysis buffer (10 mM Tris-HCl, pH 7.4; 10 mM EDTA, pH 8.0; 0.5% Triton X-100) at 4°C for 15 min and centrifuged at $15,000 \times g$ for 20 min. The supernatants were incubated with $40 \mu\text{g/ml}$ proteinase K (Merck, Darmstadt, Germany) and $40 \mu\text{g/ml}$ RNase A (Sigma) at 37°C for 2 h. The lysate was extracted with 0.5 M NaCl and 50% 2-propanol, incubated at -20°C overnight, and then centrifuged at $15,000 \times g$ for 20 min. The pellets were suspended in TE buffer (10 mM Tris-HCl, pH 7.4 and 1 mM EDTA, pH 8.0). DNA was separated by 2% agarose gel electrophoresis at 100 V for 40 min and stained with $0.1 \mu\text{g/ml}$ ethidium bromide (20).

Caspase-1 activity assay

HeLa cells were cultured at subconfluence and treated with or without dracorhodin perchlorate at $60 \mu\text{M}$ for different time periods. Both adherent and

non-adherent HeLa cells (2×10^6) were collected. After brief rinsing in PBS, pellets were frozen at -80°C until analysis. The pellets were resuspended in $50 \mu\text{l}$ ice-cold lysis buffer (Calbiochem, San Diego, CA, USA), placed on ice for 10 min, and added to $50 \mu\text{l}$ of $2 \times$ reaction buffer containing 10 mM DTT (Calbiochem). Five microliters of YVAD-AFC (Calbiochem) was then added to give a final concentration of $50 \mu\text{M}$. The plates were incubated in a 5% CO_2 incubator at 37°C for 2 h. Amounts of released AFC were measured by using a fluorescence spectrophotometer (Hitachi, Tokyo) with excitation wavelength at 400 nm and the emission wavelength at 505 nm.

Caspase-3, caspase-8, and caspase-9 activities assay

HeLa cells were cultured at subconfluence and treated with or without dracorhodin perchlorate at $60 \mu\text{M}$ for different time periods. Both adherent and non-adherent HeLa cells were collected. After brief rinsing in PBS, pellets were frozen at -80°C until analysis. The pellets were suspended in ice-cold lysis buffer obtained from Alexis (San Diego, CA, USA), placed on ice for 10 min, and then centrifuged at $13,000 \times g$ at 4°C for 15 min. A $100\text{-}\mu\text{g}$ sample of protein was diluted in $50 \mu\text{l}$ of lysis buffer and added to $40 \mu\text{l}$ of reaction buffer. A $10\text{-}\mu\text{l}$ aliquot of the appropriate caspase substrate was then added to give a final concentration of $100 \mu\text{M}$. The plates were incubated in a 5% CO_2 incubator at 37°C for 3 h. The substrates for each caspase were as follows: Ac-DEVD-pNA for caspase-3-like activity (Alexis), Ac-LEHD-pNA (Calbiochem) for caspase-9 activity, and Z-IETD-pNA (Calbiochem) for caspase-8 activity. The substrates were prepared as 1 mM stocks according to the manufacturer's instructions and stored at -20°C . Amounts of released *p*-nitroanilide (pNA) were measured by measuring absorbance at 405 nm.

Western blotting analysis

Immunoblotting of cell lysates was performed as previously described (21). Both adherent and non-adherent cells were harvested; washed twice with ice-cold PBS; and then lysed in lysis buffer (50 mM Hepes pH 7.4, 1% Triton X-100, 2 mM sodium orthovanadate, 100 mM sodium fluoride, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, $10 \mu\text{g/ml}$ aprotinin and $10 \mu\text{g/ml}$ leupeptin) at 4°C for 60 min. The lysate was centrifuged at $15,000 \times g$ for 10 min and the supernatant was used for Western blotting 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

Bcl-2, Bax, inhibitor of caspase-dependent DNase (ICAD), Bcl-X_L (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 visualized by using 3,3'-diaminobenzidine tetrahydrochloride as the HRP substrate.

Measurement of intracellular ROS

HeLa cells at subconfluence were treated with or without dracorhodin perchlorate at 60 μ M for different time periods and then were washed twice and resuspended at a concentration of 5×10^5 cells/ml with PBS. For loading 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA) (Sigma) into the cells, the cells were incubated with the dye at a final concentration of 10 μ M at 37°C for 2 h. The cell suspension was well stirred and fluorescence (excitation wavelength at 485 nm and the emission wavelength at 530 nm) was monitored using a fluorescence spectrophotometer (Hitachi) (22).

