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(±)-3′-*O*, 4′-*O*-dicynnamoyl-*cis*-khellactone, a derivative of (±)-praeruptorin A, reverses P-glycoprotein mediated multidrug resistance in cancer cells

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Abstract—P-glycoprotein (Pgp) is an ATP-driven membrane exporter for a broad spectrum of hydrophobic xenobiotics. Pgp-overexpression is a common cause of multidrug resistance (MDR) in cancer cells and could lead to chemotherapeutic failure. Through an extensive herbal drug screening program we previously showed that (±)-praeruptorin A (PA), a naturally existing pyranocumarin isolated from the dried root of *Peucedanum praeruptorum* Dunn., re-sensitizes Pgp-mediated MDR (Pgp-MDR) cancer cells to cancer drugs. A number of PA derivatives were synthesized and one of these, (±)-3′-*O*, 4′-*O*-dicynnamoyl-*cis*-khellactone (DCK), was more potent than PA or verapamil in the reversal of Pgp-MDR. In Pgp-MDR cells DCK increased cellular accumulation of doxorubicin without affecting the expression level of Pgp. In Pgp-enriched membrane fractions DCK moderately stimulated basal Pgp-ATPase activity, suggesting some transport substrate-like function. However, DCK also inhibited Pgp-ATPase activity stimulated by the standard substrates verapamil or progesterone with decreased V_{max}s but K_ms were relatively unchanged, suggesting a primarily non-competitive mode of inhibition. While the binding of substrates to active Pgp would increase the reactivity of the Pgp-specific antibody UIC2, DCK decreased UIC2 reactivity. These results suggest that DCK could bind simultaneously with substrates to Pgp but perhaps at an allosteric site and thus affect Pgp–substrate interactions.

1. Introduction

Multidrug resistance (MDR) in cancer cells could lead to chemotherapeutic failure. The molecular mechanisms leading to MDR include the activation of transport and detoxification systems, enhancement of target repair activities, alterations of drug targets, and disregulation of cell death pathways.[1] MDR may be resulted from the over-expression of transporter proteins such as P-glycoprotein (Pgp), multidrug resistance associated protein (MRP1), lung resistance protein (LRP), and breast cancer resistance protein (BRCP).[2,3] Pgp is a 170-kDa ATP-dependent membrane transporter protein encoded by MDR1 gene and is active against a spectrum of functionally unrelated hydrophobic drugs.[4] Pgp antagonists have been shown to reverse MDR by increasing intracellular drug availability. A few MDR antagonists, such as curcumin, may act by modulating Pgp expression.[5] Direct inhibitors of Pgp belong to various groups of chemicals including calcium channel blockers, calmodulin inhibitor, coronary vasodilators, hormones, cyclosporines and indole alkaloids, quinolines, surfactants, and antibodies.[6] Several pharmacophore models of Pgp inhibitors have been built through three-dimensional quantitative structure–activity relationship analysis of various Pgp substrates and inhibitors.[7–10] In general, these Pgp models consist of hydrophobic centers (aromatic rings), hydrogen bond acceptors (O or N atom), and hydrogen bond donors (OH or NH group) in certain spatial separation.

(±)-Praeruptorin A (PA), a naturally existing pyranocumarin isolated from the dried root of a medicinal plant *Peucedanum praeruptorum* Dunn., was shown to re-sensitize Pgp-overexpressing MDR (Pgp-MDR) cells by transiently depleting ATP and/or suppressing Pgp expression.[11] DCK was derived from PA by replacing the two aliphatic acyloxys at C-3′ and C-4′ with two cynnamoyloxys that could form hydrophobic centers in a more planar configuration. Our data show that

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DCK is a potent MDR reversal agent and may directly inhibit Pgp by an non-competitive mechanism which interferes with the binding of transport substrates and/or the transport process.

