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3′-O, 4′-O-aromatic acyl substituted 7,8-pyranocoumarins: a new class of P-glycoprotein modulators

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Keywords
(±)-Praeruptorin A; 7, 8-pyranocoumarin; multidrug resistance; P-glycoprotein

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Abstract

Objectives P-glycoprotein (Pgp) overexpression in tumour cells leads to multidrug resistance (MDR) and causes failure in cancer chemotherapy. We have previously identified (±)-praeruptorin A (PA) as a potential lead compound for Pgp modulators. In this study we investigated the MDR-reversing activities of PA derivatives.

Methods Series 7,8-pyranocoumarins with various C-3′ and C-4′ side chains had been semi-synthesized and their MDR-reversing activity was investigated in Pgp-overexpressing MDR tumour cell line HepG2/Dox and in a KB V1 xenograft animal model.

Key findings All 7,8-pyranocoumarins exhibited equal or higher activity in modulating Pgp. DCK (**12**), DMDCK (**15**), **16**, **21**, **23** and **24** at 4 μM achieved 91%~99% decrease in IC50 value (concentration inhibiting cell growth by 50%) of anticancer agents vinblastine, doxorubicin, puromycin and paclitaxel, and were more active than others. DMDCK also remarkably enhanced the growth inhibitory effect of paclitaxel on KB V1 xenografts ($P < 0.05$), showing a potency required for clinical usage. Mechanistic studies suggested that these 7,8-pyranocoumarins might reverse Pgp-MDR through directly binding to substrate binding site(s) or allosteric site(s) on Pgp therefore impairing Pgp-mediated drug transport.

Conclusions Results from the study suggested that 3′-O, 4′-O-aromatic acyl substituted 7,8-pyranocoumarins could serve as a new class of Pgp modulator. Acyls play an important role in maintaining and enhancing the Pgp-modulating ability of pyranocoumarins. 3,4-Dimethoxyl substituted aromatic acyls, bearing a methoxy that might interact with Pgp as hydrogen bond accepter, were shown to be the most potent for reversing MDR.

Introduction

P-glycoprotein (Pgp) is an ATP-dependent membrane transporter protein encoded by the MDR1 gene. Pgp plays the role of bodyguard by extruding xenotoxics out of cells thus protecting living organs and tissues from damage. However, overexpression of Pgp in tumour cells confers multidrug resistance (MDR).[1–4] In Pgp overexpressing cells, Pgp decreases the cellular concentration of anticancer drugs that are Pgp substrates by actively transporting them out. Pgp inhibitors (or modulators) restore the chemosensitivity of MDR cells via impairing Pgp-directed drug transport. Since verapamil was found to have MDR-reversing activity in 1981,[5] thousands of natural and synthesized compounds have been studied for their Pgp-modulating activity. Hundreds of compounds with Pgp-inhibiting properties were investigated *in vitro* or *in vivo*, which led to the discovery of highly active Pgp inhbitors, including valspodar (PSC 833, a ciclosporin A derivative), dexverapamil (verapamil derivative), tariquidar (XR9576), zosuquidar (LY335979) and biricodar (VX-710).[6,7] Though some Pgp inhibitors are undergoing phase II or III clinical study, there are now currently no reversal agents clinically available.

Identification of target sites on Pgp has been challenging because of the difficulty in obtaining crystallized Pgp as well as the structural diversity of molecules that Pgp recognizes and transports. Therefore, great effort has been made for many years to identify common characteristics on Pgp substrates and inhibitors by structure–activity relationship (SAR) studies. Some commonly shared structural features were rapidly recognized, such as the amphiphilic character of the molecules, the presence of aromatic rings, hydrogen bond

accepter or electron donor (O or N atom), and hydrogen bond donor (OH or NH group) *et al*., in certain spatial arrangements.[8–11]

We have shown that (±)-praeruptorin A (PA), a natural 7,8-pyranocoumarin from *Peucedanum praeruptorum* Dunn., reverses Pgp-mediated MDR in cancer cells.[12] To find more potent Pgp modulators, a number of PA derivatives were prepared. These compounds had the same 7,8-pyranocoumarin core structure but different C-3′-O and C-4′-O acyls in *cis*- or *trans*-configuration. We have previously reported that one of these new semi-synthetic 7,8-pyranocoumarins (±)-3′-O,4′-O-dicynnamoyl-*cis*-khellactone (DCK, or **12**), is more potent than PA or verapamil in reversing Pgp-MDR.[13] Further studies revealed that 3,4-dimethoxylation on cinnamoyl remarkably enhanced the MDR-reversing activity of DCK.[14] It was found in our study that the type of 3′-acyl and 4′-acyl seems to play a very important role in keeping or enhancing the ability of 7,8-pyranocoumarins in modulating Pgp. Side chains altered the drug–Pgp interaction mode. To explore the role of acyls, the structure–activity relationship of these pyranocoumarins was studied by comparing their ability to reverse Pgp-mediated drug resistance in human tumours. The mode of drug–Pgp interaction was also investigated. DMDCK,[15] which shows the highest MDR-reversing ability *in vitro*, was further studied *in vivo*, for its potential medical usage as a chemo-sensitizer of paclitaxel.

Materials and Methods

Doxorubicin, vinblastine, puromycin, paclitaxel, verapamil, sulforhodamine B (SRB), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and other chemicals were purchased from Sigma/Aldrich Co. (St Louis, MO, USA). RPMI 1640 medium, MEM medium, fetal bovine serum (FBS) and antibiotic–antimycotic (100 ×) were Gibco products (Brooklyn, NY, USA). Anti-MDR1 monoclonal UIC2 (UIC2 mouse monoclonal anti-human MDR1) was from Immunotech (Marseilles, France). Goat anti-mouse IgG2a-PE was from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

NMR spectra were recorded on a Varian NMR-300 MHz spectrometer in $CDCl_3$. Optical rotations were measured on a PE343 polarimeter. MS were carried out on a LCQ Advantage mass spectrometer.

Semi-synthesis of 7,8-pyranocoumarins

7,8-Pyranocoumarins were prepared in a two-step reaction as described previously with minor modification.[13–16] Firstly the basic hydrolysis of PA provided intermediates **2**, **3** and **4** (Figure 1) and secondly acylation of **2**, **3** and **4** provided the target compounds (Figures 2–4).

Basic hydrolysis was carried out by adding 200 ml of 0.5 M KOH to 15.5 mmol PA (6.0 g in 500 ml of dioxane) and the mixture was stirred at 60°C for 20 min. After cooling, the pH was adjusted to 2~3 by 10% H_2SO_4 and the mixture was stirred for 2 h at room temperature and extracted three times with $CHCl_3$ (300 ml, 200 ml, 200 ml). The combined $CHCl_3$ fraction was washed with 300 ml of saturated $NaHCO_3$ followed by 300 ml of water and dried over Na_2SO_4. After solvent removal, the residue was separated by repeated flash chromatography on silica gel ($CHCl_3$–EtOAc, 3:1) and fractions were monitored by thin-layer chromatography (TLC) at 365 nm. Yield of **2**, **3** and **4** was 400 mg (7%), 1.0 g (22%) and 900 mg (20%) respectively.

