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## Anti-AIDS agents 89. Identification of DCX derivatives as anti-HIV and chemosensitizing dual function agents to overcome P-gp-mediated drug resistance for AIDS therapy

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### Abstract

In this study, 19 dicamphanoyl-dihydropyranochromone (DCP) and dicamphanoyl-dihydropyranoxanthone (DCX) derivatives, previously discovered as novel anti-HIV agents, were evaluated for their potential to reverse multi-drug resistance (MDR) in a cancer cell line over-expressing P-glycoprotein (P-gp). Seven compounds fully reversed resistance to vincristine (VCR) at 4  $\mu$ M, a 20-fold enhancement compared to the first generation chemosensitizer, verapamil (4  $\mu$ M). The mechanism of action of DCPs and DCXs was also resolved, since the most active compounds (**3**, **4**, and **7**) significantly increased intracellular drug accumulation due, in part, to inhibiting the P-gp mediated drug efflux from cells. We conclude that DCPs (**3** and **4**) and DCXs (**7**, **11**, and **17**) can exhibit polypharmacologic behavior by acting as dual inhibitors of HIV replication and chemoresistance mediated by P-gp. As such, they may be useful in combination therapy to overcome P-gp-associated drug resistance for AIDS treatment.

### Keywords

pyranochromone derivatives; chemoresistance; P-glycoprotein (P-gp) inhibitors; anti-HIV agents; dual function inhibitors

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The first and best characterized mammalian drug-efflux pump, the ATP-binding cassette (ABC), P-glycoprotein (P-gp), is known to play a significant role in anti-HIV drug absorption and disposition.<sup>1</sup> Even after several years of highly active anti-retrovirus treatment (HAART), failure to eradicate HIV in P-gp distributed reservoirs, such as the brain and HIV-infected lymphocytes, as well as multi-drug resistance (MDR),<sup>2–4</sup> are both attributed in part to P-gp expression. Among current anti-HIV drugs, most protease inhibitors (PIs) and selected non-nucleoside reverse transcriptase inhibitors (NNRTIs) have

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been identified either as P-gp substrates or as inducers of P-gp expression.<sup>5</sup> It is the expression of P-gp that accounts for reductions in both intracellular concentration and bioavailability of AIDS drugs.<sup>6</sup> Therefore, a novel anti-HIV agent having MDR reversal action should, in principle, have a dual benefit for combination (HAART) therapy since it would target HIV replication and improve the bioavailability of other anti-HIV agents used in the combination that are P-gp substrates.

Pyranochromone derivatives, analogs of dicamphanoyl-dihydropyranochromone (DCP) and dicamphanoyl-dihydropyranoxanthone (DCX) (Figure 1), were previously identified in one of our laboratories as potent anti-HIV agents against both wild-type and drug-resistant HIV strains, with unique molecular structures and mechanism of action.<sup>7, 8</sup> Since the structural features, including the conjugated planar system and bulky side chains at the 3' and 4' positions, share certain similarities to current pharmacophore model for P-gp inhibitors,<sup>9</sup> we hypothesised that these compounds might serve as P-gp inhibitors to reverse P-gp efflux potency and function in P-gp over-expressed cancer cells. To evaluate our hypothesis, DCP and DCX analogs with high anti-HIV selectivity were synthesized<sup>7, 8, 10</sup> and tested by following a two-step bio-assay to (1) determine their capacity to reverse VCR resistance of MDR-1 cancer cells and (2) measure P-gp-mediated drug efflux. As reported herein, we have successfully identified dual-function anti-HIV agents using this approach.

DCP and DCX analogs were synthesized following literature methods reported earlier, as illustrated in Scheme 1.<sup>7, 8, 10</sup> Briefly, reaction of **20** or **23** with ethyl alkanoates or hydroxybenzoic acids under appropriate conditions gave hydroxylated chromone (**21**) and xanthone (**24**), respectively. Reaction of the resulting compounds with a pyrano moiety through alkylation and cyclization yielded **22** and **25**. After asymmetric dihydroxylation and esterification, the final products **1–19** were obtained.

Compounds **1–19** are potent anti-HIV agents with activity starting from 0.06  $\mu\text{M}$  and 0.07  $\mu\text{M}$  against wild-type and multidrug-resistant HIV strains, respectively, and with low selective cytotoxicity against the host TZM-bl cell line ( $\text{IC}_{50}$  generally over 10  $\mu\text{M}$ ).<sup>7, 8</sup> In this study, the ability of these anti-HIV compounds to reverse MDR using a P-gp over-expressed cancer cell line (KB-vin) was evaluated by measuring the reversal of vincristine (VCR) resistance in the presence of 4  $\mu\text{M}$  of DCP or DCX analogs. Verapamil, a first generation chemosensitizer and P-gp competitive inhibitor, was used as the positive control. The results are shown in Table 1. In general, the DCP and DCX analogs dramatically reversed the VCR resistance in the KB-vin cells by lowering the  $\text{GI}_{50}$  values of VCR under co-treatment conditions.

DCP analogs **3** and **4**, with a methyl group substituted at  $\text{R}^2$ , afforded a ten-fold better chemosensitization than their counterparts **1** and **2**, as shown in Table 1. (The  $\text{GI}_{50}$  of VCR is 2.9 or 3.9 nM in combination with compound **3** or **4**, versus 30 or 20 nM in combination with compound **1** or **2**.) This suggests that  $\text{R}^2$ -methyl in DCP is important for optimizing the activity. The small alkyl group is also tolerated well at  $\text{R}^1$  in DCP. Both **3** and **4** exerted high activity significantly better than verapamil. Both compounds also showed potent anti-HIC activity. Significantly, compound **3** fully reversed the VCR resistance in KB-vin under the co-treatment condition (with  $\text{GI}_{50}$  of 2.0 nM for VCR in the parent drug-sensitive KB cell line).