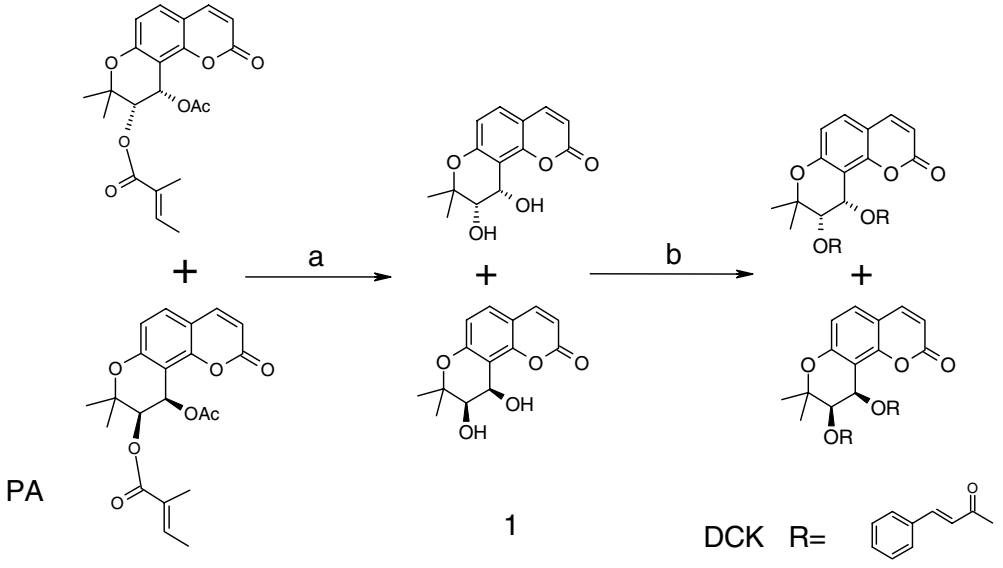
2. Results and discussion

2.1. Preparation and identification of DCK

DCK was prepared by a two-step reaction (see Scheme 1). Basic hydrolysis of PA by 0.5 M KOH in dioxane provided the intermediate (±)-*cis*-khellactone (**1**), and dicynnamoylation of (**1**) catalyzed by dimethylaminopyridine (DMAP) in $CHCl_2$ gave DCK.[12] The NMR and MS data of DCK were identical with those of (+)-3′-*O*,4′-*O*-dicynnamoyl-*cis*-khellactone reported by Wu et al.[12] Its zero specific rotation indicates that the product was (±)-3′-*O*,4′-*O*-dicynnamoyl-*cis*-khellactone.

2.2. DCK resensitized Pgp-MDR cells

In this study, human hepatoma cell line HepG2, leukemia cell line K562, and their Pgp-MDR sublines HepG2/Dox and K562/Dox were employed for testing MDR reversal activity of DCK. Compared to their parental drug-sensitive cells by IC_{50} values (concentration inhibiting 50% of cell growth), K562/Dox cells were 227.3 and 129.2 times more resistant to Pgp substrate anticancer drugs vinblastine and doxorubicin, whereas HepG2/Dox cells were 1174.2 and 211.8 times more resistant to the two drugs, respectively. Both HepG2/Dox and K562/Dox showed no drug resistance to non-Pgp substrate drug cisplatin (substrate of MRP2, MRP3), camptothecin (substrate of BCRP), and arsenic (substrate of MRP1), indicating that the overexpressed Pgp was the main reason for the MDR phenotype in the two cell lines used in this study (Table 1). DCK by itself showed little growth inhibitory effect (IC_{50} > 18 μM). However, the addition of 4 μM DCK to treatments with Pgp substrate drugs vinblastine, doxorubicin, puromycin or paclitaxel increased the drug sensitivity of HepG2/Dox cells, as indicated by changes in IC_{50}, by 28.2- 20.8- 17.6- and 23.5-fold, respectively (Table 2). Under similar conditions the increase in drug sensitivity of K562/Dox cells was 28.6- 15.0- 11.3- and 108.3-fold, respectively (Table 3). DCK showed a much stronger MDR reversing activity than PA and was



Scheme 1. Reagents and conditions: (a) 0.5 M KOH, dioxane, 60 °C, 20 min, 15%; (b) cynnamic acid, DMAP, DCC, CHCl2, reflux, 2.5 h, 61%.

Table 1. Cytotoxicity of anticancer agents, PA and DCK

Treatment	K562 IC_{50}[a]	K562/Dox IC_{50}[a]	RR[c]	HepG2 IC_{50}[b]	HepG2/Dox IC_{50}[b]	RR[c]
Vinblastine	8.80 ± 2.07 ($\times10^{-4}$)	0.20 ± 0.07	227.3	2.64 ± 0.67 ($\times10^{-4}$)	0.31± 0.05	1174.2
Doxorubicin	0.24 ± 0.11	31.02 ± 17.07	129.2	0.17 ± 0.09	36.00 ± 2.46	211.8
Cisplatin	2.58 ± 0.01	2.01	0.8	3.00 ± 0.79	2.07 ± 1.12	0.7
Camptothecin	0.10 ± 0.00	0.09 ± 0.01	0.9	0.02 ± 0.01	0.02 ± 0.01	1.0
Arsenic	3.34 ± 0.25	3.47 ± 0.61	1.0	4.52 ± 0.97	2.88 ± 0.60	0.6
PA	>50	30.14 ± 6.09	—	>50	14.42 ± 2.52	—
DCK	30.61 ± 5.43	52.59 ± 8.74	1.7	18.4 ± 4.28	34.67 ± 5.39	1.8

Cells were exposed to drugs for 72 h and IC_{50} (μM drug concentration inhibiting 50% growth) was determined. Values presented as means ± SD of three experiments.

[a] IC_{50} was determined by MTT assay.

[b] $IC_{50}$was determined by SRB assay.

[c] RR = Resistant ratio = $IC_{50\ (MDR\ cell)}/IC_{50(parental\ cell)}$.

