Effects of Polyphenolic Anthrone Derivatives, Resistomycin and Hypericin, on Apoptosis in Human Megakaryoblastic Leukemia CMK-7 Cell Line

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A tetrahydroxyanthrone derivative, resistomycin, was isolated from the culture broth of *Streptomyces sulphureus* and a similar polyphenolic dianthraquinone, hypericin, was isolated from an extract of *Hypericum perforatum* L. as modulators for apoptosis. Resistomycin inhibited apoptosis induced by actinomycin D (AD) with or without acceleration by colcemid (CL) in human megakaryoblastic leukemia CMK-7 cells. IC_{50} for inhibition against AD-induced apoptosis was about 0.5 μM and IC_{50} for inhibition against AD plus CL-induced apoptosis was about 1 μM. CL alone induced weak apoptosis in cells, which was enhanced by resistomycin. Hypericin did not inhibit AD-induced apoptosis and slightly enhanced CL-induced apoptosis. Emodin, corresponding to 1 of 2 anthraquinone units in hypericin, did not show any effect on this apoptotic system. AD-induced apoptosis was inhibited by the antioxidative flavonoid, luteolin (IC_{50} 45 μM), and a protein kinase C (PKC) inhibitor, staurosporine (IC_{50} 1.5 μM), but these compounds did not affect the CL-induced apoptosis. Hypericin and resistomycin scavenged superoxide anion radicals at the same rate as luteolin. PKC in CMK-7 cells was inhibited by hypericin and luteolin, but not significantly inhibited by resistomycin. This result suggests that the inhibition of AD-induced apoptosis by resistomycin is at least partly correlated with its antioxidative activity, and that the enhancement of CL-induced apoptosis by this compound depends upon the lack of PKC inhibitory activity. Though the mechanism is not clear, the enhancement of the CL-induced apoptosis might be hindered by PKC inhibition in the case of hypericin and luteolin.

Introduction

Apoptosis has been widely studied in cell biology and medicine, especially in cancer chemotherapy (Bamford *et al.*, 2000). We previously reported that a concomitant addition of actinomycin D (AD) and a tubulin polymerization inhibitor, colcemid (CL), caused rapid apoptosis in human megakaryoblastic leukemia cell line CMK-7 (Yamazaki and Tsuruga *et al.*, 2000). The time course study showed that a decrease of mitochondrial transmembrane potential and a release of cytochrome *c* into cytosol occurred prior to the cleavage of procaspase-9 and procaspase-3. This series of events is the usual way to activate an executioner enzyme, caspase-3, via constructing apoptotic machinery (apoptosome) with cytochrome *c*, Apaf-1, and procaspase-9 in drug-induced apoptosis (Li *et al.*, 1997). Cytochrome *c* release was markedly accelerated by CL, indicating that cytoskeletal disruption led to a loss of organelle membrane integrity and liberation of cytochrome *c* for apoptosis. However, AD was essential for this apoptosis. It did not proceed rapidly unless AD treatment was done along with or prior to CL treatment. The detailed mechanism combining the effects of AD and CL is not known. Apoptosis is a very complex system including many signal mediators such as reactive oxygen species (ROS) (Verhaegen *et al.*, 1995), protein kinases (Cross *et al.*, 2000), and caspases (Bratton *et al.*, 2000). Our preliminary experiments with α-tocopherol and luteolin (Yamazaki and Dang *et al.*, 2000) and cyclopiazonic acid and geldanamycin (Shiono *et al.*, 2001) showed that ROS and ATPase-like enzymes were involved in the signaling of this apoptosis. New compounds affecting apoptosis are therefore potential therapeutic drugs and become useful

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tools for investigating the signaling mechanism. We screened microorganisms and plant metabolites for effectors of apoptosis, and found that an anthrone (and benzo[*c,d*]pyrenedione) derivative, resistomycin, isolated from 1 species of actinomycetes had a unique property affecting this apoptosis.

Materials and Methods

Materials

AD, Ac-DEVD-amc, staurosporine, emodin, and a standard specimen of hypericin, and rat brain protein kinase C were purchased from Sigma Chemical Co. (St. Louis, MO), luteolin was from EXTRASYNTHES (GENAY, France), fetal bovine serum (FBS) was from Cansera International Inc. (Rexdals, Canada), and CL was from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Stock solutions of all reagents were prepared in dimethylformamide (DMF).

