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Reversal of multidrug resistance in cancer cells by pyranocoumarins isolated from Radix Peucedani

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Abstract

The pyranocoumarins, (±)-3′-angeloyl-4′-acetoxy-*cis*-khellactone, were isolated from Radix Peucedani, the dry root of *Peucedanum praeruptorum* Dunn, through bioassay-guided fractionation. The chemical structure of pyranocoumarins was determined by mass spectrometry and nuclear magnetic resonance (NMR) spectroscopy. X-ray crystallography showed that there are eight molecules (i.e. two each of four conformers) in each unit cell with their optical activities equally cancelled out. The four conformers are 3′(*R*)-angeloyl-4′(*R*)-acetoxy-khellactone in two conformational forms, and 3′(*S*)-angeloyl-4′(*S*)-acetoxy-khellactone in two conformational forms. Pyranocoumarins caused apoptotic cell death with IC_{50} of 41.9 ± 2.8 and 17.3 ± 8.2 μM for drug-sensitive KB-3-1 and multidrug resistant (MDR) KB-V1, respectively. The two- to threefold sensitivity difference between the two cell lines is interesting considering that the same ratio for doxorubicin is 50–300. Strong synergistic interactions were demonstrated when pyranocoumarins were combined with common anti-tumor drugs including doxorubicin, paclitaxel, puromycin or vincristine in MDR KB-V1 cell line, but not in drug-sensitive KB-3-1 cells. Pyranocoumarins increased doxorubicin accumulation in KB-V1 cells by about 25% after 6 h of incubation. Pyranocoumarins treatment for 24 h down-regulated the expression of P-glycoprotein in KB-V1 cells at both protein and mRNA levels. Pyranocoumarins also transiently reduced the cellular ATP contents in KB-V1 cells in a dose-dependent manner. Our results suggest that pyranocoumarins could be a potential MDR reversing agent.

Keywords: *Peucedanum praeruptorum* Dunn; Pyranocoumarins; Multidrug resistance; KB-V1; P-glycoprotein; Apoptosis

1. Introduction

Resistance of cancer cells to chemotherapeutic agents remains one of the major obstacles in achieving an effective treatment for cancer. The molecular mechanisms of multidrug resistance (MDR) in cancer cells may involve overexpression of membrane drug efflux pumps, *p53* mutations, and up-regulation of bcl-2, DNA repair or cellular detoxification enzymes (Gottesman et al., 2001). One of the better understood areas concerns with drug efflux transporters, such as P-glycoprotein, multidrug resistance associated protein (MRP1), lung resistance protein (LRP) and breast cancer resistance protein (BCRP). P-glycoprotein is a 170-kDa trans-membrane glycoprotein, which is overexpressed in various MDR cell lines. It functions as an ATP-dependent drug efflux pump that rapidly extrudes a variety of hydrophobic anti-tumor drugs from target cancer cells, and thereby prevents the drugs from exerting their cytotoxic effects (Gottesman et al., 2002; Tan et al., 2000). Potential P-glycoprotein inhibitory agents have been developed with some of them on clinical trail (Goldstein, 1995; Thomas and Coley, 2003). The first generation of P-glycoprotein inhibitory agents being tested clinically include the calcium-channel blocker, verapamil, and the immuno-suppressant, cyclosporin A (Hindenburg et al., 1987; Thomas and Coley, 2003). Although having some efficacy, these agents are relatively weak P-glycoprotein inhibitors (EC_{50}, 2–10 μM) and exhibit dose-limiting side effects that severely restrict their clinical utility (Ozols et al., 1987; Tan et al., 2000). Thus there is considerable interest in the search for new P-glycoprotein inhibitors that

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do not elicit significant toxicity at doses required for P-glycoprotein inhibition.

Plant materials have a long history of being successfully used in the treatment of cancer, both as chemotherapeutic agents and as complementary treatments. In our laboratory, we have been screening various Chinese herbal drugs with specific actions in inducing the preferential death of MDR cancer cells or reversing MDR phenotype in cancer cells. Bioassay-guided fractionation of extracts from Radix Peucedani (the dried root of *Peucedanum praeruptorum* Dunn, Fam. Umbelliferae, also known as "Baihua Qianhu" in Chinese medicine) has led to the isolation of the pyranocoumarin compounds, (±)-3′-angeloyl-4′-acetoxy-cis-khellactone. The herb is well-known for the treatment of respiratory diseases and pulmonary hypertension. It contains a number of angular-type, nonglycosidic pyranocoumarins. The first isolation of praeruptorin A, B, C and D from the herb was reported by Chen et al. (1979). Praeruptorin A was found to be racemic to praeruptorin C, and praeruptorin B to D. The chemical structure of one of the enantiomer of praeruptorin A, thought to be praeruptorin C, was reported to be 3′(*S*)-angeloyloxy-4′(*S*)-acetoxy-3′,4′-dihydroseselin. Four seselin-type coumarins, called Pd-Ia, Pd-Ib, Pd-II and Pd-III, were isolated from the ether-soluble fraction of Radix Peucedani (Okuyama and Shibata, 1981).