Table 2. Effects of PA, DCK, and verapamil on drug sensitivity of HepG2 and HepG2/Dox cells

	HepG2/Dox		HepG2	
	IC_{50} (μM)	Sensitivity increased[a]	IC_{50} (μM)	Sensitivity increased[a]
Vinblastine	0.31 ± 0.05		2.64 ± 0.67 ($\times 10^{-4}$)	
With PA	0.13 ± 0.05	2.4	2.41 ± 0.88 ($\times 10^{-4}$)	1.1
With DCK	0.011 ± 0.004	28.2	2.45 ± 0.52 ($\times 10^{-4}$)	1.1
With verapamil	0.063 ± 0.023	4.9	3.38 ± 1.07 ($\times 10^{-4}$)	0.8
Doxorubicin	36.00 ± 2.46		0.17 ± 0.09	
With PA	25.43 ± 4.23	1.4	0.15 ± 0.03	1.1
With DCK	1.73 ± 0.52	20.8	0.15 ± 0.04	1.1
With verapamil	6.28 ± 3.33	5.7	—	—
Puromycin	104.41 ± 10.87		0.21 ± 0.03	
With PA	61.03 ± 6.32	1.7	0.20 ± 0.03	1.1
With DCK	5.93 ± 0.90	17.6	0.17 ± 0.07	1.2
With verapamil	13.49 ± 2.49	7.7	—	—
Paclitaxel	4.23 ± 0.05		5.94 ± 2.68 ($\times 10^{3}$)	
With PA	1.33 ± 0.54	3.2	5.42 ± 1.91($\times 10^{3}$)	1.1
With DCK	0.18 ± 0.14	23.5	9.78 ± 1.22 ($\times 10^{3}$)	0.6
With verapamil	0.86 ± 0.12	4.9	—	—

HepG2 and HepG2/Dox cells were exposed to anticancer drugs in the presence of 4 μM PA, DCK or verapamil for 72 h and IC_{50} (μM drug concentration inhibiting 50% growth) was determined by SRB assay. Values are means ± SD of three experiments.

[a] Folds increased in drug sensitivity = (IC_{50} of control)/(IC_{50} in the presence of modulator).

Table 3. Effects of PA, DCK, and verapamil on drug sensitivity of K562/Dox cells

Drug	IC_{50}	Sensitivity increased[a]
Vinblastine	0.20 ± 0.07	
With PA	0.057 ± 0.020	3.5
With DCK	0.007 ± 0.003	28.6
With verapamil	0.017 ± 0.005	11.8
Doxorubicin	31.02 ± 17.07	
With PA	19.70 ± 2.44	1.6
With DCK	2.08 ± 0.72	15.0
With verapamil	3.36 ± 0.93	9.3
Puromycin	47.69 ± 9.00	
With PA	30.13 ± 6.50	1.6
With DCK	4.23 ± 1.08	11.3
With verapamil	6.62 ± 1.49	7.2
Paclitaxel	3.25 ± 0.45	
With PA	1.44 ± 0.18	2.2
With DCK	0.03 ± 0.01	108.3
With verapamil	0.12 ± 0.02	27.1

K562/Dox cells were exposed to anticancer drugs in the presence of 4 μM PA, DCK or verapamil for 72 h and IC_{50} (μM drug concentration inhibiting 50% growth) was determined by MTT assay. Values are means ± SD of three experiments.

[a] Folds increased in drug sensitivity = (IC_{50} of control)/(IC_{50} in the presence of modulator).

active at concentrations as low as 2 μM. The decreases in IC_{50} values were not observed in drug-sensitive cells (Table 2), implying that DCK mainly acted on the overexpressed Pgp.

2.3. DCK enhanced doxorubicin-induced G2/M arrest in HepG2/Dox cells

Doxorubicin is a topoisomerase II inhibitor which induces G2/M arrest in cell cycle. As shown in Figure 1, in drug-sensitive HepG2 cells doxorubicin achieved almost a complete G2/M arrest at 0.2 μM but in Pgp-MDR HepG2/Dox cells the concentration required was 50 μM. DCK at concentrations as high as 20 μM had no effect on the cell cycle of HepG2/Dox cells. If DCK reverses MDR by maintaining cellular doxorubicin concentration as proposed, the effectiveness of doxorubicin on cell cycle arrest should be restored by DCK. Indeed, DCK significantly enhanced doxorubicin-induced G2/M arrest in Pgp-MDR cells but not in drug-sensitive cells. For example, 4 μM DCK reduced the required doxorubicin concentration from 50 to 2 μM. This result supported the suggestion that DCK was functioning as a Pgp modulator.

2.4. DCK increased drug uptake and reduced drug efflux in MDR cells

Flow cytometry analysis showed that DCK increased cellular concentration of doxorubicin, a Pgp substrate. After an 1-h incubation with doxorubicin, the presence of 10 μM DCK increased cellular doxorubicin accumulation by 30%, compared to a 25% increase caused by 15 μM of verapamil (Fig. 2A). The increase in drug accumulation was probably due to a decreased drug efflux since DCK inhibited doxorubicin efflux from HepG2/Dox cells pre-loaded with doxorubicin as efficient as verapamil (Fig. 2B). DCK also decreased the efflux of another fluorescent Pgp substrate Rh-123 in HepG2/Dox cells but in this case DCK was less active than verapamil (data not shown). The differential inhibitory effect is conceivable if these drugs are transported by different transport sites on Pgp.[13–18]

2.5. DCK did not affect Pgp expression

It was previously noted that PA might decrease both MDR1 mRNA level and Pgp level in Pgp-overexpressing KB V1 cells. Pgp expression was monitored by