Cell culture and screening

The human magakaryoblastic luekemia cell line CMK-7 was kindly provided by Dr. T. Sato (Sato *et al.*, 1989). Cells were cultured in RPMI-1640 containing 10% FBS at 37 °C in humidified atmosphere containing 5% CO_2. Screening was done with 96-well plates in which were placed CMK-7 cell cultures (0.1 ml/well) containing 1 μM AD and 1 μM CL with samples. After incubation for 7 hr, apoptosis was checked by microscopically observing cell fragmentation.

Isolation of resistomycin and hypericin

A slant culture of *Streptomyces sulphureus* ISP 5104 was inoculated in 500-ml Sakaguchi flasks containing 100 ml of seed culture medium: 1% glucose, 0.5% beaf extract, 0.5% polypepton, and 0.3% sodium chloride in water (pH 7.0 before autoclaving). Cultivation was conducted at 30 °C for 3 days on a reciprocal shaker. Fermentation was then done in 500 ml Sakaguchi flasks, each containing 100 ml of the same medium. The seed culture (2 ml) was added to each flask, and cultivation was conducted at 30 °C for 5 days on a reciprocal shaker. A 2-liter fermentation broth was centrifuged at 2000 rpm for 10 min at 4 °C. The mycelium cake was extracted with EtOH. The extract was filtered and concentrated *in vacuo* to an aqueous solution. The condensed solution was mixed with the supernatant and was extracted with an equal volume of EtOAc (1 liter). The EtOAc extract was concentrated under reduced pressure to give a residue (400 mg). The EtOAc extract was subjected to Wakogel C-300 column chromatography (*n*-hexane-EtOAc by stepwise elution) to give 3 active fractions (40–60% EtOAc eluates, 12.2 mg). Fractions were combined and crystallized from EtOH to yield resistomycin (4.6 mg) as yellow needles, mp > 300 °C [lit. dec. 315 °C (Brockmann and Schmidt-Kastner, 1951)]; ^{1}H-NMR spectral data (acetone-d_6); δ 1.63 (6H, s, Me-12, 13), 3.05 (3H, s, Me-14), 6.31 (1H, s, H-4), 7.08 (1H, s, H-8), 7.32 (1H, s, H-11), 14.36, 14.55, 14.79 (phenolic OHs); ^{13}C-NMR spectral data (DMSO-d_6); 46.3 (C-1), 205.1 (C-2), 102.8 (C-2a), 170.5 (C-3), 100.6 (C-4), 170.0 (C-5), 106.1 (C-5a), 184.8 (C-6), 106.8 (C-6a), 168.2 (C-7) 119.7 (C-8), 152.3 (C-9), 114.5 (C-9a), 162.9 (C-10), 110.0 (C-11), 152.7 (C-11a), 107.5 (C-11b), 139.9 (C-11c), 128.9 (C-11d), 28.8 (C-12, 13), 25.9 (C-14); FAB-MS; *m/z* 377 ($M+H^+$).

Dry whole plants (8.5 g) of *Hypericum perforatum* L. were soaked with EtOH. The extract was concentrated and partitioned between CH_2Cl_2 and H_2O. The aqueous layer was extracted with EtOAc. The EtOAc extract (120 mg) was chromatographed on Wakogel C-300 ($CHCl_3$–EtOAc–MeOH) to obtain 30% MeOH eluate (18.8 mg). The fraction was subjected to column chromatography on Wakogel 100C18 (H_2O–MeOH by stepwise elution). 100% MeOH eluate yielded hypericin (3.9 mg) as a red amorphous solid. ^{1}H-NMR and FAB-MS data was consistent with that for commercial hypericin (Sigma).

Effect of compounds on apoptosis

CMK-7 cells (1×10^6 cells/ml) were treated with 1 μM AD, 1 μM AD plus 1 μM CL, or 1 μM CL with or without a test sample for 8 to 24 hr. Concentrations of the sample are given in Fig. 2. Cells (5×10^5 for caspase-3 assay and 1×10^6 for DNA cleavage assay) were collected by centrifugation, washed with cold PBS, and stored in a deep freeze until use. Assays were conducted as described elsewhere (Yamazaki and Tsuruga *et al.*, 2000).