Praeruptorin A was reported to have biological effects such as the calcium antagonistic action (Okuyama and Shibata, 1981; Zhao et al., 1999), calcium channel blocking effect (Chang et al., 1993; Li et al., 1994; Hao et al., 1996; Feng et al., 1998) and antagonistic effect on platelet aggregation induced by platelet activating factor (Aida et al., 1995). In the present study, we report a MDR reversal activity of these pyranocoumarins in human cancer cells.

2. Materials and methods

2.1. Chemical analyses

Melting points were determined on an Electrothermal 8100 melting point apparatus and are uncorrected. Optical rotations were measured on a Jasco DIP-370 Digital Polarimeter. Fourier transform infrared spectroscopy (FTIR) spectra were recorded on a Perkin-Elmer 1600 spectrophotometer, and UV spectra were taken on a Shimadzu UV-3100 spectrophotometer. NMR spectra were recorded in $CDCl_3$ solution on a Varian NMR-300 MHz spectrometer, using tetramethylsilicon as an internal standard. Silica gel 60 (200–300 mesh) was used for column chromatography. C18 Rocket Silicon Column and PE Series 200 Micro Pump was used for high performance liquid chromatography (HPLC). Sixty percent methanol was used as the solvent system and the UV detector was set at 320 nm. Liquid chromatography/mass spectrometry (LC/MS/MS) was recorded on a PE SCIEX API 365 LC/MS/MS system.

2.2. Plant material, extraction and fractionation

Radix Peucedani was purchased from Anhui Province of China in one lot. The herbarium specimen (Accession No. QH004A) is deposited at the City University of Hong Kong, Kowloon, Hong Kong. The dry root was powdered and extracted with 50% cold ethanol for 72 h. The ethanolic extract was filtered through Whatman Filter Paper No. 2 and ethanol removed by a rotary evaporator. The residue of ethanolic extract was then freeze-dried and weighed. The ethanolic extract was extracted by chloroform/water (1:1), and chloroform in the organic fraction was removed by a rotary evaporator. The residue was purified by a silica gel column using methanol-diethyl ether (1:5) as the eluting solvent. The eluate was dried and washed with petroleum ether. Repeated re-crystallization in ethanol at −20 °C produced white crystals of (±)-3′-angeloyl-4′-acetoxy-*cis*-khellactone (pyranocoumarins).

Pyranocoumarins were obtained as white needles (ethanol). Mp 153–154 °C. $[\alpha]_D^{20}$ 0° (*c*0.03, $CHCl_3$). UV (ethanol) λ_{max} (logε) at 25 μg/ml 324 (0.984), 255 (0.24), 210 (1.472). IR υ_{max}^{KBr} cm^{-1} 1740.86 (carbonyl stretching in α-pyrone), 1654, 1608, 1492 (C=C skeletal vibrations in aromatic ring), 1285, 1236, 1148, 1115 (C–O stretching vibrations in aryl ester groups), 847 (αβ-unsaturated ester moiety). ^1H NMR ($CDCl_3$, 300 MHz) δ 7.60 (1 H, d, J=9.53 Hz, H-4), 7.36 (1 H, d, J=8.51 Hz, H-5), 6.80 (1 H, d, J=8.51 Hz, H-6), 6.60 (1 H, d, J=4.99 Hz, H-4′), 6.24 (1 H, d, J=9.53 Hz, H-3), 6.13 (1 H, qq, J=7.33, 1.47 Hz, H-3′a), 5.41 (1 H, d, J=4.99 Hz, H-3′), 2.11(3 H, s, H-2″), 1.96 (3 H, dq, J=7.33, 1.47 Hz, H-4′a), 1.87 (3 H, qu, J=1.47 Hz, H-2′b), 1.47 and 1.43 (3 H, s, *gem*-$(Me)_2$). EIMS *m*/*z* (rel. int.): 409 $[M+Na]^+$ (100), 349.2 (13), 327.2 (20), 284.2 (13), 227.2 (46), 198.9 (7), 83 (43), 55 (78). CHN elemental analysis, *anal.* C 65.00%, H 6.08%, calcd for $C_{21}H_{22}O_7$, C 65.28%, H 5.74%.

Single crystals suitable for X-ray diffraction studies were grown by the slow diffusion of *n*-hexane into the concentrated ethanol and kept at −20 °C. $C_{21}H_{22}O_7$, Mr=386.40; primitive monoclinic, space group $P2_1/c$(#14), a=16.970(1) Å, b=12.5520(7) Å, c=18.671(1) Å, β=97.94(1) Å, V=3938.9(4) $Å^3$. D_c=1.303 g/cm^3; F_{000}=1632.00; μ(MoKα)=0.98 cm^{-1}; specimen: 0.22×0.110.07 mm; n_v=505; $|\Delta\rho$ max$|$=0.14 e $Å^{-3}$. All calculations were performed using the teXsan crystallographic software package of Molecular Structure. A total of 24,404 reflections were collected in ω-Scan mode to $2\theta_{max}$=55.1°, of which 9255 were unique. The structure was solved by direct methods and expanded using Fourier techniques. The non-hydrogen atoms were refined anisotropically. Hydrogen atoms were included but not refined. The final cycle of full-matrix least-squares refinement was based on 3635 observed reflections (I>1.50$\sigma(I)$) and 505 variable parameters and converged (largest parameter shift was 0.05 times its esd). The standard deviation of an observation of unit weight was 1.28. The weighting scheme was based on

counting statistics and included a factor ($p = 0.040$) to downweight the intense reflections. The maximum and minimum peaks on the final difference Fourier map corresponded to 0.50 and -0.36 $e^-/Å^3$, respectively. Atomic coordinates, bond lengths and angles, and thermal parameters may be obtained from the Cambridge Crystallographic Data Center.