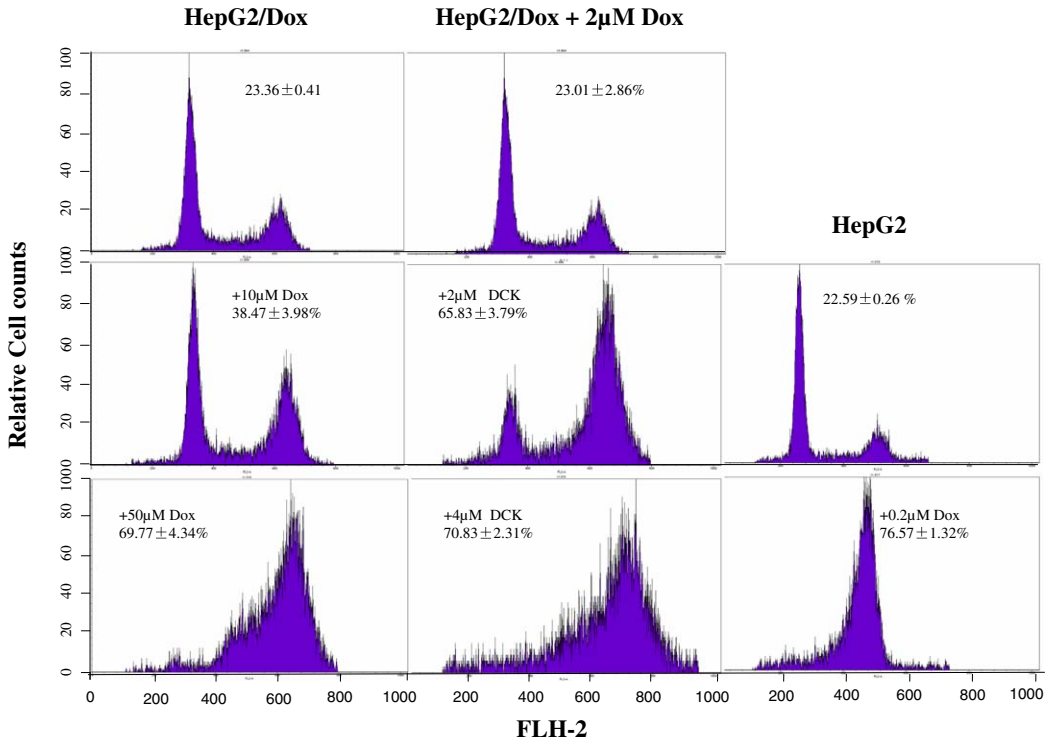


Figure 1. DCK enhanced doxorubicin-induced G2/M arrest in HepG2/Dox cells. Cells were incubated with doxorubicin with or without addition of DCK for 48 h. Flow cytometry results of one typical experiment are shown. Numerical data are percent of G2/M cells and are presented as means ± SD of three independent experiments.

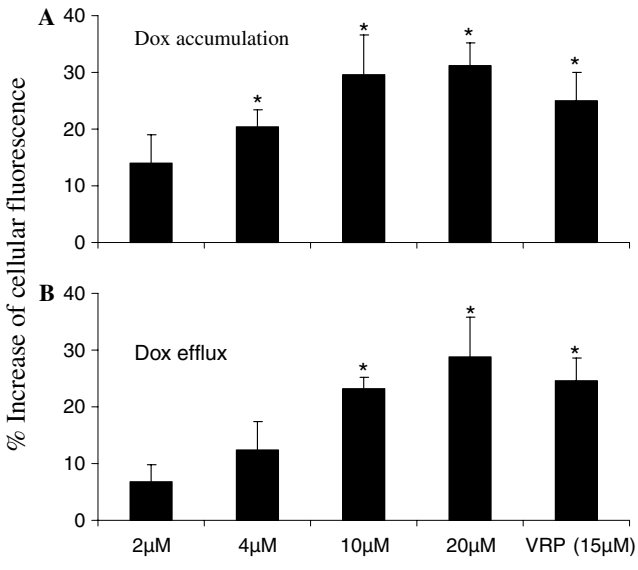


Figure 2. Effect of DCK on doxorubicin retention in HepG2/Dox cells. (A) Cellular doxorubicin accumulation assay. Cells were incubated with 5 μM doxorubicin with or without addition of DCK or verapamil (VRP) for 1 h. Effect of DCK and verapamil on cellular doxorubicin accumulation was analyzed by flow cytometry. Increase of cellular fluorescence is shown in percentage. (B) Doxorubicin efflux assay. Cells were treated with 5 μM doxorubicin for 1 h followed by an additional hour in doxorubicin-free medium with DCK or verapamil. Cellular fluorescence was analyzed by flow cytometry and increase of cellular fluorescence is shown in percentage. Results are mean values of three experiments. Significantly different at $*P < 0.05$ compared with baseline control.

Western blot analysis and our results showed that both K562/Dox cells and HepG2/Dox cells expressed high level of MDR1, and a 72 h treatment with 20 μM DCK did not affect the expression level (Fig. 3).

2.6. DCK inhibited transport substrate-stimulated Pgp-ATPase activity

Pgp hydrolyzes ATP to support drug transport activity therefore the Pgp-catalyzed ATP hydrolysis rate reflects indirectly the transport activity of Pgp. We studied Pgp-ATPase activity catalyzed by the membrane fractions prepared from Pgp-MDR cells while suppressing the activities of other major membrane ATPases. As shown in Figure 4, verapamil and progesterone, two standard Pgp transport substrates, stimulated ATP hydrolysis in a dose dependent manner. DCK by itself also slightly stimulated basal Pgp-ATPase activity (Fig. 4). But in the presence of 5 μM DCK, the verapamil- and progesterone-stimulated ATPase activity was notably inhibited with decreased V_{max}s but K_ms were decreased or unchanged.