Antioxidative activity and PKC inhibition

Superoxide anion scavenging activity was measured with a cytochrome *c* reduction system as described elsewhere (Hyland and Auclair, 1981). Superoxide anion radicals were generated by adding 0.5 M of NaOH in dimethylsulfoxide (DMSO) (1% (v/v)). To each sample (50 µl ethanol solution containing a 30 to 450 µM test sample) was added 0.85 ml of 0.2 M phosphate buffer (pH 8.6) containing 180 µM of EDTA (Na_2) and 18 µM of cytochrome *c*. Then, 0.4 ml of the radical solution or 1% water/DMSO solution was added to the mixture. After incubation at room temperature for 30 min, antioxidative activity was determined by measuring absorbance at 535, 550, and 565 nm. % scavenging = $100 - [\{ A_{550} - (A_{535} + A_{565}) / 2 \} / \{B_{550} - (B_{535} + B_{565}) / 2 \} \times 100$, where A represents absorbance for the test sample and B represents absorbance for the control without it.

PKC inhibition was evaluated with a commercial ELISA kit (MBL, Tokyo, Japan) with a CMK-7 cell extract as the enzyme. Calibration curve (absorbance increase at 490 nm in 5 min vs. enzyme unit) was prepared with a standard PKC specimen from Sigma (Cat. No. P0329, 103 U/ml). PKC activity in the CMK-7 cell extract was determined by reference to the calibration curve. Thus, a definite amount of CMK-7 cell enzyme equivalent to 7 mU of the standard PKC from Sigma (1U = 1 nmol phosphate transfer per min at 30 °C) was applied to each well in the ELISA experiment.

Results

In screening of about 600 microbial cultures and 200 plant extracts, the culture broth of *S. sulphureus* showed the strongest inhibitory activity against AD and CL-induced apoptosis. Fermentation was conducted in a medium as described in experiments. The ethyl acetate extract of the entire culture broth was purified by bioassay-directed fractionation using a combination of silica gel column chromatography and chrystallization to yield the compound (4.6 mg) as yellow needles. The ^{1}H-NMR spectrum of the compound showed signals assignable to 3 hydrogen-bonded phenolic hydroxyl protons (δ 14.36, 14.55, and 14.79), to 3 methyl protons (δ 1.63 (6H) and 3.05 (3H)), and to 3 aromatic protons (δ 6.31, 7.08, and 7.32). These signals were a useful starting point for determining the structure. Further inspection of ^{1}H- and ^{13}C-NMR data indicated a match with NMR data reported for resistomycin (Brockmann *et al.*, 1969; Höfle and Wolf, 1983), allowing the compound to be identified as resistomycin. Resistomycin has a benzo[*c,d*]pyrene ring, which was first isolated from *Streptomyces resistomycificus* as an antimicrobial metabolite (Brockmann and Schmidt-Kastner, 1951). This compound inhibited RNA polymerase (Haupt *et al.*, 1975) and HIV-1 protease (Roggo *et al.*, 1994), but its inhibition of apoptosis is reported here for the first time to our knowledge.

We had already found that an EtOH extract of *H. perforatum* L. induced marked cell elongation in apoptotic CMK-7 cells. The similarity between the structure of resistomycin and that of hypericin reported in *H. perforatum* L. (Piperopoulos *et al.*, 1997) prompted us to isolate hypericin and related metabolites from the plant. Morphological change-inducing activity was confirmed with isolated hypericin (data not shown).