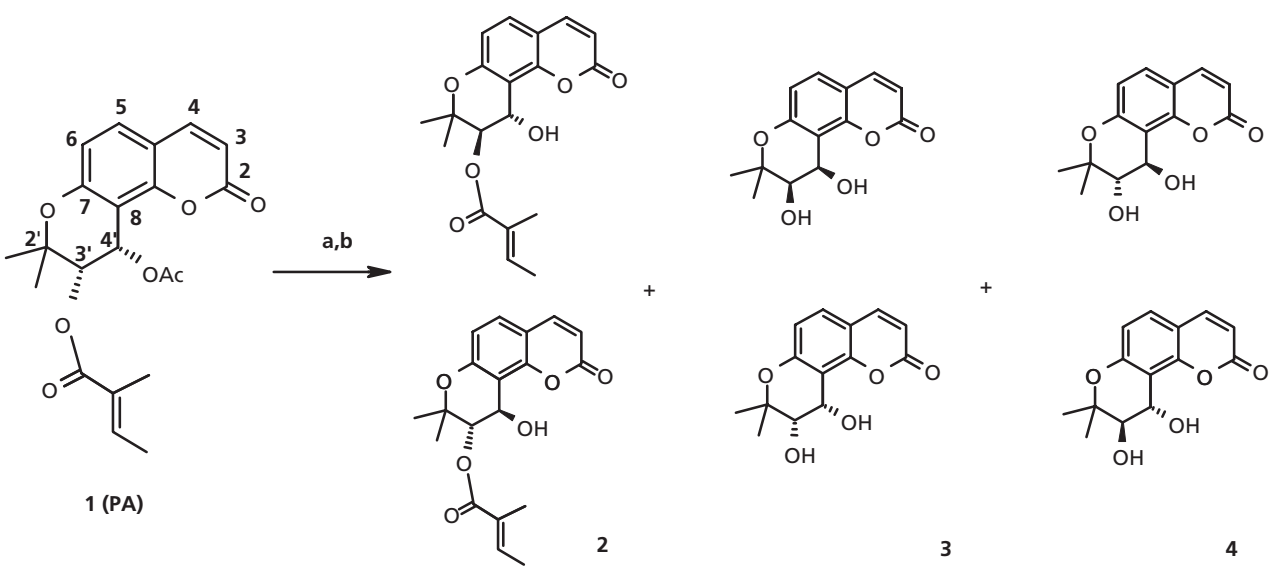

Figure 1 Scheme for preparation of intermediates **2**, **3** and **4**. Reagents and conditions: (a) 0.5 M KOH, dioxane, 60°C; (b) 10% H_2SO_4, pH 2~3, rt.

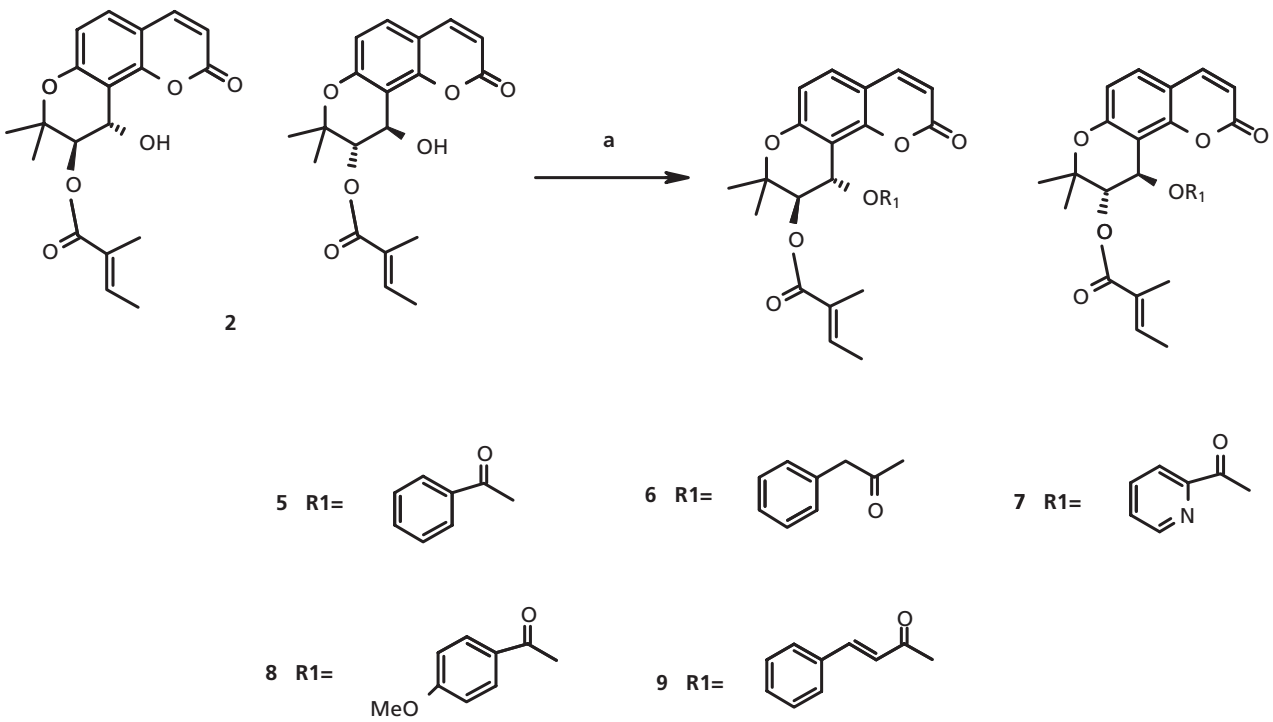

Figure 2 Scheme for preparation of **5~9**. Reagents and conditions: (a) CH_2Cl_2, DCC, DMAP, RnOH, reflux.

Acylation of **2** (Figure 2) was carried out by adding, separately, 1.2 mmol benzoic acid (145 mg), 1.2 mmol benzeneacetic acid (160 mg), 1.2 mmol picolinic acid (145 mg), 1.2 mmol 4-methoxybenzoic acid (180 mg) and 1.2 mmol cinnamic acid (180 mg) to a mixture of **2** (80 mg or 0.233 mmol) in dichloromethane ($CHCl_2$) 5 ml, *N*,*N*'-dicyclohexylcarbodiimide (DCC) 206 mg or 1 mmol, and 4-dimethylaminopyridine (DMAP) 3 mg or 0.025 mmol. The mixture was stirred/refluxed for 3 h, cooled to room temperature, filtered and the filtrate was separated and purified by repeated flash silica gel 60 column chromatography (petroleum ether–EtOAc, 4:1). Fractions were monitored by TLC under 365 nm and purified **5** (40 mg, 38%), **6** (49 mg, 46%), **7** (35 mg, 33%), **8** (46 mg, 41%), **9** (54 mg, 49%) were obtained, respectively.