2.7. DCK affecting UIC2 antibody reactivity

The mAb UIC2 is a conformation-sensitive antibody which preferentially binds to an external epitope of Pgp that is associated with substrate or competitive inhibitor.[18] UIC2 reactivity was monitored by the use of a fluorescent secondary antibody. Figure 5 shows that cyclosporin A, a Pgp substrate, increased UIC2 labeling

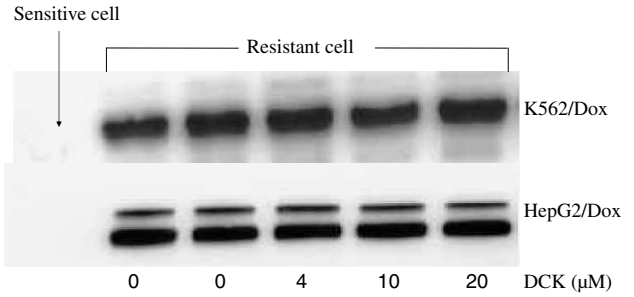


Figure 3. DCK did not affect Pgp expression within MDR cells. HepG2/Dox cells and K562/Dox cells were incubated with or without DCK for 72 h. Lysed cell extracts containing 50 μg of total cellular protein were separated on 10% SDS–PAGE. Pgp was detected by antibody labeling. Samples in lane 1 were from untreated K562 and HepG2 cells.

of HepG2/Dox cells. The blocking of the ATP site by sodium vanadate (Na_3VO_4), and thus blocking the transportation process, also decreased UIC2 labeling. Unlike substrates, DCK decreased UIC2 reactivity like Na_3VO_4.

2.8. DCK enhanced growth inhibitory effect of doxorubicin in soft-agar colony-forming assay

Cancer cell proliferation in a chronic cytotoxicity environment can be evaluated by the soft-agar colony formation assay. As shown in Figure 6, 4 μM DCK or 2 μM doxorubicin individually had no appreciable effect on colony formation of HepG2/Dox cells. However, when the two drugs were combined (4 μM DCK and 2 μM doxorubicin), few HepG2/Dox colonies could be formed.

3. Conclusion

Although the mother compound PA suppresses MDR1 gene expression, our results show that DCK is a more potent MDR modulator that inhibits Pgp directly.

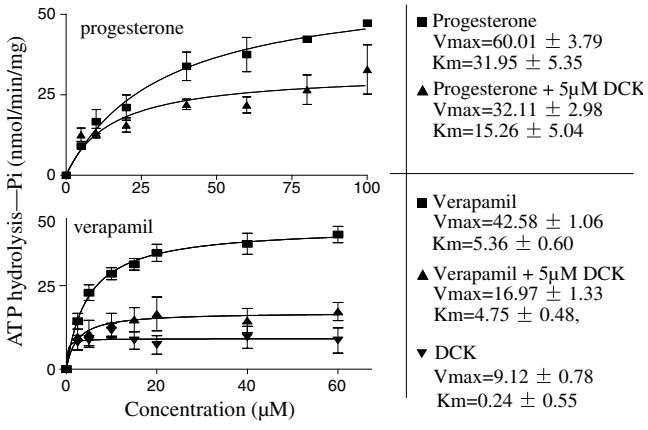


Figure 4. Effect of DCK on basal and substrate-stimulated ATPase activity of Pgp. Membrane vesicles were prepared from drug-resistant Pgp-overexpressing K562/Dox cells. Pgp ATPase activity was measured with other major membrane ATPases suppressed.

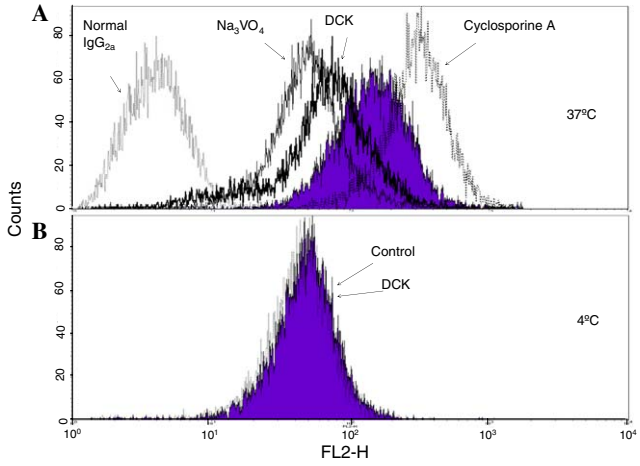


Figure 5. Effect of DCK on mAb UIC2 binding reactivity to Pgp. (A) HepG2/Dox cells reacted with mAb UIC2 at 37 °C, in the presence or in the absence (filled histogram) of 1 mM Na_3VO_4 (thin line), 5 μM DCK (thick line), or 5 μM cyclosporine A (broken line). Normal IgG_{2a} was used as negative control (dotted line). UIC2 binding affinity to Pgp can be detected by labeling with a fluorescent secondary antibody and analyzed by flow cytometry. 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 DCK or 5 μM cyclosporin A. (B) HepG2/Dox cells reacted with mAb UIC2 at 4 °C in the presence or in the absence (filled histogram) of 5 μM DCK (dotted line), the cellular fluorescence kept unaltered.