2.3. Cell lines and reagents for bioactivity study

Human oral epidermoid carcinoma cell line KB-3-1 and its multidrug resistant subline KB-V1 were generously provided by Dr. Michael Gottesman, National Institute of Health, Bethesda (Shen et al., 1986). The KB-V1 line was derived from ethyl methanesulfonate treated KB-3-1 cells by a series of step selections for vinblastine resistance. KB-3-1 cells were maintained in minimum essential medium (Gibco, MD, USA) containing 10% fetal bovine serum. KB-V1 cells were grown in the same medium, with the addition of 150 ng/ml of vinblastine to maintain the MDR phenotype. Vinblastine, vincristine, doxorubicin, puromycin, paclitaxel, actinomycin D and verapamil were purchased from Sigma (St. Louis, USA).

2.4. Cytotoxicity assay

IC_{50} of pyranocoumarins and doxorubicin to KB-3-1 and KB-V1 cells was determined by Sulforhodamine B cytotoxicity assay (Skehan et al., 1990). Briefly, 3×10^3 of KB-3-1 or KB-V1 cells were seeded into 96-well plates and cultured overnight. Pyranocoumarins or doxorubicin was then added and incubated further for 72 h. The colour intensity of Sulforhodamine B produced, which had been confirmed to positively correlate to the number of cells, was estimated by determining OD at 515 nm. Solvent (for pyranocoumarins, 20% DMSO and 80% ethanol) and blank medium were included as control. Each set of experiments was independently performed at least three times.

2.5. Doxorubicin accumulation assay

Approximately 5×10^6 of KB-3-1 or KB-V1 cells were collected by trypsinization and re-suspended in 2 ml Hanks' balanced salted solution (HBSS). After being treated by pyranocoumarins at IC_{50} for different durations of time (0, 2, 4 and 6 h), cells were incubated with 2 μM of doxorubicin for 30 min at 37 °C in a humidified 5% CO_2 incubator. The doxorubicin inside the cells was estimated by a FACSCalibur flow cytometer (Becton Dickinson) at excitation 488 nm/emission 600 nm. Data were collected and analyzed using the CellQuest Software. Mean fluorescence intensities of cell samples containing 1×10^4 of either drug-treated or untreated cells were obtained. Verapamil, a well-known inhibitor of the P-glycoprotein pump, was used as a positive control. Each experiment was independently performed at least three times.

2.6. Synergistic effects of pyranocoumarins with various anti-tumor drugs on KB-V1 cells

Approximately 3×10^3 of KB-3-1 or KB-V1 cells were seeded into 96-well plates and cultured overnight. The culture medium was replaced with fresh medium containing serial dilutions of various combinations of pyranocouamrins with doxorubicin, vincristine, paclitaxel, puromycine, actinomycin D or mytomycin C, and further incubated for 72 h. Sulforhodamine B assay was performed and the isobologram was constructed (Chou and Talalay, 1984) to determine the combination index (CI) of pyranocoumarins and other anti-tumor drugs. Mutually nonexclusive equations were used to determine the combination index values. $CI>1$, $CI=1$, and $CI<1$ indicate antagonism, additive effect, or synergism, respectively. All the experiments were repeated at least three times.

2.7. Estimation of MDR1 mRNA expression by reverse transcriptase PCR (RT-PCR)

Total RNA from pyranocoumarins treated (for 24 h) or untreated KB-3-1 or KB-V1 cells was isolated using High Pure™ RNA Isolation Kits (Roche) according to the manufacturer's instruction. RT-PCR was performed as described previously (Fong et al., 2001). cDNA was synthesized from 1 μg of total RNA by incubation for 1.5 h at 42 °C in a reaction mixture containing 100 ng random primers, 1 × reaction buffer (50 mM Tris–HCl, 100 mM NaCl, 1 mM EDTA, 0.05% polydocanol v/v, 50% glycerol v/v, pH 8.4), 10 mM dNTP, 100 mM dithiothreitol and 50 units/ml Expand™ Reverse Transcriptase (Roche). PCR amplifications were carried out using Expand™ Long Template PCR System (Roche). Primers for MDR1 were 5′-CCCATCATTGCAATAGCAGG-3′ (sense) and 5′-GTTCAAACTTCTGCTCCTGA-3′ (antisense); primers for β-actin were 5′-GATGATATCGCCGCGCTCGTCGTCGAC-3′ (sense) and 5′-AGCCAGGTCCAGACGCAGGATGGCATG-3′ (antisense). Five microcuries of [α-^{32}P]dATP was added to the 25-μl amplification reaction mix. PCR was performed in a Gene Cycler™ (Bio-Rad) for 27 cycles with each cycle consisting of 1 min at 94 °C, 1 min at 57 °C and 1 min at 72 °C. PCR products were separated on 10% polyacrylamide gel.