For the preparation of **10~24** (Figures 3 and 4), 1.5 mmol benzoic acid (180 mg), 1.5 mmol benzeneacetic acid (200 mg), 1.5 mmol cinnamic acid (220 mg), 1.5 mmol 4-methoxybenzeneacetic acid (250 mg), 1.5 mmol 4-methoxycinnamic acid (270 mg), 1.5 mmol 3,4-dimethoxycynnamic acid (310 mg), 1.5 mmol 3,4-dimethoxybenzoic acid (270 mg) and 1.5 mmol 4-methoxybenzoic acid (230 mg) were separately added to a mixture of **3** (80 mg or 0.31 mmol) in $CHCl_2$ 5 ml, DCC 206 mg and DMAP 4 mg, and stirred under refluxing condition for 3 h. The mixture was then treated by following the preparation steps for compounds **5~9** described above to give **10** (46 mg, 32%), **11** (52 mg, 34%), **12** (also DCK, 81 mg, 50%),[13] **13** (62 mg, 36%), **14** (also MMDCK, 17 mg, 7%),[14] **15** (also DMDCK, 22 mg, 11%),[14] **16** (46 mg, 25%) and **17** (19 mg, 12%). The same treatment of **4** (80 mg) separately with 15 mmol of benzoic acid, benzeneacetic acid, cinnamic acid, 4-methoxybenzeneacetic acid, 4-methoxycinnamic acid, 3,4-dimethoxycynnamic acid and 3,4-dimethoxybenzoic acid gave **18** (38 mg, 26%), **19** (43 mg, 28%), **20** (51 mg), **21** (41 mg, 24%), **22** (20 mg, 11%), **23**(30 mg, 15%) and **24** (13 mg, 7%), respectively.

Structural determination of above compounds was based on their MS data and ^{1}H NMR data, and on comparison with data reported in the literature.[15,16]

Cell lines and cell culture

Pgp-overexpressing human tumour cell lines HepG2/Dox (hepatocarcinoma), K562/Dox (leukaemia) and KB V1 (epidemoid carcinoma) were generously provided by Dr Judy Chan of the Chinese University of Hong Kong, Dr Morjani of the University of Reims Champagne-Ardenne, France and Dr Michael Gottesman of the National Institute of Health, Bethesda, USA, respectively. HepG2/Dox cells and K562/Dox cells were routinely cultured at 37°C, 5% CO_2, in RPMI-1640 medium supplemented with 10% FBS and 100 U antibiotics. KB V1 cells were maintained in MEM containing 10% FBS and 100 U antibiotics at 37°C in a humidified 5% CO_2

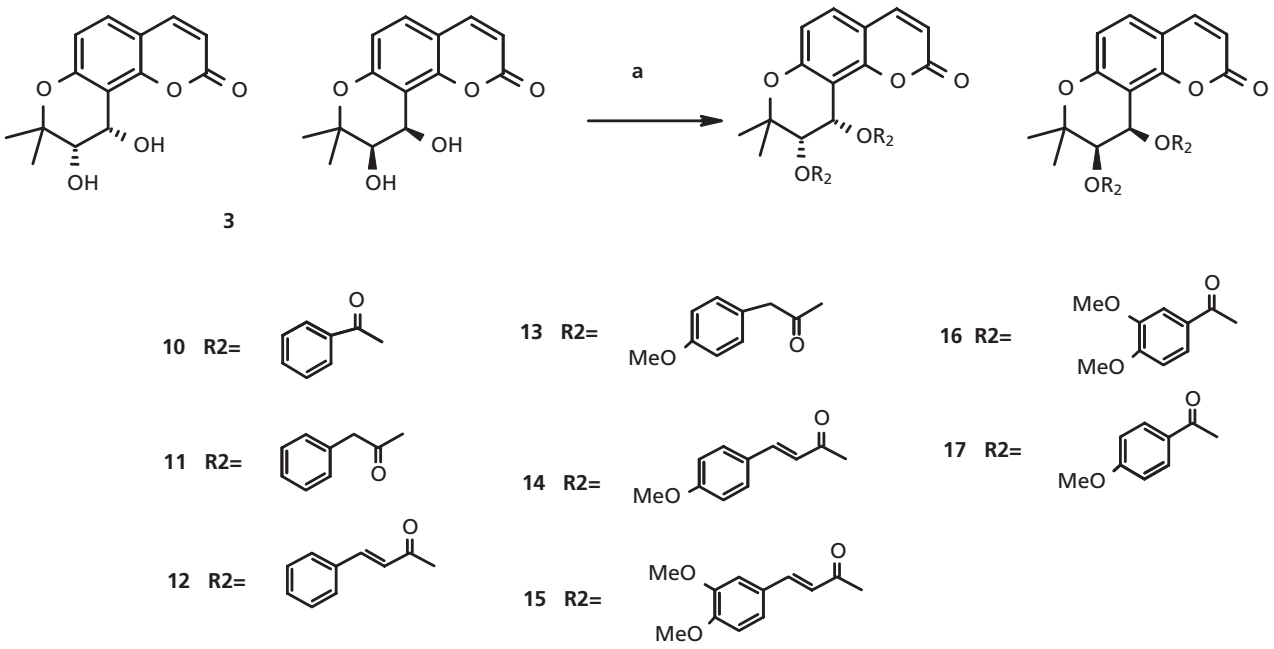

Figure 3 Scheme for preparation of **10~17**. Reagents and conditions: (a) CH_2Cl_2, DCC, DMAP, RnOH, reflux.

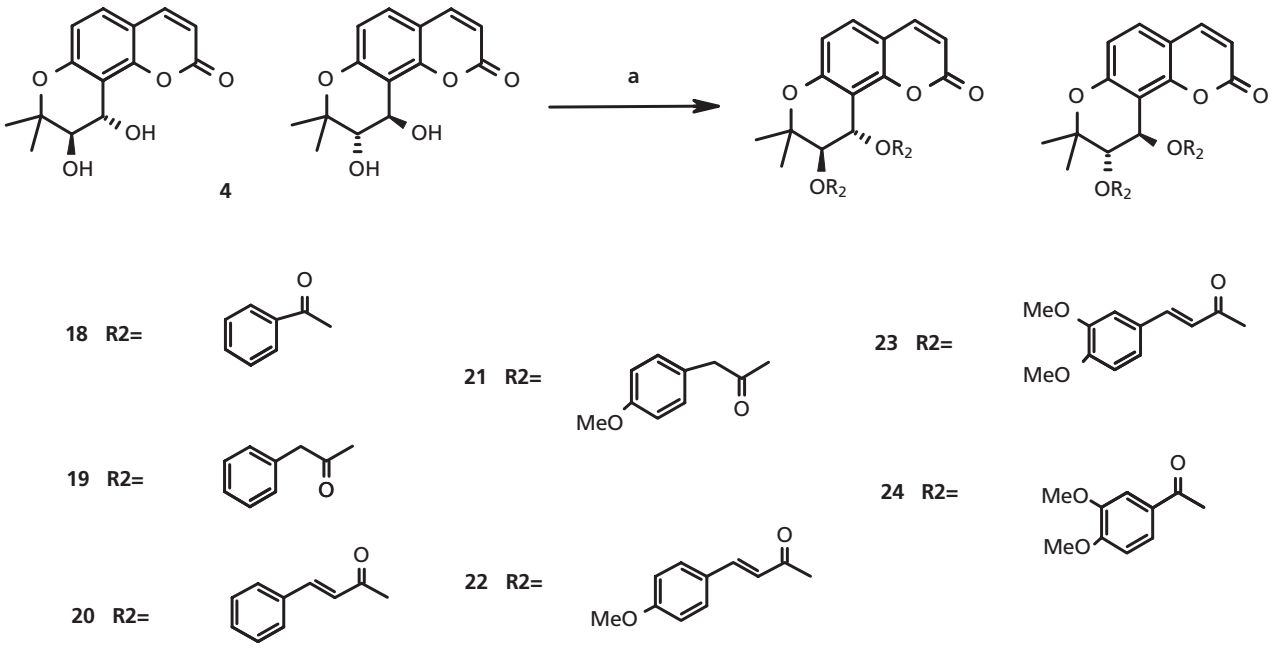

Figure 4 Scheme for preparation of **18~24**. Reagents and conditions: (a) CH_2Cl_2, DCC, DMAP, RnOH, reflux.