We compared the effects of resistomycin, hypericin, a related anthraquinone derivative, emodin, and a typical antioxidant, luteolin, on AD and CL-

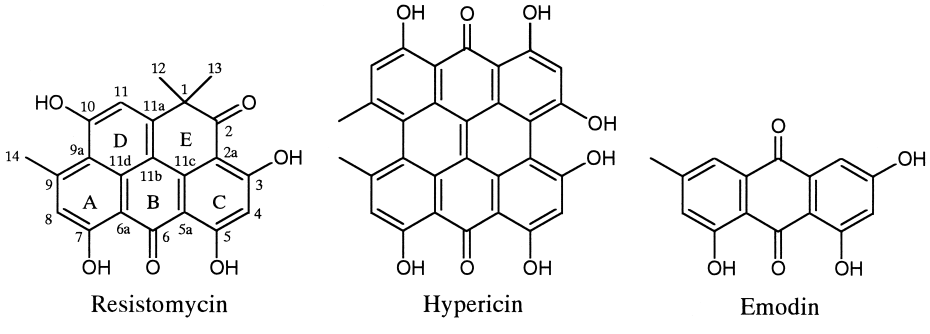

Fig. 1. Structure of resistomycin, hypericin, and emodin.

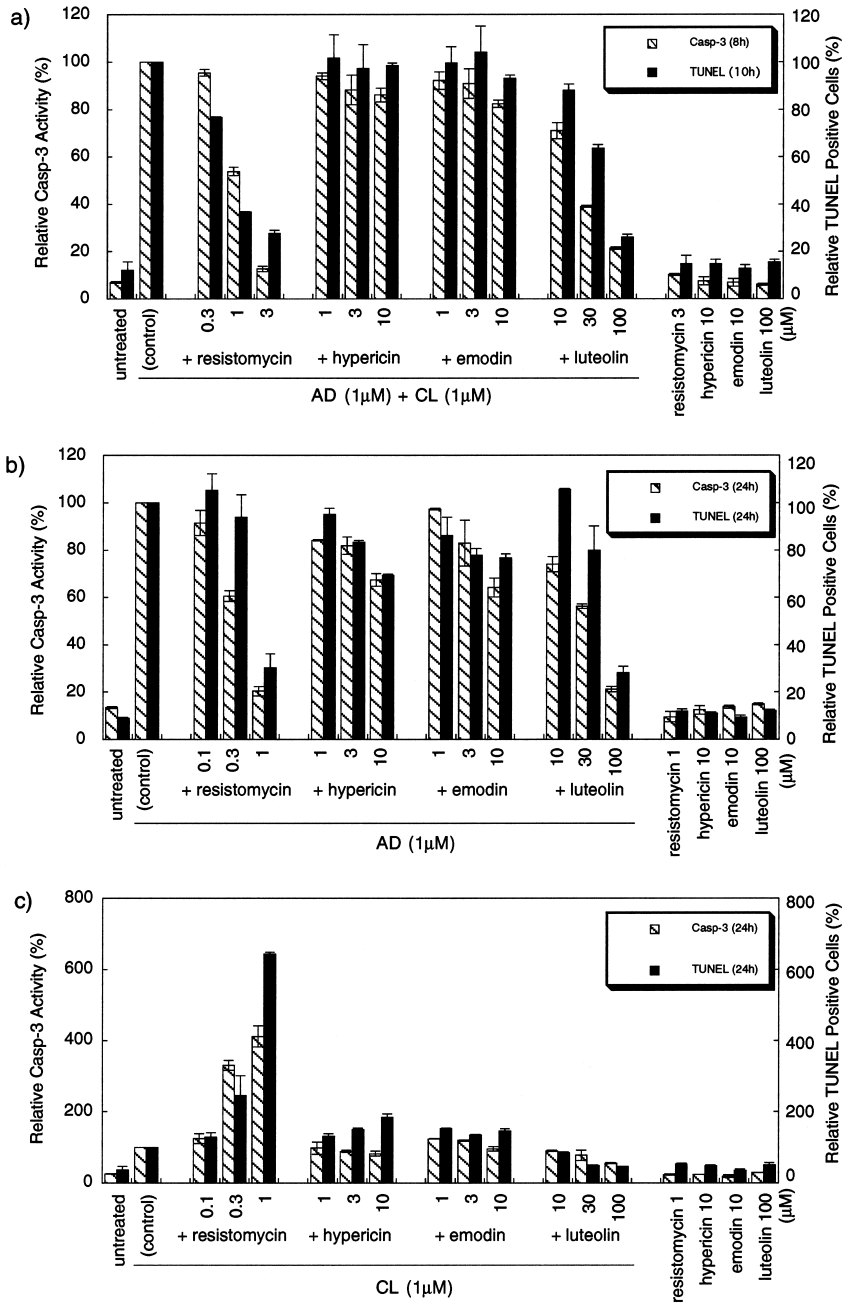

Fig. 2. Effect of resistomycin, hypericin, emodin, and luteolin on caspase-3 activity and the TUNEL positive cell ratio in (a) AD and CL-induced apoptosis, (b) AD-induced apoptosis, and (c) CL-induced apoptosis. CMK-7 cells (1×10^6 cells/ml) were incubated with or without 1 μM AD and/or 1 μM CL in RPMI-1640 medium containing 10% fetal bovine serum (FBS) in the absence or presence of test compound at different concentrations. Incubation time: (a) 8 hr for caspase-3 and 10 hr for TUNEL assays. (b and c) 24 hr for caspase-3 and TUNEL assays. Data is presented as% of the value obtained with the control mixture (with AD and CL but without the compound). Control values are 5.5, 5.5, and 1.1 nmol/hr/10^6 cells for caspase-3 activity, and 52, 56, and 16% for TUNEL positive cell ratio, for (a), (b), and (c), respectively. Data shown is the mean ± s.d. of triplicate samples in a representative experiment repeated twice with comparable results.

induced apoptosis in CMK-7 cells. Luteolin was tested as a reference because resistomycin and hypericin showed antioxidative activity (see below). Apoptosis was evaluated by caspase-3 activity and the DNA cleavage ratio (TUNEL positive cell ratio) (Yamazaki and Tsuruga *et al.*, 2000). As shown in Fig. 2a, resistomycin and luteolin dose-dependently suppressed apoptosis, while hypericin and emodin failed to suppress it at concentrations tested. Suppression by resistomycin was found at a lower concentration (IC_{50} of 1 μM for caspase-3 activity and 0.8 μM for TUNEL positive cell ratio)