2.8. Western blotting of P-glycoprotein

KB-V1 cells were treated with pyranocoumarins for 24 h, harvested and lysed in ice-cold lysis buffer (1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 1% Triton X-100, 2 mM phenylmethylsulphonyl fluoride, 10 μg/ml aprotinin). Protein concentration was determined by the Bradford assay and 50 μg samples were subjected to 8% SDS-polyacrylamide gel electrophoresis and electro-transferred to nitrocellulose membranes. Membranes were blocked with 5% nonfat milk/0.1% Tween-20/Tris-buffered

saline (TBS) (10 mM Tris pH7.5, 100 mM NaCl) then incubated with anti-P-glycoprotein antibody (Calbiochem) for 1 h at room temperature before incubation with horseradish-peroxidase-conjugated secondary antibody (Gibco BRL) for 1 h at room temperature. The protein was detected by the chemiluminescence assay system (Amersham, NJ, USA). β-actin was used as a control for equal sample loading and was detected by a polyclonal antibody (Oncogene Science, Uniondale, NY, USA). KB-3-1 cell extract was also included as a basal expression level control.

2.9. ATP assay

Approximately 10^6 KB-V1 cells were seeded in a 100-mm dish and incubated overnight. Various amounts of pyranocoumarins were added and cells were incubated further for 2 or 4 h. Cells were harvested, counted and lysed by the ATP assay buffer. The cellular ATP content was measured using an ATP-luciferase kit (Bioluminescent Somatic Cell Assay Kit) according to the manufacturer's instruction. Fluorescence was recorded by Moonlight 1500 (Analytical Luminesence Laboratory). Ten measurements were taken for each sample and averaged by the luminometer. The cellular ATP concentration was calculated from the ATP standard calibration curve.

2.10. Statistic

Each experimental result value is expressed as mean ± standard deviation (S.D.) and data were analyzed by Student's *t*-test. Differences from the respective controls at $P < 0.05$ were considered significant.

3. Results

3.1. Characterization of pyranocoumarins

Traditionally, plant pyranocoumarins have been isolated upon successive extractions with solvents of increasing polarity including light petroleum, diethyl ether, benzene, ether, acetone, and methanol. We started with a 50% ethanol extraction of the plant material. After drying and re-extraction with chloroform/water (1:1), organic products were purified by silica gel chromatography and re-crystallized in cold ethanol. Crystals of sufficient size for crystallography were obtained from ethanol-*n*-hexane re-crystallization.

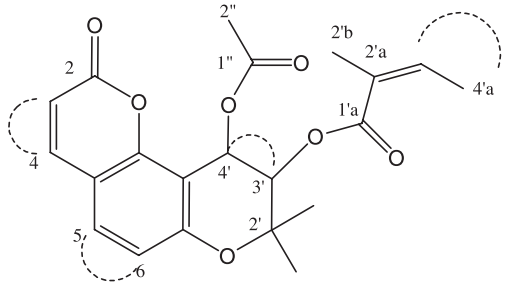

Fig. 1. Significant correlations observed in the 1H–1H COSY NMR spectra.

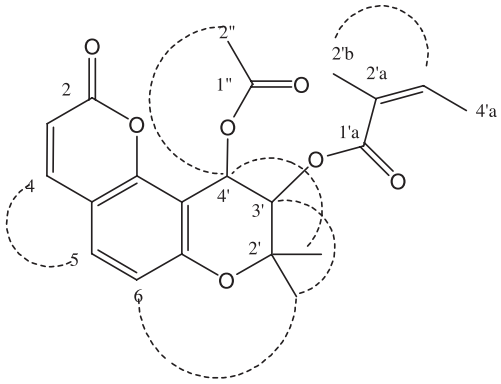

Fig. 2. Significant correlations observed in the NOESY NMR spectra.

The ^{1}H NMR spectrum of pyranocoumarins contains two pairs of doublets at $\delta = 6.24$ and 7.60 (each 1 H, $J = 9.53$ Hz) and at $\delta = 7.36$ and 6.80 (each 1 H, $J = 8.51$ Hz), which are in agreement with the 3-H and 4-H signals of the α-pyrone ring system and a significant *o*-coupling signal due to 5-H and 6-H on the coumarin ring. A pair of doublets at $\delta = 5.41$ and 6.60 ppm (each 1 H, d, $J = 4.99$ Hz) was assigned to the methine protons at C-3′ and C-4′ of *cis*-khellactone which showed a characteristic splitting pattern ($J = 4.99$ Hz). A quartet coupling at $\delta = 6.13$ (1 H, qq, $J = 7.33$, 1.47 Hz) to a doublet quartet coupling at $\delta = 1.96$ (3 H, dd, $J = 7.33$, 1.47 Hz) was assigned to H-3′a and H-4′a, respectively. The signal at $\delta = 2.11$ ppm (3 H, s) was due to an acetyl group at H-2″ and the quintet signal at $\delta = 1.87$ ppm (3 H, qu, $J = 1.47$ Hz) resulted from the H-2'b at 2-methyl-butyrate moiety. Two close singlets at $\delta = 1.47$ and 1.43 ppm ($\Delta = 0.04$ ppm), due to the 2′-*gem*-$(Me)_2$ groups of a dihydropyran ring, indicate a *cis*-configuration at C-3′ and C-4′ (Gonzalez et al., 1979). The assignments of ^{1}H NMR resonance were confirmed through analysis of its ^{1}H–^{1}H COSY and NOESY data (Figs. 1 and 2). With an optically inactive chemical property, we established the structure as (±)-*cis*-3′-angeloyl-4′-acetoxy-khellactone (Fig. 3).