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 $\pm$ S.D., and they were considered significant at a *P* of less than 0.05. All *P*s are two tailed.

Results

Cytotoxic effects of dracorhodin perchlorate on tumour cells

As shown in Fig. 2, dracorhodin perchlorate inhibited cell growth of all the four human tumour cell lines,

HeLa, A375-S2, U937, and MCF-7, in a dose- and time-dependent manner; and after 24 h treatment, the IC₅₀ for dracorhodin perchlorate in each cell was less than that of 5-FU (Table 1). By 48 h after treatment with 60 μ M dracorhodin perchlorate, HeLa cell death rate reached nearly 100% (Fig. 2).

Dracorhodin perchlorate induces HeLa cell death through apoptotic pathways

In order to determine whether dracorhodin perchlorate-induced HeLa cell death was through apoptotic pathways, the morphological changes and DNA fragmentation were observed. When HeLa cells were cultured with 60 μ M dracorhodin perchlorate for 24 h, marked morphological changes were observed as compared with the untreated control. The majority of cells became round, and some of these cells showed membrane

Fig. 2. Dose-dependent inhibitory effect of dracorhodin perchlorate on HeLa cells. The cells were cultured for 12 (closed diamond), 24 (closed square), 36 (closed triangle), and 48 (open square) h with different concentrations of dracorhodin perchlorate. *n* = 3. Mean $\pm$ S.D.

Table 1. Inhibitory effects of dracorhodin perchlorate on U937, A375-S2, MCF-7, and HeLa cells

Compound	Concentration (μ M)	Cell death rate (%)			
		U937	A375-S2	MCF-7	HeLa
Dracorhodin perchlorate	20	56.14 $\pm$ 2.23	41.42 $\pm$ 4.80	18.05 $\pm$ 2.79	30.01 $\pm$ 1.10
	40	79.32 $\pm$ 3.05	59.16 $\pm$ 2.97	22.50 $\pm$ 0.61	65.21 $\pm$ 4.12
	60	92.27 $\pm$ 2.44	57.12 $\pm$ 2.71	46.08 $\pm$ 2.98	75.06 $\pm$ 5.61
	80	91.14 $\pm$ 8.98	57.56 $\pm$ 9.61	51.69 $\pm$ 2.54	89.76 $\pm$ 11.71
5-FU	20	28.41 $\pm$ 1.65	55.67 $\pm$ 5.74	26.57 $\pm$ 1.73	25.54 $\pm$ 0.47
	40	37.95 $\pm$ 4.02	52.47 $\pm$ 4.11	29.49 $\pm$ 2.52	31.19 $\pm$ 0.57
	60	59.55 $\pm$ 13.2	54.22 $\pm$ 6.18	30.03 $\pm$ 2.26	26.19 $\pm$ 2.05
	80	75.45 $\pm$ 11.20	58.87 $\pm$ 4.90	36.48 $\pm$ 3.97	36.90 $\pm$ 0.28

The cells were cultured with different concentrations of dracorhodin perchlorate and 5-FU for 24 h. *n* = 3. Mean $\pm$ S.D.

Fig. 3. Dracorhodin-perchlorate-induced apoptotic morphological changes and DNA fragmentation of HeLa cells. **A:** HeLa cells were incubated in the medium alone for 24 h (a) or the medium containing 60 μ M dracorhodin perchlorate for 24 h (b) (magnification = $\times 200$). Arrow indicates multi-blebbing cells and apoptotic bodies. Scale bar represents 100 μ m. **B:** HeLa cells were incubated in the medium alone for 36 h (a) (magnification = $\times 200$) or the medium containing 60 μ M dracorhodin perchlorate for 24 h (b) (magnification = $\times 200$, arrow indicates vesicle like area) and 36 h (c) (magnification = $\times 400$, arrow indicates fragmented nuclei). Hoechst 33258 staining. Scale bar represents 100 μ m. **C:** HeLa cells were cultured with dracorhodin perchlorate at 60 μ M for 0 (a), 12 (b), 24 (c), 36 (d), and 48 (e) h. Hae-III-digested phage ϕ x-174 DNA fragments were used as molecular markers (M).

blebbing (Fig. 3A). The morphological changes of nuclei were observed by Hoechst 33258 staining. In the control group, HeLa cell nuclei were round in shape

and stained homogeneously with Hoechst 33258. After 24-h treatment with 60 μ M dracorhodin perchlorate, a vesicle-like area is found near nuclei, which was seldom

Fig. 4

reported in the previous studies. By 36 h, apoptotic characteristics in HeLa cells were observed with marked blebbing nuclei (Fig. 3B). After 24-, 36-, or 48-h treatment with 60 μ M dracorhodin perchlorate,

HeLa cells exhibited a typical DNA fragmentation that is another hallmark of apoptosis (Fig. 3C). Thus, dracorhodin perchlorate induced HeLa cell death through apoptotic pathways.