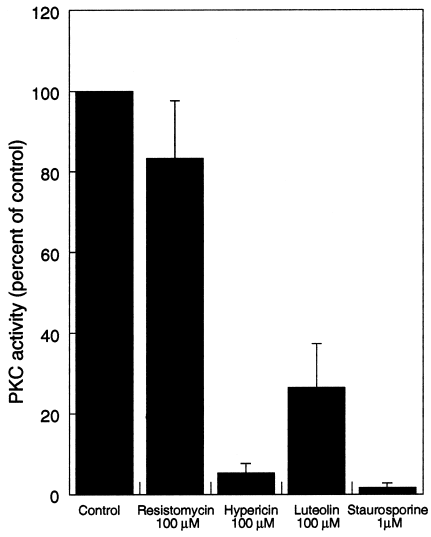

Fig. 3. Inhibition of protein kinase C (PKC) activity by resistomycin, hypericin, luteolin, and staurosporine. PKC activity was expressed by absorbance at 490 nm due to the peroxidase reaction (5 min), and given as% relative to the control ($A_{490\,nm}$ = 0.457 obtained with 7 mU PKC). Data shown is the mean ± s.d. of triplicate samples in a representative experiment repeated twice with comparable results.

than that by luteolin (IC_{50} of 23 μM for caspase-3 activity and 44 μM for TUNEL positive cell ratio). Each compound itself showed neither cytotoxic nor apoptotic effect on cells in the experimental time at the concentration in Fig. 2. When cells were treated only with AD, apoptosis was also suppressed by resistomycin and luteolin. Hypericin and emodin showed weak inhibitory activity at 10 μM in this case (Fig. 2b). CL alone slowly induced apoptosis in CMK-7 cells. Surprisingly, resistomycin enhanced caspase-3 activation and DNA cleavage. DNA cleavage was also somewhat enhanced by hypericin. Luteolin showed no effect on this CL-induced apoptosis (Fig. 2c). Hypericin is known to induce apoptosis under light irradiation and is used in photodynamic therapy (Agostinis *et al.*, 2002). We took care to avoid long exposure to light with the sample during assay, so hypericin itself did not show apoptosis in our experiment.

Hypericin and emodin were reported to have oxygen radical scavenging (Rahimipour *et al.*, 2001; Matsuda *et al.*, 2001). We evaluated the antioxidative activity of resistomycin together with hypericin, emodin, and luteolin. All 4 compounds showed antioxidative activity and the EC_{50} values were 2.0, 7.2, 7.5, and > 38.5 μM for hypericin, luteolin, resistomycin, and emodin, respectively.