Recrystallization of pyranocoumarins from ethanol-*n*-hexane yielded a crystal of sufficient quality suitable for X-ray structure analysis, which on completion led to the solution of two enantiomers with two half-chair conformers in respect of their dihydropyran ring. Fig. 4 shows the perspective drawings with absolute stereostructure of one

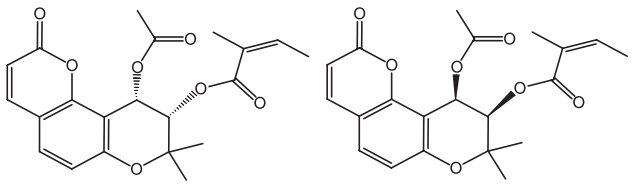

Fig. 3. Chemical structures of pyranocoumarins.

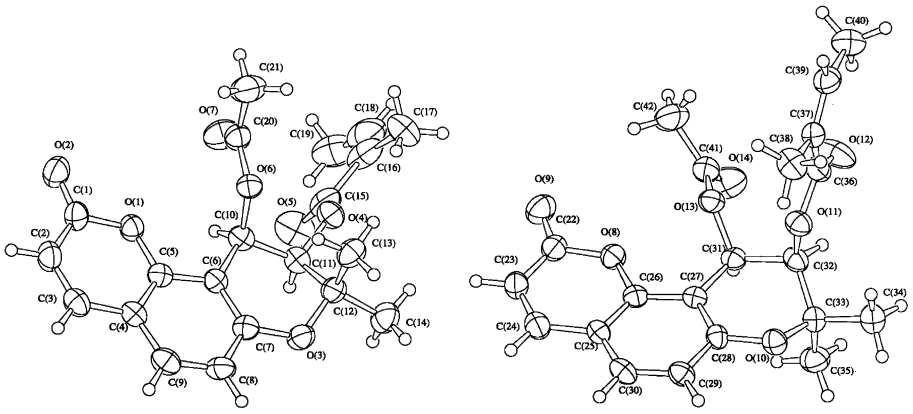

Fig. 4. Perspective view of conformers of the C-3′(*R*), C-4′(*R*) enantiomers.

of the enantiomers with its half-chair conformers. It is a compound with two asymmetric centers at C-3′ and C-4′. The compound crystallizes with two conformational isomers in space group $P2_1/c$, which is a centrosymmetric primitive monoclinic unit cell with *c*-glide plant $\perp 2_1$ axis $\| b$. This means that in each unit cell, in addition to two symmetry related copies of the two molecules illustrated in Fig. 4, there are two more molecules with an enantiomorphic chiral relation to the two illustrated with two symmetry related copies, produced by crystallographic centers of symmetry in the space group. As such, there are eight molecules (i.e. four molecules with their symmetry related copies) in each unit cell and their optical activities are equally cancelled out. The four molecules are 3′(*R*)-angeloyl-4′(*R*)-acetoxy-khellactone in two conformational forms, and the 3′(*S*)-angeloyl-4′(*S*)-acetoxy-khellactone in two conformational forms.

3.2. Cytotoxicity of pyranocoumarins on KB-3-1 and KB-V1 cells

Cytotoxic activity of pyranocoumarins on KB-3-1 and KB-V1 cells was studied using Sulforhodamine B cytotoxicity assay. The IC_{50} values obtained were 17.26 ± 8.24 μM for KB-V1 and 41.91 ± 2.80 μM for KB-3-1, respectively (Table 1). In other words, KB-V1 cells are more sensitive to these compounds than KB-3-1 cells. However, under the same experimental conditions KB-V1 cells are highly resistant to cancer drugs such as doxorubicin, with an IC_{50} value of 3.05 ± 0.28 μM. In comparison, the IC_{50} value of doxorubicin to the drug sensitive KB-3-1 cells was 0.06 ± 0.01 μM, showing a sensitivity difference of over 50 folds. DNA fragmentation analysis further revealed that the pyranocoumarins-induced cell death was associated with apoptosis. After being treated with various amounts of pyranocoumarins for 24 h, DNA ladder was detected in both KB-V1 and KB-3-1 cells in a dose-dependent manner, but DNA ladder was more visible in KB-V1 cells than in KB-3-1 cells (Fig. 5).

Table 1
The IC_{50} for doxorubicin and pyranocoumarins to KB-3-1 and KB-V1 cells

Cell line	IC_{50} (μM ± S.D.)	
	Doxorubicin	Pyranocoumarins
KB-3-1	0.0639 ± 0.0106	41.9153 ± 2.8016
KB-V1	3.0510 ± 0.2846	17.2656 ± 8.2441
Ratio[a]	47.7	0.41

[a] The IC_{50} ratio of MDR KB-V1 cells to drug-sensitive KB-3-1 cells indicated the degree of resistance of MDR cells towards a particular drug.