DCK stimulates the basal Pgp ATPase activity and this has some substrate-like activity but it is an effective non-competitive inhibitor that decreases the V_{max}s of verapamil and progesterone with the K_ms relatively unchanged. As a non-competitive inhibitor it is likely that DCK might bind to Pgp simultaneously with other substrates. The interaction would cause Pgp conformational changes that could hinder intramolecular movements necessary for the transportation process as well as transport-associated ATP hydrolysis.

DCK was derived from PA by replacing the two acyls at C-3′-*O* and C-4′-*O* site with two cynnamoyls. The new derivative has two bulky polar groups on the same plane and is more active than PA and verapamil in resensitizing Pgp-MDR cells. Our results provide a positive foundation for future PA based drug development.

4. Experimental

4.1. General

Doxorubicin, vinblastine, puromycin, paclitaxel, cisplatin, camptothecin, arsenic, verapamil, sulforhodamine B (SRB), 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT), MgATP, cell culture grade agarose, and other reagent grade chemicals were purchased from Sigma/Aldrich Co. (St. Louis, MO, USA). Propidium iodide (PI) was from Molecular Probes (OR, USA). Cell culture media and supplements were products of GibcoBRL (MD, USA). Nitrocellulose membranes and secondary antibody (horseradish–

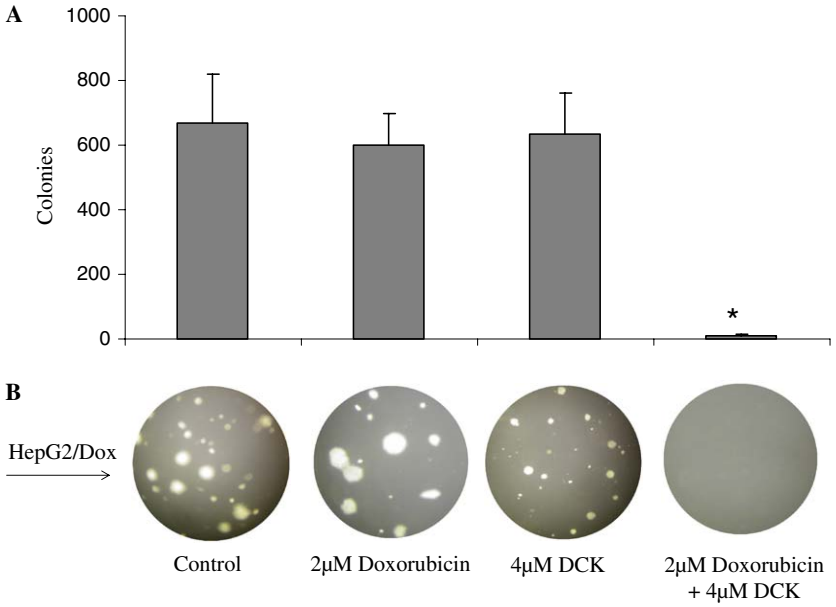


Figure 6. DCK enhanced growth inhibitory effect of doxorubicin in soft-agar colony-forming assay. (A) Colony numbers from three independent experiments are shown as means ± SD. Significantly different to the untreated control group at $*P < 0.05$. (B) Pictures from a typical experiment showing the colonies formed in untreated plate (control), and the plates treated with 2 μM doxorubicin, or 4 μM DCK, or combination of 2 μM doxorubicin and 4 μM DCK.

peroxidase-conjugated anti-rabbit IgG) were from Bio-Rad (CA, USA), and the anti-Pgp antibody was from Calbiochem (La Jolla, USA). Anti-MDR1 monoclonal UIC2 (UIC2 mouse monoclonal anti-human MDR1) was from Immunotech (PA, USA). Goat anti-mouse IgG2a-PE and normal IgG2a were from Santa Cruz Biotechnology (CA, USA).

Nuclear magnetic resonance spectra (NMR) were recorded on a Varian NMR-300 MHz spectrometer in CDCl3. Optical rotation was measured on a PE343 polarimeter. Mass spectra (MS) were recorded on a Thermo-Finnigan LCQ advantage mass spectrometer.

4.2. Preparation of DCK

Basic hydrolysis was carried-out by adding 60 mL of 0.5 M KOH to PA (1.0 g in 150 mL of dioxane) and the mixture was stirred at 60 °C for 20 min. After cooling, pH was adjusted to 2–3 by 10% H_2SO_4 and the mixture was stirred for 2 h at room temperature and extracted with $CHCl_3$ and trice (100, 100, and 60 mL). The combined $CHCl_3$ fraction was washed with 100 mL of saturated $NaHCO_3$ followed by 100 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 a thin-layer chromatograph (TLC) at 365 nm. The yield was 150 mg (15%) of (**1**). Compound (**1**) appeared to be white amorphous solid with zero specific rotation. ^{1}H NMR δ ppm ($CDCl_3$-300 MHz): 7.67 (1H, d, $J = 9.5$ Hz, 4-H), 7.33 (1H, d, $J = 8.6$ Hz, 5-H), 6.81 (1H, d, $J = 8.6$ Hz, 6-H), 6.23(1H, d, $J = 9.6$ Hz, 3-H), 5.21 (1H, d, $J = 4.9$ Hz, 4′-H), 3.88 (1H, d, $J = 5.0$ Hz, 3′-H), 1.48 and 1.41 [3H each, s, 2′-$(Me)_2$]. MS (ESI) for $C_{14}H_{14}O_5$: 285 $[M+Na]^+$.