incubator. Into a medium of HepG2/Dox, K562/Dox and KB V1 cells, 1.2 μM doxorubicin, 0.1 μM doxorubicin and 500 ng/ml vinblastine were added separately to maintain the drug resistance phenotype. All MDR cells were grown in drug-free medium for at least seven days before testing.

Growth inhibitory assay

Approximately 5000 cells per well were seeded in 96-well plates and incubated overnight. Cells were then treated with various concentrations of drug for 72 h. Cell growth inhibi-

tory effects of drugs were determined by SRB assay and were evaluated according to their respective IC50 values (concentration of compound inhibiting 50% of cell growth) as previously described.[13] Each experiment was performed independently at least three times and results were expressed as mean ± standard deviation (SD). Solvents and media were included as blank control.

Assessment of Pgp-multidrug resistance reversing activity

IC50 values of Pgp substrate anticancer agents vinblastine, doxorubicin, puromycin and paclitaxel were tested in the presence or absence of a Pgp modulator separately. The ability of a pyranocoumarin in modulating Pgp was evaluated by percentage reduction of IC50 value of an anticancer agent achieved by 4 μM of the pyranocoumarin and was expressed as $R\% = [1 - IC50_{(absence)}/IC50_{(presence)}] \times 100$. The more potent a Pgp modulator, the closer the %R is to 100%. Verapamil was used as a positive control.

Drug accumulation assay

Approximately 1×10^6 K562/Dox cells were suspended in 1 ml of medium containing 10 μM doxorubicin and various concentrations of test substances and incubated at 37°C for 1 h. Cells were then washed with ice-cold PBS twice and resuspended in 1 ml of ice-cold PBS. Cellular fluorescence of doxorubicin was measured on a FACSCalibur flow cytometer (Becton Dickinson, Mountain View, CA, USA). Verapamil was the positive control.

MDR1 reactivity shift assay

Pgp reactivity to the conformation-sensitive mAb UIC2 was performed as described previously.[13] HepG2/Dox cells were pretreated with 5 μM ciclosporin (Pgp substrate), 1 mM sodium vanadate (ATP inhibitor) or 5 μM pyranocoumarin at 37°C for 10 min. UIC2 was then added to the mixture and reacted at 37°C for another 10 min. After removal of the unconjugated UIC2 molecules by washing and centrifuging, UIC2 molecules that bound to Pgp were labelled with goat anti-mouse IgG_{2a}-PE and detected by using a FACSCalibur flow cytometer. Normal mouse IgG_{2a} served as a negative control. Pgp reactivity to UIC2 was evaluated by variation in fluorescence intensity compared to modulator-free control.

Pgp-ATPase activity assay

Pgp-enriched membrane vesicles were prepared from Pgp-overexpressing K5652/Dox cells. The effect of **15**, **16**, **23** and **24** on both basal and substrate-stimulated ATPase activity of Pgp was assayed based on phosphate-release catalysed by the membrane vesicle with all other major membrane ATPases inhibited as described previously.[13] Baseline controls contained 100 μM Na_3VO_4, pH 10.0 and/or ethanol (vehicle) at a maximum final concentration of 2% v/v. All experiments were repeated three times.

Animals studies

BALB/c nude mice (female, 4–5 weeks old) were provided by Experiment Animal Center of Guangzhou University of TCM. All animal experiments were approved by the Animal Ethics Committee at Guangzhou University of Chinese Medicine (Document No. syxk(Yue)2008-0001) and performed following Animal Care and Use guidelines set by NIH (National Institute of Health, USA). After one week of acclimatization to laboratory conditions, mice were subcutaneously injected with 3×10^6 of KB V1 cells on the back to establish tumour xenografts. Two days after tumour cell injection, mice were randomized into four groups and were treated by intraperitoneal injection of 10 mg/kg of paclitaxel every other day for five doses (group B, $n = 7$), or 50 mg/kg of DMDCK daily for ten doses (group D, $n = 8$), or a combination of five doses of 10 mg/kg of paclitaxel with ten doses of 50 mg/kg of DMDCK (group C, $n = 8$). Mice treated with vehicle (8.6% of Cremophor EL, 8% of EtOH and 5% of DMSO in 0.9% saline, 10 ml/kg) were used as drug negative control (group A, $n = 7$). Tumour size was monitored by measuring two perpendicular diameters with a caliper. Tumour volume (V) was calculated from: $V = 1/2(L \times W^2)$, where L is the length and W is the width in millimeters.

Statistical analysis

All results were expressed as mean ± SD unless otherwise noted. Effects of various treatments were analysed by using the Kruskal–Wallis test. Bonferroni procedure was then used to evaluate individual differences between treatments. The Bonferroni procedure-adjusted P-values < 0.05 were considered statistically significant.

Results

Structural identification

Compounds **5–24** were enantiomers with zero specific optical rotation value. Structural determination of pyranocoumarins was based on the MS and ^{1}H NMR data, and on comparing the NMR data with that of compounds reported in the literature.[15,16] MS data and NMR data of newly synthesized compounds **13**, **16**, **21**, **23** and **24** are presented as follow.

13. $C_{32}H_{30}O_9$. MS (ESI): 581 $[M+Na]^+$. ^1H NMR δppm ($CDCl_3$-300 MHz): 7.61 (1H, d, J = 9.6 Hz, 4-H), 7.36 (1H, d, J = 8.4 Hz, 5-H), 7.31~7.16 (10H, m, Ar-H), 6.78 (1H, d, J = 8.4 Hz, 6-H), 6.56 (1H, d, J = 4.8 Hz, 4′-H), 6.25 (1H, d, J = 9.6 Hz, 3-H), 5.23(1H, d, J = 4.8 Hz, 3′-H), 3.76, 3.75 (3H each, s, 2×Ar-OCH_3), 3.65, 3.50, 3.43 and 3.35 (1H each, d, J = 15.2 Hz, 2×Ar-CH_2-CO), 1.27 and 1.24 [3H each, s, 2′-$(Me)_2$].