3.3. Pyranocoumarins increased doxorubicin accumulation in KB-V1 cells

Because of a high express level of the P-glycoprotein drug efflux pump, MDR KB-V1 cells accumulate and retain less anti-tumor drugs when compared with drug-sensitive KB-3-1 cells. To determine whether pyranocoumarins have any effect on the retention of anti-tumor drugs, KB-V1 cells were treated with 17.3 μM pyranocoumarins for 2, 4, 6 and 8 h before being incubated with 2 μM doxorubicin. The fluorescence of intracellular doxorubicin was measured by flow cytometer. As shown in Fig. 6, pyranocoumarins significantly increased the intracellular accumulation of doxorubicin in KB-V1 cells after 4 h of treatment, and after 6 h of pyranocoumarins treatment doxorubicin fluorescence intensity was increased by 25%. However, in the drug-sensitive KB-3-1 cells in which P-glycoprotein pump activ-

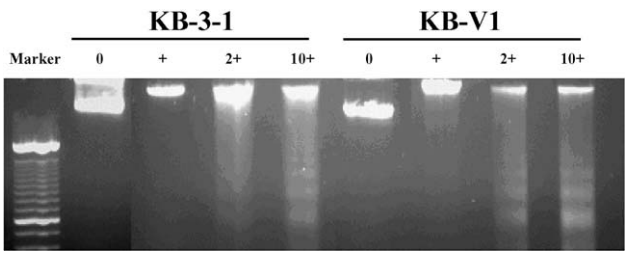

Fig. 5. Pyranocoumarins induced apoptosis in KB-3-1 and KB-V1 cells. KB-3-1 and KB-V1 cells were treated with pyranocoumarins (0: control; +: $1 \times IC_{50}$; (17.3 μM) 2+: $2 \times IC_{50}$; (35 μM) 10+: $10 \times IC_{50}$ (173 μM)) for 24 h. Cellular DNA was extracted and subject to electrophoresis on 2% agarose gel. Similar results were obtained in three independent experiments.

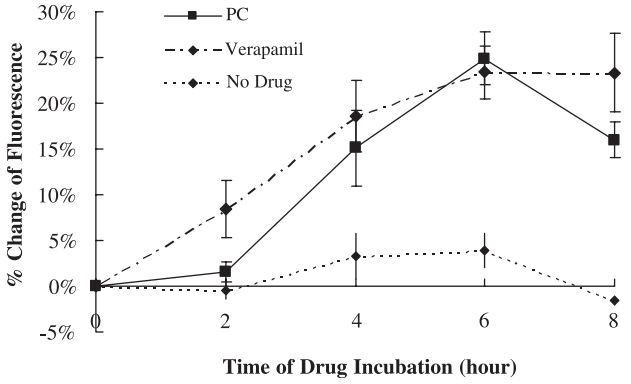

Fig. 6. Accumulation of doxorubicin in pyranocoumarins-treated KB-V1 cells. Cells were incubated with doxorubicin and pyranocoumarins and cellular doxorubicin fluorescence of 1×10^4 cells were measured. Controls were untreated cells at the beginning of experiments (time 0) and verapamil-treated cells. Similar results were obtained in two independent experiments. PC, pyranocoumarins.

ity is relatively low, pyranocoumarins had no significant effect on doxorubicin accumulation (data not shown).

3.4. Synergistic effect of pyranocoumarins with anti-tumor drugs

We next asked whether pyranocoumarins could augment the cytotoxic effect of anti-tumor drugs. The interaction of pyranocoumarins with common anti-cancer drugs was experimentally quantified by the plotting of combination index curves (Chou and Talalay, 1984). Pyranocoumarins were mixed with doxorubicin, paclitaxel, vincristine, puromycin, actinomycin D or mitomycin C individually at ratios equal to the concentration ratios of respective IC_{50}, while varying the total concentration for each pyranocoumarins/cancer drug pair. The combined cytotoxicity was assessed in both KB-3-1 and KB-V1 cells by Sulforhodamine B assay. Combination index curves for both mutually exclusive and