Fig. 4. Involvement of caspases during dracorhodin-perchlorate-induced apoptosis in HeLa cells. A: Effects of caspase inhibitors on 60 μ M dracorhodin-perchlorate-induced HeLa cell death. The cells were cultured in the absence or presence of caspase inhibitors, 1 h prior to the addition of dracorhodin perchlorate, and then incubated for 12 h. * P <0.05, ** P <0.01 vs dracorhodin perchlorate at 60 μ M group. B: Activities of caspase-3 (diamond), -8 (square), and -9 (triangle) in dracorhodin-perchlorate-treated HeLa cells. The cells were assayed after treatment with dracorhodin perchlorate at 60 μ M for 12 and 24 h. n = 3. Mean $\pm$ S.D. ** P <0.01, *** P <0.001 vs without dracorhodin perchlorate group. C: Activities of caspase-1 in dracorhodin-perchlorate-treated HeLa cells. The cells were assayed after treatment with dracorhodin perchlorate at 60 μ M for 12 and 24 h. n = 3. Mean $\pm$ S.D. ** P <0.01 vs without dracorhodin perchlorate. D: Degradation of ICAD and PARP in dracorhodin-perchlorate-treated HeLa cells. The cells were treated with dracorhodin perchlorate at 60 μ M for the indicated times. ICAD and PARP were analyzed by Western Blotting.

Caspases were activated during dracorhodin-perchlorate-induced HeLa cell death

Caspases inhibitors, z-VAD-fmk, Ac-YVAD-cmk, z-DEVD-fmk, z-IETD-fmk, and z-LEHD-fmk, at doses ranging from 5 to 20 μ M effectively inhibited dracorhodin-perchlorate (60 μ M)-induced HeLa cell death. However, the caspase-10 inhibitor, z-AEVD-fmk, blocked the cell death at a higher dose (Fig. 4A).

After administration of dracorhodin perchlorate, caspase-3, -8, and -9 significantly activated at 12 h: caspase-3 activity was 11 times the negative control group, and caspase-8, -9 activity reached 2 and 4 times the control group, respectively. By 24 h, the initiator caspase-8, -9 activities began to decline, but caspase-3

activity continued to increase and reached levels 18 times higher than that of the control group (Fig. 4B). Dracorhodin perchlorate also induced activation of caspase-1 after 12-h treatment (Fig. 4C).

ICAD, the inhibitor of a caspase-dependent DNase referred to as CAD (DEF40/CPAN), is a caspase-3 substrate that is inactivated by cleavage and allows the DNase to execute the characteristic fragmentation of DNA. ICAD can be expressed as two isoforms, 45 kDa (ICAD-L/DFF45) and 35 kDa (ICAD-S/DFF35). Although both ICAD-L/DFF45 and ICAD-S/DFF35 binds to and inhibit CAD, only ICAD-L/DFF45 was reported to be functional (23, 24). Thus, the ICAD-L/DFF45 expression in HeLa cells was examined to

further confirm the activation of caspase-3. After exposure to 60 μ M dracorhodin perchlorate for 24 h, ICAD-L was barely detected. Another characteristic associated with the execution phase of the apoptosis pathway is the specific PARP cleavage by caspases. This cleavage leads to its inactivation, thus preventing futile DNA repair cycles (25). It has been identified that caspase-3 is the most efficient processing enzyme for PARP (26). Cleavage of PARP was examined after treatment with dracorhodin perchlorate. As expected, by 24 h, the amount of 116 kDa protein declined and the 85 kDa degraded product increased (Fig. 4D). These results were consistent with the caspase-3 activation preceding this time period and the obvious DNA fragmentation occurring after this time period.

Taken together, these results suggest that activation of the caspase cascade occurred in HeLa cells after exposure to 60 μ M dracorhodin perchlorate.

Involvement of Bcl-X_L and Bax in dracorhodin-perchlorate-induced apoptosis

Since the Bcl-2 family members are critical regulators of the mitochondrial pathway, we assessed the expression of the Bcl-2 family proteins, Bcl-2, Bcl-X_L, and Bax. At 6 h after treatment with dracorhodin perchlorate, the expression of Bcl-X_L began to decrease, but that of Bax began to increase at 24 h, while the level of Bcl-2 remained stable compared with that of Bcl-X_L (Fig. 5).