16. $C_{32}H_{30}O_{11}$. MS (ESI): 613 $[M+Na]^+$. ^1H NMR δppm ($CDCl_3$-300 MHz): 7.71 (2H, d, J = 8.5 Hz, 2×Ar-6-H), 7.57 (1H, d, J = 9.6 Hz, 4-H), 7.54 (2H, s, 2×Ar-2-H), 7.46 (1H, d, J = 8.5 Hz, 5-H), 6.90 (1H, d, J = 4.9 Hz, 4′-H), 6.87 (2H, d, J = 8.5 Hz, 2×Ar-5-H), 6.85 (1H, d, J = 8.5 Hz, 6-H), 6.14 (1H, d, J = 9.6 Hz, 3-H), 5.61 (1H, d, J = 4.9 Hz, 3′-H), 3.90 (3H, s, Ar-OCH_3), 3.89 (3H, s, Ar-OCH_3), 3.84(6H, s, 2×Ar-OCH_3), 1.62 and 1.47 [3H each, s, 2′-$(CH_3)_2$].

21. $C_{32}H_{30}O_9$. MS (ESI): 581 $[M+Na]^+$. ^1H NMR δppm ($CDCl_3$-300 MHz): 7.61 (1H, d, J = 9.4 Hz, 4-H), 7.36 (1H, d, J = 8.5 Hz, 5-H), 7.20 and 7.12 (2H each, d, J = 8.0 Hz, Ar-H), 6.82~6.76 (4H, m, Ar-H), 6.78 (1H, d, J = 8.5 Hz, 6-H), 6.25 (1H, d, J = 4.8 Hz, 4′-H), 6.24 (1H, d, J = 9.4 Hz, 3-H), 5.22 (1H, d, J = 4.8 Hz, 3′-H), 3.78 (6H, s, 2×Ar-OCH_3), 3.68 and 3.55 (1H each, d, J = 15.4 Hz, Ar-CH_2-CO), 3.50 (2H, s, Ar-CH_2-CO), 1.21 [6H, s, 2′-$(CH_3)_2$].

23. $C_{36}H_{34}O_{11}$. MS (ESI): 665 $[M+Na]^+$. ^1H NMR δppm ($CDCl_3$-300 MHz): 7.66 (2H, d, J = 15.9 Hz, 2×-OCOCH=), 7.61 (1H, d, J = 9.6 Hz, 4-H), 7.41 (1H, d, J = 8.4 Hz, 5-H), 7.06 (2H, d, J = 8.4 Hz, 2×Ar-6-H), 7.04 and 7.00 (1H each, s, 2×Ar-2-H), 6.86 (1H, d, J = 8.4 Hz, 6-H), 6.82 and 6.81 (1H each, d, J = 8.4 Hz, 2×Ar-5-H), 6.41 (1H, d, J = 3.9 Hz, 4′-H), 6.30 (2H, d, J = 15.9 Hz, 2×Ar-CH=), 6.22 (1H, d, J = 9.6 Hz, 3-H), 5.51 (1H, d, J = 3.9 Hz, 3′-H), 3.90, 3.88, 3.87, 3.86 (3H each, s, 4×Ar-OCH_3), 1.53 and 1.43 [3H each, s, 2′-$(CH_3)_2$].

24. $C_{32}H_{30}O_{11}$. MS (ESI): 613 $[M+Na]^+$. ^1H NMR δppm ($CDCl_3$-300 MHz): 7.76 (2H, d, J = 8.4 Hz, 2×Ar-6-H), 7.57 (2H, s, 2×Ar-2-H), 7.54 (1H, d, J = 9.6 Hz, 4-H), 7.42 (1H, d, J = 8.1 Hz, 5-H), 6.90 (2H, d, J = 8.4 Hz, 2×Ar-5-H), 6.80 (1H, d, J = 8.1 Hz, 6-H), 6.58 (1H, d, J = 3.6 Hz, 4′-H), 6.19 (1H, d, J = 9.6 Hz, 3-H), 5.62 (1H, d, J = 3.6 Hz, 3′-H), 3.93 (9H, s, 3×Ar-OCH_3), 3.88 (3H, s, OCH_3), 1.58 and 1.47 [3H each, s, 2′-$(CH_3)_2$].

Pgp-multidrug resistance was reversed by aromatic acyl substituted 7, 8-pyranocoumarins

The effects of **5–24** on cell proliferation were tested firstly in HepG2/Dox cells (Table 1). Compound **13** was then excluded from follow-up study because it strongly inhibited tumour cell growth (IC50 < 5 μM). The ability of other pyranocoumarins to reduce Pgp-mediated drug resistance in MDR cells was evaluated in HepG2/Dox cells by their ability to decrease the IC50 value of anti-tumour agents that are Pgp substrates and the results re presented in Table 2. PA was the least active Pgp modulator tested. R% values of vinblastine, doxorubicin, puromycin and paclitaxel achieved by 4 μM of PA were 30.9%, 15.5%, 37.5% and 68.8%, respectively. Verapamil, a known Pgp modulator, showed activity higher than PA and the corresponding R% values achieved by 4 μM of verapamil were 60.6%, 70.9%, 87.7% and 79.6%, respectively. In general, all semi-synthesized pyranocoumarins exhibited MDR-reversing ability higher than, or equivalent to, PA. Compounds **12**, **15**, **16**, **23** and **24** achieved R%s of all four anticancer agents by more than 90%, and were shown to be the most active group of pyranocoumarins. Among these, compounds **15** and **16** were the most active because they achieved R%s of all four anticancer agents that were not only significantly greater than PA ($P < 0.001$), but also significantly greater than verapamil ($P < 0.01$). Compounds **10**, **17** and **21**, though less active than the above five, also exhibited remarkable ability to reduce resistance of all four anticancer drugs when compared with PA. Compounds **7**, **19** and **20** were the least active pyranocoumarins because they exhibited equivalent activity to PA.

Table 1 Growth inhibitory effect of pyranocooumarins against HepG2/Dox cells

Compound No.	IC50 (μM)	Compound No.	IC50 (μM)
PA (1)	10.84 ± 1.58	**14**	72.62 ± 0.30
5	15.01 ± 3.60	**15**	76.33 ± 2.22
6	18.57 ± 2.48	**16**	58.36 ± 2.81
7	29.23 ± 6.81	**17**	45.69 ± 7.28
8	22.02 ± 5.19	**18**	46.35 ± 3.83
9	20.11 ± 4.29	**19**	16.48 ± 0.06
10	19.87 ± 10.46	**20**	28.66 ± 2.02
11	18.73 ± 1.49	**21**	49.54 ± 10.45
12	26.31 ± 1.41	**22**	62.54 ± 4.17
10	19.87 ± 10.46	**23**	86.38 ± 2.48
13	2.48 ± 0.52	**24**	72.29 ± 5.56

IC50, concentration inhibiting cell growth by 50%. Cells were exposed to drugs for 72 h. Values are means ± SD of three experiments.