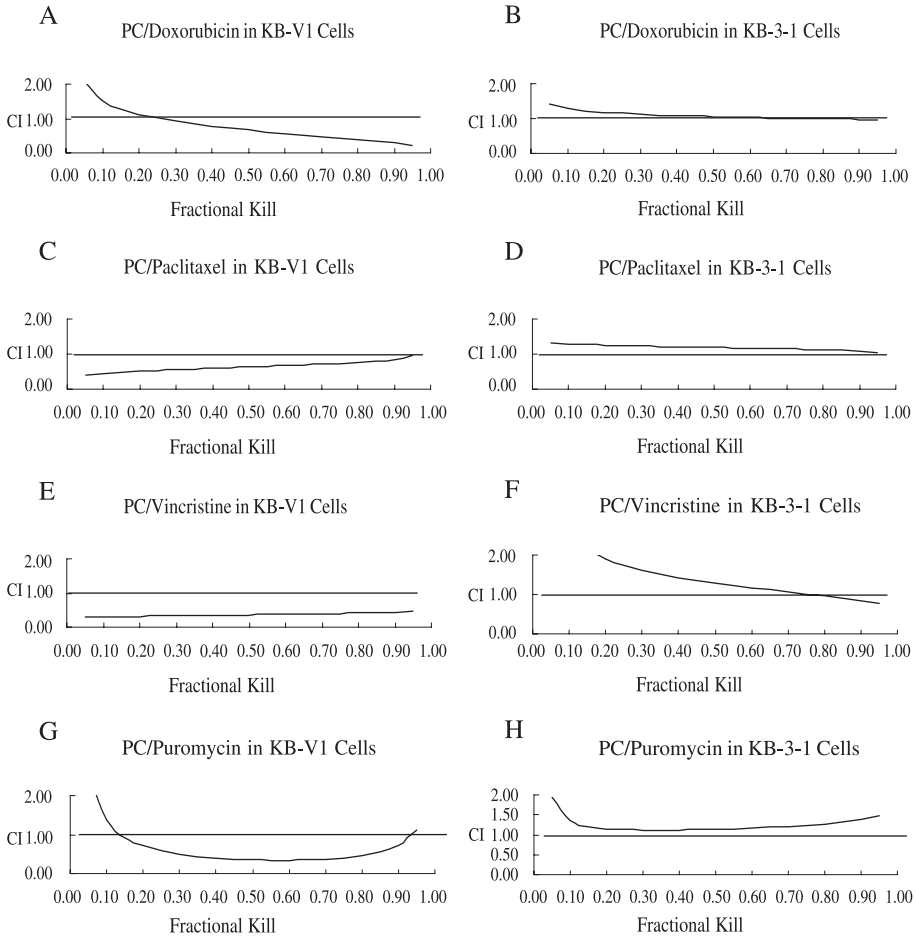

Fig. 7. Synergism between pyranocoumarins and doxorubicin, paclitaxel, vincristine, or puromycin. KB-V1 cells in 96-well plates were treated with combinations of pyranocoumarins (PC) with doxorubicin (A), vincristine (C), paclitaxel (E) or puromycin (G) for 72 h and then cell survival was assessed by SRB assay. Combination index (CI) curves were generated and since similar conclusions could be drawn from mutually exclusive and non-exclusive curves, only mutually non-exclusive curves are shown here. KB-3-1 cells were studied as a control (B, D, F and H). These data represent the averages from three separate experiments.

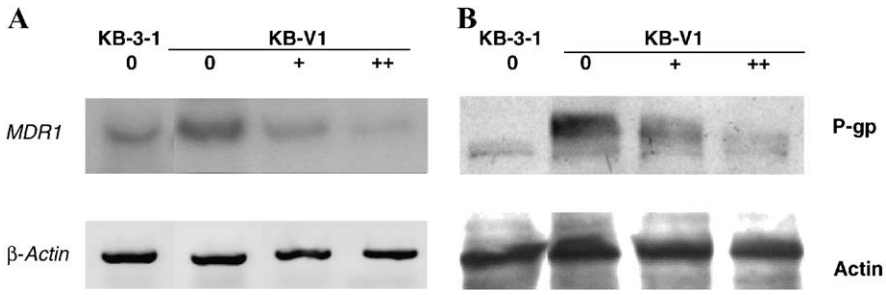

Fig. 8. P-glycoprotein expression in Pyranocoumarins-treated cells. *MDR1* mRNA and P-glycoprotien protein were detected by RT-PCR (A) and Western blot (B), respectively. KB-V1 cells were treated with various amounts of pyranocoumarins (0: control; +: 1 × IC_{50}; (17.3 μM) ++: 2 × IC_{50} (35 μM)) for 24 h and total RNA or protein was studied. Actin and β-actin were included as internal standards. KB-3-1 cells were studied as the control. Similar results were obtained in two independent experiments.

nonexclusive modes of interaction were generated. In our experiments variations between the two curves did not affect our conclusion and for simplicity only mutually nonexclusive curves are shown here. Significant synergistic effects were observed when pyranocoumarins were mixed with either doxorubicin, paclitaxel, vincristine or puromycin in KB-V1 cells (Fig. 7A,C,E,G), but not with actinomycin D or mitomycin C (data not shown). On the other hand, in KB-3-1 cells only additive or antagonistic effects were observed (Fig. 7B,D,F,H).

3.5. RT-PCR and western blot analysis of MDR1 gene and P-glycoprotein expression

Consistent with the previous reports, RT-PCR (Fig. 8A) and Western blotting (Fig. 8B) showed that KB-V1 cells expressed higher levels of *MDR1* mRNA and P-glycoprotein protein as compared to KB-3-1. However, when KB-V1 cells were treated with various doses of pyranocoumarins for 24 h, the levels of *MDR1* mRNA and P-glycoprotein were markedly decreased (Fig. 8A,B).

3.6. Pyranocoumarins diminished cellular ATP content in KB-V1 cells

As P-glycoprotein is an ATP-dependent membrane transporter, altering the cellular ATP content would likely affect the function of P-glycoprotein. KB-V1 cells were treated with 1 × IC_{50} (17.3 μM) and 2 × IC_{50} (35 μM) of pyranocoumarins for various durations and cellular ATP concentrations were then determined. Table 2 shows that the average cellular ATP content in KB-V1 cells dropped more than 50% after being treated with 2 × IC_{50} (35 μM) of pyranocoumarins for just 2 h. In untreated KB-V1 cells the ATP content was found to be fairly constant over the entire course of the experiment.