Cinnamic acid (220 mg or 15 mmol) was added to a mixture of (**1**) (80 mg, 0.31 mmol), dichloromethane (5 mL), *N*, *N*′-dicyclohexylcarbodiimide (206 mg, 1 mmol), and 4-dimethylaminopyridine (4 mg, 0.032 mmol). The mixture was stirred/refluxed for 3 h, cooled to room temperature, filtered, and the filtrate was separated and purified by a repeated flash silica gel 60 column chromatograph (petroleum ether/EtOAc, 4:1). Fractions were monitored by TLC at 365 nm and purified DCK (81 mg, 52% yield) was obtained.

DCK appeared to be white amorphous solid with a zero specific rotation. ^{1}H NMR δppm ($CDCl_3$-300 MHz): 7.68 (2H, d, $J = 15.9$ Hz, –CO–CH═), 7.61 (1H, d, $J = 9.5$ Hz, 4-H), 7.47–7.42 (4H, m, Ar–H), 7.39 (1H, d, $J = 8.6$ Hz, 5-H), 7.36–7.30 (6H, m, Ar–H), 6.86 (1H, d, $J = 8.4$ Hz, 6-H), 6.78 (1H, d, $J = 4.9$ Hz, 4′-H), 6.45 (2H, d, $J = 15.9$ Hz, Ar–CH═), 6.22 (1H, d, $J = 9.6$ Hz, 3-H), 5.53(1H, d, $J = 4.9$ Hz, 3′-H), 1.57 and 1.48(3H each, s, C-2′-Me). MS (ESI) for $C_{32}H_{26}O_7$: 545 $[M+Na]^+$.

4.3. Cell lines and cell culture

Human hepatoma cell line HepG2 and its doxorubicin-selected Pgp-overexpressing subline HepG2/Dox were kindly provided by Dr. Judy Chan, Chinese University of Hong Kong.[19] Human leukemia cell line K562 was from Gibco (ATCC No. CCL-243), whereas its doxorubicin-selected Pgp-overexpressing subline K562/Dox was kindly provided by Dr. Morjani, University of Reims Champagne-Ardenne, France.[20,21] All cell lines were grown in RPMI 1640 medium containing 10% FBS and 100 U antibiotics, at 37 °C in a humidified 5% CO_2 incubator. To maintain MDR phenotype,

1.2 and 0.1 μM doxorubicin were added to HepG2/Dox and K562/Dox cultures, respectively. All MDR cells were maintained in drug-free medium for at least 7 days before used.

4.4. Cell growth inhibition assay

Drug concentrations inhibiting 50% growth (IC_{50}) were determined in 72 h cultures in the 96-well format. The cell number of anchorage dependent HepG2 and HepG2/Dox cells was estimated by SRB cellular protein assay.[22] Each well was seeded with 5000 cells in 50 μl medium per well and incubated overnight. Cells were incubated with fresh medium containing drugs for 72 h, fixed in 50 μl ice-cold 15% trichloroacetic acid, and washed with triple-distilled water five times. Cellular protein was stained by adding 50 μl of 0.4% SRB in 1% acetic acid for 10 min, rinsed with 1% acetic acid five times, and air-dried. After adding 100 μl of 10 mM Tris base (pH 10.5), optical density (OD) at 515 nm was obtained.

MTT cytotoxic assay[23] was employed to monitor viable cells in suspended K562 and K562/Dox cell cultures. Cells were seeded and grown in 96-well plates as above, treated with drugs for 68 h, and 10 μl of 5 mg/mL MTT solution was added to each well. After 4 h, 100 μl of a stop solution (50% isobutanol, 0.04 N HCl, and 10% SDS) was added and plates were incubated overnight at room temperature. OD was determined at 570 nm. Each experiment was repeated three times and results were expressed as means ± standard deviation (SD). Solvents and media were included as blank control.

4.5. MDR reversing activity

MDR reversing activity of a test compound was evaluated by comparing IC_{50} values of an anticancer drug in the presence and absence of 4 μM of the test compound. Verapamil, a Pgp modulator, was used as the positive control and medium control was subtracted as background. All experiments were repeated at least three times.

4.6. Cellular doxorubicin accumulation

About 2×10^6 cells were suspended in 2 mL of medium containing 5 μM doxorubicin. Various concentrations of a test substance were added and incubated at 37 °C for 40 min. Cells were washed with ice-cold PBS twice and resuspended in 1 mL of ice-cold phosphate-buffered saline (PBS). Verapamil was included as a positive control. Cellular doxorubicin concentration was monitored by a FACSCAN flow cytometer (Becton–Dickinson Immunocytometry Systems, San Jose, CA, USA).

4.7. Drug efflux assay

About 2×10^6 cells were suspended in 2 mL of medium containing 5 μM doxorubicin and incubated at 37 °C for 1 h. Cells were washed with ice-cold PBS twice, resuspended in fresh medium containing the test modulator, and incubated at 37 °C for 1 h. Cells were washed with ice-cold PBS twice and resuspended in 1 mL of ice-cold PBS. Cellular fluorescence intensity was monitored on a FACSCAN flow cytometer.