3′-O, 4′-O-aromatic acyl substituted 7, 8-pyranocoumarins increased doxorubicin accumulation within K562/Dox cells

We had reported previously that DCK[12] and DMDCK (**15**) might increase cellular drug accumulation and slow down Pgp-mediated drug efflux within tumour cells by directly interacting with Pgp.[13,14] In this study, the effect of PA and **10–24** on doxorubicin accumulation within K562/Dox cells was investigated and compared. Cells were incubated with

Table 2 Percentage reduction of IC50 values of anticancer drugs in HepG2/Dox cells

Compound	Vinblastine	Doxorubicin	Puromycin	Paclitaxel
PA (1)[a]	30.9 ± 12.8	15.5 ± 2.8	46.3 ± 8.7	68.6 ± 2.2
5	77.6 ± 3.6*	52.6 ± 12.5	51.8 ± 13.0	71.1 ± 18.9
6	76.7 ± 5.8*	33.3 ± 22.5	58.4 ± 9.5	67.5 ± 32.0
7	69.9 ± 14.3	49.5 ± 8.0	56.9 ± 4.4	79.6 ± 16.4
8	70.3 ± 9.0	69.1 ± 3.0***	66.1 ± 4.9	77.4 ± 6.7
9	74.5 ± 12.9*	57.9 ± 2.2*	78.0 ± 0.6***	85.8 ± 5.7
10	88.1 ± 4.1*** ##	86.1 ± 5.8***	78.1 ± 4.9***	95.5 ± 1.5*** #
11	77.8 ± 8.5**	75.5 ± 6.6***	77.5 ± 6.2***	91.4 ± 2.5*
12[a]	96.2 ± 1.6***###	93.8 ± 0.9*** ##	94.3 ± 0.6***	95.9 ± 3.3*** ##
14[a]	79.6 ± 5.4**	94.6 ± 1.9***###	91.0 ± 1.6***	87.3 ± 3.8
15[a]	99.3 ± 0.2***###	99.4 ± 0.0***###	99.2 ± 0.3***###	99.3 ± 0.2*** ###
16	98.7 ± 0.2***###	99.1 ± 0.1***###	98.6 ± 0.5*** ##	98.4 ± 0.1*** ###
17	84.61 ± 7.1*** #	92.1 ± 3.9*** ##	83.7 ± 1.8***	91.9 ± 1.2**
18	70.7 ± 9.2	71.8 ± 6.3***	56.6 ± 10.0	85.7 ± 2.4
19	40.4 ± 20.5	39.8 ± 5.6	37.5 ± 10.9	69.7 ± 7.3
20	39.4 ± 11.0	41.2 ± 15.6	64.6 ± 7.4	66.2 ± 7.9
21	96.0 ± 2.8***###	87.6 ± 2.3***	96.1 ± 1.2***	99.6 ± 0.1*** ###
22	65.5 ± 3.2	85.9 ± 2.8***	83.4 ± 8.3***	74.9 ± 5.3
23	91.7 ± 3.8***###	98.2 ± 0.6***###	96.1 ± 0.8***	96.2 ± 2.0*** ##
24	93.9 ± 2.7***###	96.1 ± 4.1***###	96.5 ± 0.0***	97.8 ± 0.3*** ##
Verapamil	60.6 ± 7.0	70.9 ± 5.2***	86.1 ± 2.3***	79.6 ± 2.5

R% = [1 − $IC50_{(absence)}$/$IC50_{(presence)}$] × 100, in which $IC50_{(presence)}$ is IC50 (concentration inhibiting cell growth by 50%) of a drug in the presence of 4 μM of a Pgp modulator, $IC50_{(absence)}$ is IC50 of a drug in the absence of any Pgp modulator. Values are means ± SD of three experiments. *P < 0.05, **P < 0.01, or ***P < 0.001, compared with PA; #P < 0.05, ##P < 0.01, and ###P < 0.001, compared with verapamil. [a]Data derived from Fong *et al*.[14]

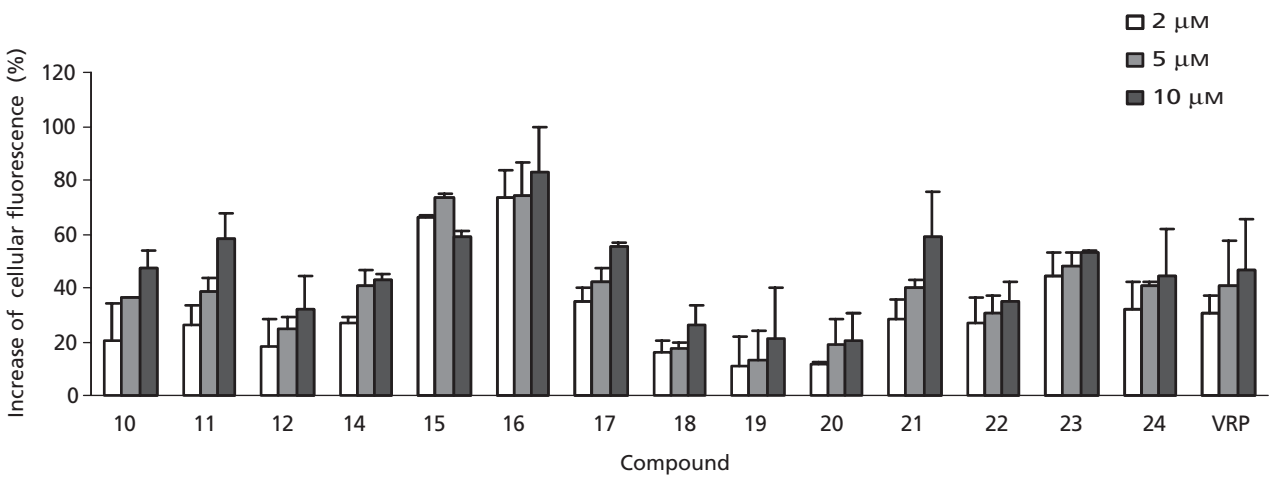

Figure 5 Increased cellular doxorubicin accumulation by (±)-praeruptorin A (PA) derivatives or verapamil in K562/Dox cells. Cells were incubated with 10 μM doxorubicin with or without addition of 2 μM, 5 μM or 10 μM PA derivative or verapamil at 37°C for 1 h. The effect of PA derivatives and verapamil on cellular doxorubicin accumulation was analysed by flow cytometry. Increase of cellular fluorescence is shown as a percentage. Results are means ± SD values of three experiments.

10 μM doxorubicin in the presence or absence of 2 μM, 5 μM or 10 μM of pyranocoumarin or verapamil at 37°C for 1 h. Fluorescence intensity of cellular doxorubicin was analysed. Figure 5 shows that all tested pyranocoumarins increased cellular fluorescence in a dose-dependent manner. On the whole, the ability of these compounds to increase cellular doxorubicin accumulation was positively correlated with their ability to reverse doxorubicin resistance in K562/Dox cells. For example, **15** or **16** at 5 μM increased cellular doxorubicin fluorescence by more than 70%, whereas the corresponding increase achieved by **18**, **19** or **20** was less than 20%. The above result suggested that these pyranocoumarins

might restore the drug sensitivity of Pgp-MDR cells through increasing cellular drug accumulation.