Table 2
Change of ATP content in KB-V1 cells

Pyranocoumarins	ATP content		
	0 h	2 h	4 h
untreated	0% ± 0	0.26% ± 0.03	3.36% ± 1.1
1 IC_{50} (17.3 μM)	1.10% ± 0.1	4.57% ± 2.1	− 5.20% ± 1.8[a]
2 IC_{50} (35 μM)	2.70% ± 0.2	− 62.80% ± 12.9[a]	− 23.05% ± 8.5[a]

[a] Significant difference at $P < 0.05$ when compared with untreated control at the same time point.

4. Discussion

Praeruptorin A and Pd-Ia have been considered as the same *cis*-dihydroseselin compound isolated from BaiHua QianHu, but their absolute stereostructures have not been reported. In the present study, we isolated the pyranocoumarin compounds, (±)-3′-angeloyl-4′-acetoxy-*cis*-khellactone, from this herb and for the first time determined its crystal structure. Pyranocoumarins agreed mostly with praeruptorin A in Infra Red, Melting Point, Optical Rotation, and elementary analysis as reported by Chen et al. (1979), although there are some deviations in ^{1}H NMR. Our crystallography study revealed that there are eight molecules within each unit cell (two each of four types of conformers).

Relative to its parental KB-3-1 cells, the P-glycoprotein over-expressing KB-V1 cells were 200-fold more resistant to the cytotoxic action of vinblastine (Watanabe et al., 1995) and showed a strong cross-resistance to doxorubicin. Our cytotoxicity study revealed that although pyranocoumarins are less effective than doxorubicin, MDR KB-V1 cells showed a little more sensitive to pyranocoumarins (IC_{50} 17.3 μM) than drug-sensitive KB-3-1 cells (IC_{50} 41.9 μM). This suggested that pyranocoumarins possess the ability to circumvent the MDR phenotype conferred by the over-expression of P-glycoprotein.

We demonstrated that pyranocoumarins-caused cell death involved apoptotic mechanisms (Fig. 5). Previous reports suggested that some MDR cancer cells underwent apoptosis much more readily than drug-sensitive cells under certain conditions such as exposure to 2-deoxy-D-glucose (Bell et al., 1998) or inhibitors of glucosylceramide synthase (Nicholson et al., 1999). We are in the process of identifying the molecular events leading to the apoptotic cell death of pyranocoumarins-treated cells.

In the drug combination studies and the plotting of the combination index curves, we demonstrated a synergistic effect in the killing of KB-V1 cells by combining pyranocoumarins with doxorubicin, vincristine, paclitaxel or puromycin. We first found that pyranocoumarins increased intracellular doxorubicin accumulation in P-glycoprotein over-expressing KB-V1 cells, but not in drug sensitive KB-3-1 cells, suggesting the immediate target of pyranocoumarins was the drug efflux pump (Wu et al., 1996). Furthermore, when compared with verapamil, a well-established P-glycoprotein inhibitor, pyranocoumarins seems to be as effective in enhancing doxorubicin accumulation. Verapamil, a calcium channel blocker, could also block the function of P-glycoprotein (Krishna and Mayer, 2000). Previous studies on the bioactivities of coumarins from *P. praeruptorum* Dunn have shown that they exhibited some calcium channel blocking actions (Kozawa et al., 1981). Further studies on the mechanism of pyranocoumarins action may help in the understanding of the function of P-glycoprotein.

Pyranocoumarins treatment rapidly and transiently reduced the cellular ATP content by approximately 50% in KB-V1 cells (Table 2). Although it is likely that this depletion of ATP could, to some extent, affect the functions of P-glycoprotein, at the present time it is difficult to assess the influence of a reduced ATP level on P-glycoprotein function and how this affects the MDR phenotype of the KB-V1 cells. In addition, it remains unclear what causes this depletion of ATP in KB-V1 cells and what other effects that this might bring.

RT-PCR and Western blot analysis showed that after pyranocoumarins treatment for 24 h the expression levels of *MDR1* mRNA and P-glycoprotein were reduced in KB-V1 cells. Among other mechanisms, the expression of P-glycoprotein has been found to be regulated by extracellular signal-regulated kinase (ERK) activity (Ding et al., 2001; Wartenberg et al., 2001). We have previously demonstrated that pyranocoumarins could activate the ERK pathway in HL-60 cells (Zhang et al., 2003). However, we cannot be sure at this stage whether pyranocoumarins could either affect the ERK signaling in KB-V1 cells or down-regulate P-glycoprotein in the similar fashion. Nonetheless, we have proved that pyranocouamrins had no effect on Glutathione *S*-transferase (GST) activity, p53 mediated apoptosis, and topoisomerase II expression in the pair of KB cells (data not shown). We postulate that pyranocoumarins might have both an early and a late effect on KB-V1 cells. The early effect (4 h after exposure to pyranocoumarins) is mediated by reducing cellular ATP content so as to slow down the efflux of doxorubicin by P-glycoprotein pump. The late effect could be mediated by down-regulation of P-glycoprotein expression which ultimately reverses the MDR phenotype as seen with other MDR modulators (Anuchapreeda et al., 2002; Sharom et al., 1999; Sikic, 1999). It is suggested that pyranocoumarins could be a potential candidate as a MDR reversing agent for cancer therapy in the future. We are now attempting to separate and study the bioactivity of the individual conformers.

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