Effect of dracorhodin perchlorate on the basal generation of ROS

Production of ROS is associated with many forms of apoptosis. The intracellular ROS level was measured after cells were exposed to 60 μ M dracorhodin perchlorate. Relative changes in intracellular ROS were monitored using a fluorescent probe, DCFH-DA. DCFH-DA diffuses through the cell membrane and is hydrolyzed by intracellular esterases to non-fluorescent DCFH, which is then rapidly oxidized to highly

fluorescent 2',7'-dichlorofluorescein (DCF) in the presence of ROS. The DCF fluorescence intensity is proportional to the amount of ROS formed intracellularly. Dracorhodin perchlorate caused a sustained generation of ROS in HeLa cell death; caspase-1 and caspase-3 inhibitors attenuated the later generation of ROS (Fig. 6).

Effect of dracorhodin perchlorate on human PMBC and human embryonic lung (HEL 299) cells

Cytotoxic effect of dracorhodin perchlorate on human PBMC and human embryonic lung (HEL 299) cells were measured and the widely used clinical antitumor drug 5-FU was used as a positive control (Figs. 7 and 8). After 12-h culture with dracorhodin perchlorate (80 μ M), the viability of PBMC from three volunteers was about 80%, 75%, and 94%; at 24 h, it was 76%, 68%, and 72%; and after 48-h treatment, it was 50%, 48%, and 60% respectively. It also exerted a higher cytotoxic effect on HEL 299 cells than 5-FU.

Discussion

Our present findings demonstrated that dracorhodin perchlorate directly inhibited the proliferation of human HeLa, A375-S2, U937, and MCF-7 cells. During 24-h culture, compared with 5-FU, dracorhodin perchlorate had stronger antiproliferative effect on all the tumor cells except for A375-S2 human melanoma cells. Based on the morphological changes of HeLa cells and DNA fragmentation, we concluded that dracorhodin perchlorate induced HeLa cell death through apoptotic pathways.

Caspases are a family of proteins that play an essential role during apoptosis. The results in this study showed that pretreatment with z-VAD-fmk, Ac-YVAD-cmk, z-DEVD-fmk, z-IETD-fmk, z-LEHD-fmk, and z-AEVD-fmk effectively inhibited dracorhodin-perchlorate (60 μ M)-induced cell death. z-AEVD-fmk inhibited

Fig. 5. The expression of BCL-X_L, Bax, and Bcl-2 in dracorhodin-perchlorate-treated HeLa cells. The cells were treated with dracorhodin perchlorate at 60 μ M for the indicated times. BCL-X_L, Bax, and Bcl-2 were analyzed by Western Blotting.

Fig. 6. Alteration of the basal ROS generation in dracorhodin-perchlorate-treated HeLa cells. **A:** Cells were treated with dracorhodin perchlorate at $60 \mu\text{M}$ for the indicated times. **B:** cells were pretreated without any inhibitor, with caspase-1 inhibitor, or caspase-3 inhibitor 1 h prior to the addition of dracorhodin perchlorate at $60 \mu\text{M}$, and then further incubated for 36 h. Black, white, striated, and dotted columns represent control, dracorhodin perchlorate, dracorhodin perchlorate plus caspase-1 inhibitor, and dracorhodin perchlorate plus caspase-3 inhibitor, respectively. Intracellular ROS was measured as described in Materials and Methods. $n=3$. Mean $\pm$ S.D. ^{###} $P<0.001$ vs control group, ^{***} $P<0.001$ vs dracorhodin-perchlorate-treated group.

cell death at a higher dose compared with other caspase inhibitors; this may be due to the difference of cell membrane permeability between them. The activity of caspases-3, -8, and -9 increased followed the degradation of caspase-3 substrates, ICAD and PARP, and the activity of caspase-1 also increased. All these observations suggest that the caspase cascade plays a critical role in dracorhodin-perchlorate-mediated HeLa cell apoptosis, and the protection by caspase-8 and

Fig. 7. Effects of dracorhodin perchlorate and 5-FU on human PBMC. The PBMC were obtained from three healthy adult volunteers and were seeded on 96-well culture plates at a density of 5×10^4 cells/well. The cells were cultured for 12 (A), 24 (B), or 48 (C) h with different concentrations of dracorhodin perchlorate and 5-FU. $n=3$. Mean $\pm$ S.D.