3′-O, 4′-O-aromatic acyl substituted 7, 8-pyranocoumarins changed Pgp reactivity to UIC2

Direct Pgp modulators may interact with Pgp on substrate site(s) and thus decrease the binding opportunity of a transport drug. They may also interact with Pgp on allosteric site(s) that causes conformational change of Pgp that is unfavorable for drug binding and transportation. The mode of interaction between Pgp and its modulator can be detected by MDR1 reactivity shift assay using conformationally sensitive mAbUIC2. UIC2 preferably binds to Pgp that is associated with a substrate or a competitive inhibitor. Binding of a compound such as ciclosporin to the substrate-binding site(s) on Pgp increases Pgp reactivity to UIC2 whereas binding of a compound such as sodium vanadate to ATP site(s) on Pgp decreases the reactivity.[17–19] We have previously reported that DCK[12] may bind to Pgp allosterically and decrease UIC2 binding whereas DMDCK[15] may bind to the Pgp substrate site and therefore increase UIC2 binding. In this study, the effect of other pyranocoumarins on UIC2 binding to Pgp was also studied and the results are shown in Figure 6. Compounds **16** and **24** increased UIC2 binding to Pgp as did **15**, exhibiting substrate-like activity, whereas the others, including PA, decreased UIC2 binding (Figure 6) as did **12**, inferring an allosteric binding to Pgp.

3′-O, 4′-O-aromatic acyl substituted 7, 8-pyranocoumarins affected Pgp ATPase activity

As an ATP-dependent membrane transporter, Pgp-mediated drug transport is accompanied by increased ATP hydrolysis, therefore Pgp-catalysed ATP hydrolysis indirectly reflects transport function of Pgp. Our previous study showed that DCK[12] and DMDCK[15] inhibited substrate-stimulated ATP hydrolysis of Pgp.[13,14] In this study, **16**, **23** and **24** had also been studied for their effects on Pgp-ATPase activity in membrane fractions prepared from Pgp-overexpressing K562/Dox cells, with activities of other major membrane ATPases suppressed. As shown in Figure 7b, verapamil, a standard Pgp substrate, stimulated ATP hydrolysis in a dose-dependent manner. In the presence of 1 μM of **16**, **23** and **24**, verapamil-stimulated Pgp-ATPase activity was inhibited with notably reduced Vmax but relatively stable Km, suggesting a primarily non-competitive inhibition. Pyranocoumarins acted differently on basal Pgp-ATPase activity. Figure 7a shows that **15** inhibited Pgp-catalysed ATP hydrolysis and **24** stimulated Pgp-catalysed ATP hydrolysis, both dose-dependently.

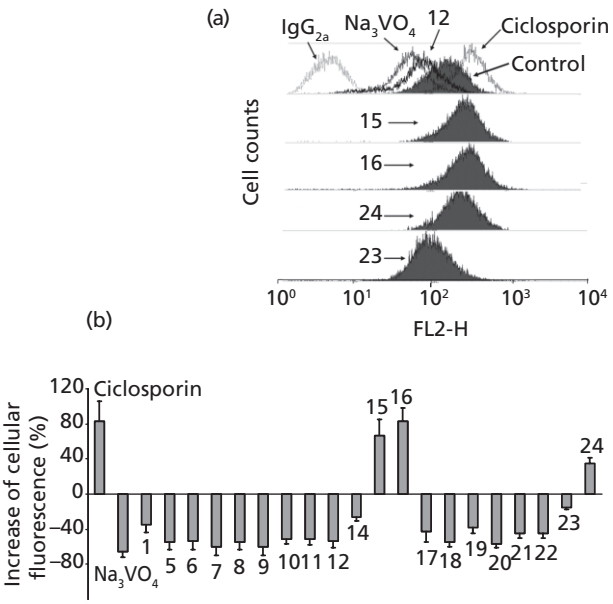

Figure 6 Effect of (±)-praeruptorin A (PA) derivatives on Pgp reactivity to mAb UIC2. HepG2/Dox cells were reacted with mAb UIC2 at 37°C, in the presence or in the absence (control) of 1 mM Na_3VO_4, 5 μM ciclosporin, or 5 μM PA derivative. Normal IgG_{2a} was used as negative control. UIC2 binding affinity to Pgp was detected by labelling with a fluorescent secondary antibody and analysed by flow cytometry. (a) Pictures from a typical experiment showed the cellular fluorescence variation in HepG2/Dox cells when cells were pretreated with 1 mM sodium vanadate, or 5 μM **12**, **15**, **16**, **23** and **24** or 5 μM ciclosporin. (b) Changes in cellular fluorescence intensity caused by 5 μM ciclosporin, 1 mM $NaVO_4$ or 5 μM PA derivative. Results are means ± SD values of three experiments.

Compound 15 enhanced paclitaxel toxicity in KB V1 xenograft model

An in-vivo experiment was performed to evaluate the ability of DMDCK[15] to enhance paclitaxel toxicity against tumour growth in nude mice bearing KB V1 xenografts. Figure 8 shows average tumour volumes in different treatment groups during a 12-day experimental period. Compared with the vehicle-treated group (A), treatment with five doses of 10 mg/kg paclitaxel only slightly decreased tumour volume (B). Treatment with ten doses of 50 mg/kg **15** did not decrease tumour volume at all (D). However, treatment with 10 mg/kg of paclitaxel every other day, together with 50 mg/kg/d of **15**, significantly decreased tumour volume (C); as shown in Figure 8, six days of treatment decreased tumour volume by 48% whereas ten days of treatment decreased tumour volume by 56% ($P < 0.05$).

Discussion

In our study, 7,8-pyranocoumarins bearing C-3′ and/or C-4′ aromatic acyl(s) exhibited an ability higher than, or

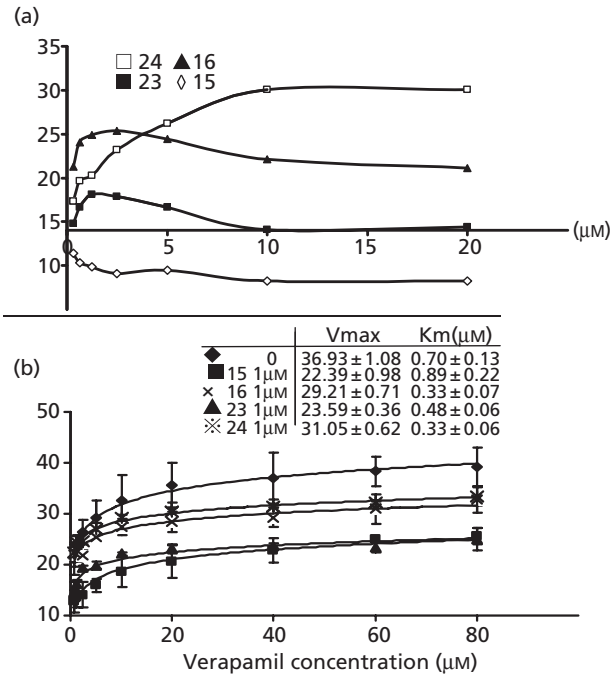

Figure 7 Effect of pyranocoumarins on basal and substrate-stimulated ATPase activity of Pgp. Membrane vesicles were prepared from Pgp-overexpressing K562/Dox cells. Pgp-ATPase activity was measured with other major membrane ATPases suppressed. (a) Inhibitory effect of **15**, **16**, **23** or **24** on verapamil-stimulated Pgp-ATPase activity. (b) Inhibitory effect of **15**, **16**, **23** or **24** on verapamil-stimulated Pgp-ATPase activity.