4.8. Cell cycle distribution

About 1×10^6 cells in 1 mL medium were seeded in a 35-mm culture dish and incubated overnight. One milliliter of medium with or without drugs of various concentrations was added and incubated for 48 h. Cells were harvested, washed with PBS twice, and fixed in 70% EtOH at −20 °C overnight. After that, the cells were washed with PBS once, resuspended in 1 mL of PBS containing 100 μg/mL Rnase A, and incubated at 37 °C for 30 min. PI staining solution was added at a final concentration of 40 μg/mL and incubated at RT for 5–10 min. Samples were then analyzed by a FACSCAN flow cytometer.

4.9. Western blot analysis for detection of Pgp expression

HepG2/Dox and K562/Dox cells were treated for 72 h with 5, 10 and 20 μM, DCK in cell growth medium and were then incubated in ice-cold lysis buffer (50 mM Tris, pH 7.4, 100 mM NaCl, 1 mM EDTA, 5% glycerol, 0.5 mM $MgCl_2$, 0.5% NP 40, 0.05% SDS, 1 mM PMSF, 1 μg/mL leupeptin, 1% aprotinin, 1% pepstatin A, 2 mM Na_3VO_4, and 50 mM NaF) for 20 min and collected by centrifugation (10,000*g*) at 4 °C for 15 min. Protein concentration of supernatants was determined by Bradford assay. Samples containing 50 μg of protein were subjected to 10% SDS–PAGE and electro-transferred to nitrocellulose membranes. Membranes were blocked with 3% non-fat milk/0.05% Tween 20/TBS (10 mM Tris, pH 7.5, 100 mM NaCl), incubated with anti-Pgp antibody for 1 h at room temperature followed by horseradish–peroxidase-conjugated secondary antibody for another 1 h at room temperature. Protein bands were detected by the ECL method. Drug-sensitive HepG2 and K562 cell extracts were also included as basal expression level control.

4.10. Pgp-enriched microsomal membrane preparation

Pgp-overexpressing K562/Dox cells (10^9–10^{10}) were washed with ice-cold PBS containing 0.1 mM PMSF and vesicle buffer (0.01 M Tris–HCl, pH 7.5, 0.25 M sucrose, and 0.2 mM $CaCl_2$) at 4 °C and collected by centrifugation (4000*g*, 10 min). Membrane vesicles were prepared by following the steps as described previously[24] and stored at −80 °C before use.

4.11. ATP hydrolysis[24–26]

Membrane preparations were thawed on ice and diluted by ice-cold ATPase buffer (50 mM Tris–Cl, pH 7.4, 50 mM KCl, 2.5 mM $MgSO_4$, and 3 mM DTT) containing 3 mM sodium azide (to inhibit the mitochondrial ATPase), 0.5 mM EGTA (to inhibit Ca-ATPase), and 2 mM ouabain (to inhibit the Na/K-ATPase). The reaction was carried out in the 96-well format and reaction mixture (60 μl per well) contained membrane preparation (10 μg of protein), drugs, and 4 mM MgATP in ATPase buffer. Plates were kept in ice and the reaction

was started by bringing the plates to 37 °C. After 30 min, the reaction was terminated by the addition of 30 μl of 10% SDS solution, 200 μl of a 4:1 mixture of 10% fresh ascorbic acid (pH 5.0) and 35 mM ammonium molybdate in 15 mM zinc acetate (pH 5.0). After 30 min at 37 °C in the dark, absorption at 750 nm was determined and the free phosphate content was determined by comparing to a standard curve. Base line controls contained 100 μM Na_3VO_4, pH 10.0, and/or ethanol (drug solvent) at a maximum final concentration of 2% v/v. All experiments were repeated three times.

4.12. Soft-agar colony formation

Each sample (5000 cells and drugs in 1.5 mL of 0.3% agar-medium) was plated onto a 1.5-mL underlayer (0.6% agar in RPMI-1640 medium and 10% FBS) in a 35-mm dish. Plates were incubated at 37 °C in humidified 5% CO_2 for 2 weeks.

4.13. UIC2 binding ability

This assay was performed as described[27] with minor modifications. HepG2/Dox Cells were washed with PBS and resuspended in UIC2 binding buffer (PBS + 1 % BSA). Approximately 1×10^6 cells in 800 μl binding buffer were pre-warmed at 37 °C for 10 min, incubated with drugs at 37 °C for another 10 min, and 1 μg of the monoclonal antibody UIC2 was added. After 15 min at 37 °C, 700 μl of ice-cold UIC2 buffer was added to stop the reaction. Cell samples were washed with cold buffer twice, resuspended in 500 μl ice-cold UIC2 buffer and 2 μl of goat anti-mouse IgG_{2a}-PE was added. After 15 min at 4 °C in the dark, samples were washed, resuspended in 1 mL ice-cold UIC2 binding buffer, and analyzed by using a FACSCalibur flow cytometer. The fluorescence intensity associated with the cells was expressed on a log scale. Normal mouse IgG_{2a} served as a negative control. UIC2 reactivity at 4 °C in DCK-treated HepG2/Dox cells was also performed as control.

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