caspase-10 inhibitors showed that death receptors may be involved in this event. A recent study on phagocytosis induced cell death highlights the role of ROS in the activation of caspase-3, -8 of the extrinsic (death receptor) pathways (27). Although caspase-1 is one of

Fig. 8. Effects of dracorhodin perchlorate and 5-FU on human embryonic lung HEL 299 cells. The cells were cultured with different concentrations of dracorhodin perchlorate for 24 (closed triangle), or 48 (closed square) h and 5-FU for 24 (open triangle) or 48 (open square) h. $n = 3$. Mean $\pm$ S.D.

the early caspases identified, its role in apoptosis is not so well understood as those of its cousins. One of our understandings of caspase-1's participation in dracorhodin-perchlorate-induced HeLa cell apoptosis is that it may increase the production of IL-1 β , which can inhibit proliferation of other cell types, including human mammary tumor cell line, murimyeloid cell line (M1), and human melanoma cells (28–30). It is demonstrated that in hypoxia and ischemia-induced neuronal cell death, caspase-1 is an apical activator of the cell death pathway in the premitochondrial collapse stage and that Rip2/Cardiak/Rick is a stress-inducible upstream modulator of procaspase-1 apoptotic activation (31). The evidence that Ac-YVAD-cmk prevented the degradation of PARP and the biochemical and morphological changes of apoptosis (25) also aroused our interest in a further study of the role of caspase-1 in dracorhodin-perchlorate-induced HeLa cell death.

A balance between members of the Bcl-2 family is thought to determine whether mitochondria remain intact or become permeabilized and release proteins that promote cell death (32). Bcl-X_L can bind to the CED-4-like portion of Apaf-1 and inhibits the association of Apaf-1 with procaspase-9 and thereby prevent caspase-9 activation (33, 34). Activation of Bax or Bak results in their oligomerization at the outer mitochondrial membrane and causes the release of a variety of pro-apoptotic molecules, including cytochrome *c*, from the intermitochondrial membrane space into the cytosol (35). Released cytochrome *c* then binds to Apaf-1, from 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 6 h after dracorhodin perchlorate treatment, the expression of the anti-apoptotic protein Bcl-X_L began to decrease and that of the pro-apoptotic protein Bax increased followed by this down-regulation. Caspase-9 was activated at 12 h, which was then followed by the degradation of the caspase-3 substrates. It is possible that dracorhodin perchlorate induces HeLa cell apoptosis by the down-regulation of Bcl-X_L and activation of Bax initiates the mitochondria-apoptosome pathways. Interestingly, the level of anti-apoptotic protein Bcl-2 remained constant; this is consistent with magnolol-induced apoptosis in human lung squamous carcinoma CH27 cells and magnolol-induced down-regulation of Bcl-2 in HepG2 cells (36). Therefore, whether this phenomenon depends on the cell type and the stimuli type must be further investigated.

Dracorhodin perchlorate caused a sustained generation of ROS in HeLa cell death; caspase-1 and caspase-3 inhibitors attenuated the later generation of ROS. It was reported that a caspase-1 inhibitor, Ac-YVAD-cmk, blocked a later burst of ROS during GSH depletion when added early in the death program (37); thus caspase-1 may also play a role in the later phase of ROS generation, and protection from apoptosis by Bcl-X_L may be related to its ability to serve as "anti-oxidant" in this system (38). Consequently, the decline of the expression of Bcl-X_L might also contribute to the delayed increase of ROS. Recently, Ricci and his coworkers reported that the oxidative burst occurring with the release of cytochrome *c* resulted from caspase mediated cleavage of complex I and II of the mitochondrial electron transport chain (39). Associated with the effects of caspase-1 and caspase-3 inhibitors on ROS generation, we assumed that increase of ROS in the present study may serve as an amplification of the death signal.

Cancer is currently a common cause of death in the world. Despite surgery, radiotherapy, and traditional chemotherapy, the prognosis of advanced cancer has not significantly improved over the past 10 years. Therefore, the development of novel agents with selectivity against critical apoptotic targets may provide a rational approach to management of cancers. Conventional Chinese medicines have been historically tested for safety and effectiveness to treat cancers, although in the present study, dracorhodin perchlorate had some cytotoxic effect on human PBMC and human embryonic lung HEL 299 cells, but it has stronger antiproliferative effect on U937, HeLa, and MCF-7 cells than 5-FU after 24-h treatment. Thus we hope the present results may provide a basic view for a further study on antitumor effects of dracorhodin perchlorate.

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