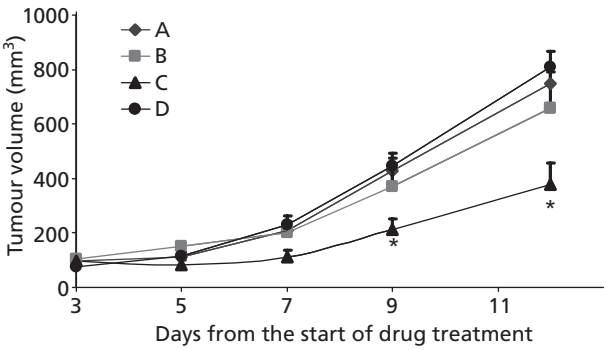

Figure 8 DMDCK enhanced growth inhibitory effect of paclitaxel on KB-3-1 tumour xenografts. Tumour-bearing mice were treated with vehicle (a), paclitaxel (b) or DMDCK (d), or the combination of paclitaxel and DMDCK (c). Tumour size was monitored by measuring two perpendicular diameters with a caliper. Tumour volume (V) was calculated from equation: $V = 1/2(L \times W^2)$, where L is the length and W is the width in millimetres. Data were expressed as means ± SEM. $^*P < 0.05$, compared with group A.

equivalent to, PA to reverse Pgp-MDR in HepG2/Dox cells (Table 2). Among the compounds, DCK,[12] DMDCK,[15] **16**, **23** and **24** showed higher activity than other pyranocoumarins and verapamil: as a matter of fact, they belong to the most active group of pyranocoumarins. When used alone at 4 μM, they did not affect cell proliferation, but significantly restored the chemo-sensitivity of HepG2/Dox cells to anticancer agents that are Pgp substrates (Table 2). In addition, DMDCK remarkably enhanced paclitaxel toxicity to KB-3-1 tumour growth *in vivo*, showing its potential for medical usage as a chemosensitizer for treatment of drug-resistant tumours.

Structure–activity relationship studies reveal that side chains at the C-3′ and C-4′ site play a very important role in keeping or enhancing MDR-reversal ability of a 7,8-pyranocoumarin. Firstly, aromatic acyls are more active than aliphatic acyls. PA has only two aliphatic acyls and is the least active whereas the other pyranocoumarins bearing aromatic acyl(s) are more or less more potent than PA. Secondly, a methoxy group on aromatic acyls also contributes to the activity. Based on data presented in Table 2, the contribution of aromatic acyloxy to MDR-reversing ability follows the order: 3,4-dimethoxycinnamoyloxy and 3,4-dimethoxybenzolyoxy > cinnamoyloxy and 4-methoxybenzeneacetyloxy > 4-methoxybenzoyloxy and 4-methoxycinamoyloxy > benzoyloxy and benzeneacetyloxy. Thirdly, the configuration of acyls may also affect the MDR-reversing ability of pyranocoumarins. Compounds **10**, **11**, **12** and **14**, bearing two identical aromatic acyls in 3′,4′-*cis*-configuration, are relatively more active than their *trans*-isomers **18**, **19**, **20** and **22**. Compounds **5**–**9**, having one aromatic acyl and one aliphatic acyl in 3′,4′-*trans*-configuration, are less active than verapamil (Table 2).

Pgp modulators have diverse structures but common characteristics such as hydrophobic points and hydrogen bond accepter points in certain special arrangements. Results from a pharmacophore search for DCK,[12] MMDCK[14] and DMDCK,[15] using a verapamil-based pharmacophore template, had been performed previously and the results might give an explanation for the role of aromatic acyls.[14] DMDCK[15] has four functional groups (two aromatic rings and two hydrogen bond accepters) simultaneously involved in interaction with Pgp in different binding modes, and therefore, are the most active group of pyranocoumarins. The 3-methoxy on cinnamoyloxy may serve as a hydrogen bond accepter whereas aromatic acyls may serve as hydrophobic points to interact with Pgp. DCK, bearing no methoxy on cinnamoyloxy, has only three groups simultaneously involved. MMDCK, though containing a methoxy on cinnamoyloxy but at a different site (4-methoxy), partially matches the pharmacophore with four functional groups. Therefore DCK and MMDCK are relatively less active than DMDCK. Compounds **16**, **23** and **24** have a 3-methoxy and 4-methoxy on aromatic acyls as has DMDCK, may also use 3-methoxy as

a hydrogen bond accepter to interact with Pgp, and thus belong to the most active of pyranocomarins. Compounds **17** and **21** just have a 4-methoxy on the aromatic acyl as does MMDCK[14] and thus have comparable ability to **14**.

Pyranocoumarins dose-dependently increased doxorubicin accumulation within Pgp-overexpressing K562/Dox cells. Further studies revealed that they may directly interact with Pgp in different manners. Results from the MDR1 reactivity shift assay, which investigated drug–Pgp binding modes, suggested that **15**, **16** and **24** might bind to Pgp substrate site(s) whereas the others may bind to Pgp allosteric site(s) and cause Pgp conformational change. Assay for basal Pgp-ATPase activity provided additional information about the drug–Pgp interaction (Figure 7a). DMDCK[15] inhibited basal Pgp-ATPase activity, implying that it may be an inhibitor of Pgp that occupies the transport substrate binding site of Pgp but can not be transported. Compound **24** dose-dependently stimulated basal Pgp-ATPase activity, acting as a typical Pgp transport substrate. Compound **16** stimulated basal Pgp-ATPase activity at a concentration less than 2 μM, but with increase in drug concentration, Pgp-catalysed ATP hydrolysis slowed down. This suggested that **16** is partly a transport substrate of Pgp. Compound **23** moderately stimulated ATP hydrolysis at a concentration lower than 1.5 μM and slowed down ATP hydrolysis at higher concentration, suggesting that **23** might be a Pgp transport substrate but at the same time also modifies Pgp-substrate intermediate complexes to a less active form. All four compounds exhibited an inhibitory effect on verapamil-stimulated ATP hydrolysis, with decreased Vmax and relatively constant Km, suggesting a non-competitive mode of inhibition. This means that pyranocoumarins may simultaneously bind with substrate drugs to Pgp but at different sites and therefore impair Pgp-mediated drug transportation.

Conclusions

We show here that 3′, 4′-aromatic acyloxy substituted 7,8-pyranoucoumarins could serve as a new class of Pgp modulator. They may reverse Pgp-MDR through directly binding to Pgp and therefore hinder the binding and transportation of substrate drug. As Pgp modulators with a 7,8-pyranocoumarin core structure, their MDR-reversal activity is closely related to the type and configuration of C-3′ and C-4′ side chains. 3,4-Dimethoxy substituted aromatic acyls are shown to be more active than other acyls in enhancing the MDR-reversal activity of pyranocoumarin. DMDCK[15] bears two 3,4-dimethoxycynamoylxoys at the C-3′ site and C-4′ site of 7,8-pyranocoumarin in *cis*-configuration, exhibited remarkable MDR-reversing activity both *in vitro* and *in vivo* and thus exhibited promising prospect in medical usage. Three newly synthesized compounds **16**, **23** and **24**, all bearing methoxy substituted aromatic acyls at the C-3′ site and C-4′ site of pyranocoumarins, also showed exciting Pgp-modulating ability and are worth further investigation.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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