Since a protein kinase C (PKC) inhibitor, staurosporine, suppressed apoptosis in CMK-7 cells (Yamazaki and Tsuruga *et al.*, 2000) and hypericin is known to have strong PKC inhibitory activity (Agostinis *et al.*, 1996), we tested the effect of resistomycin and hypericin on PKC of CMK-7 cells by ELISA. Hypericin showed strong inhibitory activity, but resistomycin did not (Fig. 3). In addition, luteolin also inhibited PKC in CMK-7 cells (Fig. 3) similarly to the previous report with bovine brain PKC (Agullo *et al.*, 1997).

Discussion

Antioxidants such as α-tocopherol and flavonoids are particularly important apoptotic inhibitors, because disadvantageous apoptosis occurring in normal organs and tissues is often induced by oxidative stress (Slater *et al.*, 1995). ROS are 1 of the earliest signal mediators in apoptosis so scavenging of ROS is more effective in avoiding apoptosis than inhibiting downstream mediators such as caspases. Actinomycin D treatment is known to generate ROS for apoptosis in HL-60 cells (Verhaegen *et al.*, 1995; Ikeda *et al.*, 1999). We also confirmed a ROS increase by AD in CMK-7 cells by flow cytometric analysis with ROS-specific dichlorodihydrofluorescein diacetate (unpublished). The inhibition of apoptosis in CMK-7 cells by α-tocopherol (Yamazaki and Dang *et al.*, 2000) and luteolin (Fig. 2a,b) supports the involvement of ROS in this apoptosis. It may thus be possible to assume that the suppression of apoptosis by resistomycin is also based on its antioxidative property. However, hypericin showed little or only weak suppression of apoptosis in AD-treated cells, (Fig. 2b) even though its antioxidative activity was stronger than that of resistomycin or luteolin. This may be due to poor uptake or unsuitable localization of hypericin in cells for its large hydrophilic structure. Emodin is also an antioxidant (Matsuda *et al.*, 2001), but its antioxidative activity would not be high enough to effectively scavenge ROS in apoptosis. If the result with hypericin and emodin excludes the possibility of ROS involvement in this apoptosis, the inhibition by resistomycin would be also conducted by a mechanism different from ROS scavenging. Hypericin is known to strongly inhibit protein kinase C (Agostinis *et al.*,

1996), which plays a role in apoptosis (Cross *et al.*, 2000). Apoptosis in CMK-7 cells was inhibited by a PKC inhibitor, staurosporine (Yamazaki and Tsuruga *et al.*, 2000). Resistomycin did not, however, show significant inhibition in the ELISA experiment (Fig. 3), suggesting that PKC is not the target molecule for resistomycin.

Interestingly, CL-induced slow apoptosis in CMK-7 cells was greatly enhanced by resistomycin (Fig. 2c), although it suppressed apoptosis in AD plus CL-treated cells. This enhancement was substantially specific to resistomycin and hardly found with hypericin, emodin, luteolin (Fig. 2c) and staurosporine (data not shown). Enhancement and inhibition are possibly done by closely related mechanisms. Precisely speaking, enhancement may be caused by resistomycin as prooxidants, because most antioxidants are also prooxidants under different conditions (Ferguson 2001; Brigelius-Flohé and Traber, 1999). The inactivity of hypericin and luteolin for the enhancement might be associated with their inhibitory activity of PKC. Little inhibition was found with resistomycin against PKC, and thus resistomycin would avoid interference with PKC on the signaling pathway. This idea bears more study. The synergistic enhancement of apoptosis is, in any case, important in combination chemotherapy for cancer (Shang *et al.*, 2001).

Although the mechanism for resistomycin to regulate apoptosis is not fully understood, a comparison of structures of the 3 compounds (Fig. 1) suggests that the trihydroxyanthrone nucleus is insufficient and the α,α-dimethyl-δ-hydroxyketone structure on rings D and E of resistomycin would be necessary to cause biological effects. In conclusion, resistomycin is a unique natural product showing antiapoptotic and proapoptotic activity in leukemia cells.

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