Compounds **5–19** are DCX analogs with diverse substitutions on the A and C rings. As shown in Table 1, most of the analogs exhibited high chemosensitization activity, indicating that a planar ring extension from chromone to xanthone is not detrimental. Compounds **5–7** bearing different substitutions on  $\text{R}^7$  afforded the antiviral compounds with a wide spectrum of chemosensitization activities. Compounds **5** and **7** with non-substitution or  $\text{R}^7$ -methoxy-

substitution exhibited comparable effects to **3** and **4**, with co-treatment GI<sub>50</sub>s as low as 2.1 and 3.4 nM, respectively. In contrast, **6**, an R<sup>7</sup>-hydroxy-DCX, failed to reverse the cytotoxicity of VCR at 4 μM. The SAR could be explained if the oxo group in the B ring functions as an H-bonding acceptor important for the interaction with the target protein, the R<sup>7</sup>-OH group may destroy the interaction by forming the intramolecular H-bonding. Compounds **8–18** contain diverse substitutions around the A-ring. Generally, an alkyl, alkoxy, or fluoride substituent at R<sup>3</sup> or R<sup>6</sup> sustained or enhanced the chemosensitization activity compared with **5** and **7**. Compounds **8**, **11**, **12**, and **16** fully reversed VCR resistance with co-treatment GI<sub>50</sub> values around 2–3 nM. Compounds with R<sup>4</sup> or R<sup>5</sup> substituents (**9**, **10**, **13**, **14**, **15** and **18**) showed no significant chemosensitization activity, although some of them were good HIV inhibitors, suggesting that chemosensitization activity is sensitive to the substitutions at R<sup>4</sup> or R<sup>5</sup>. Extension/modification on the ring system horizontally or increasing the molecular hindrance was unfavorable for the interaction of the drug molecules with the target protein(s), such as P-gp. Compound **17**, with a fluoro moiety at R<sup>5</sup>, demonstrated a comparable chemosensitization activity to the non-F-substituted analog **7** and its positional isomer **16**. This result could be attributed to the isosteric relationship of F vs H. It is interesting that introducing bromide at R<sup>8</sup> (compound **19**) significantly improved MDR reversal activity approximately two-fold compared to compound **7**, with a co-treatment GI<sub>50</sub> value as low as 1.6 nM, indicating that R<sup>8</sup> may be an important position for optimizing chemosensitization activity. Further investigation is needed to verify and extend the preliminary SAR considerations noted above.

Data shown in Table 1 demonstrated a lack of concordance between anti-HIV activity and MDR reversal potency. Some compounds, such as compounds **1**, **2**, and **9**, were potent anti-HIV inhibitors, but weaker chemosensitizers. In contrast, compounds **5**, **8**, and **12** significantly reversed VCR resistance, but only weakly inhibited anti-HIV replication. This finding is not too surprising, since different targets are presumably involved. Nevertheless, compounds **3**, **4**, **7**, **11**, and **17** exhibited significant polypharmacology and, as such, are interesting dual function anti-HIV and chemosensitization leads.

It is known that the acquired VCR resistance in KB-Vin is due to the enhanced drug efflux via P-gp over-expression.<sup>12, 13</sup> Since the addition of DCP and DCX reversed VCR's cytotoxicity as determined by phenotypic assay, we next investigated whether select compounds were active using a P-gp functional assay. The effect of DCP or DCX analogs on efflux of the P-gp substrate Doxorubicin (Dox) was determined using an established fluorometric cell-based assay. In this assay, inhibition of P-gp leads to the intracellular accumulation of Dox, a fluorescent anthracycline cancer drug, which serves as a quantitative measurement of P-gp inhibition.<sup>14–16</sup> Three compounds with the most active chemosensitization action (**3**, **4**, and **7** in Table 1) were tested for their ability to facilitate Dox influx in comparison to the positive control inhibitor, verapamil. The results are shown in Figure 2. All three compounds induced significant intracellular accumulation of Dox under co-treatment conditions, and all were more active than the positive control compound, verapamil. The results suggest that chemosensitization by DCP and DCX analogs can be attributed in part to the inhibition of P-gp efflux activity in the KB-MDR-1 cell system. Further work is needed to determine whether P-gp inhibition is the sole mechanism of chemosensitization by these novel anti-HIV compounds.

In summary, nineteen pyranochromone derivatives (DCPs and DCXs) with selective anti-HIV activity were tested using a phenotypic assay for chemosensitization activity. Most derivatives evaluated were better chemosensitizers than the control agent verapamil. Seven of them (**3**, **5**, **8**, **11**, **12**, **17** and **19**) fully reversed VCR resistance under the co-treatment condition used. In general, the SAR profiles of chemosensitization and anti-HIV activities were not concordant but five compounds, **3**, **4**, **7**, **11** and **17**, showed significant dual

pharmacologic actions. Using a P-gp functional assay, the inhibition of cancer drug efflux was demonstrated in co-treated cells, suggesting a likely mechanism for the chemosensitization action involves P-gp inhibition. Taken together, our results show that several pyranochromone derivatives possessing potent anti-HIV activity also exert substantial chemosensitization activity via P-gp inhibition. In principle, this unique, dual-function behavior would make these ideal candidates for exploring new combination (HAART) therapies since they are a novel mechanistic class of reverse transcriptase inhibitor (RTI) that could sensitize and increase efficacy of other anti-HIV drugs that are P-gp substrates. Work is ongoing to better define the mechanism of action of these pyranochromone derivatives and to establish pre-clinical anti-HIV activity in combination regimens.

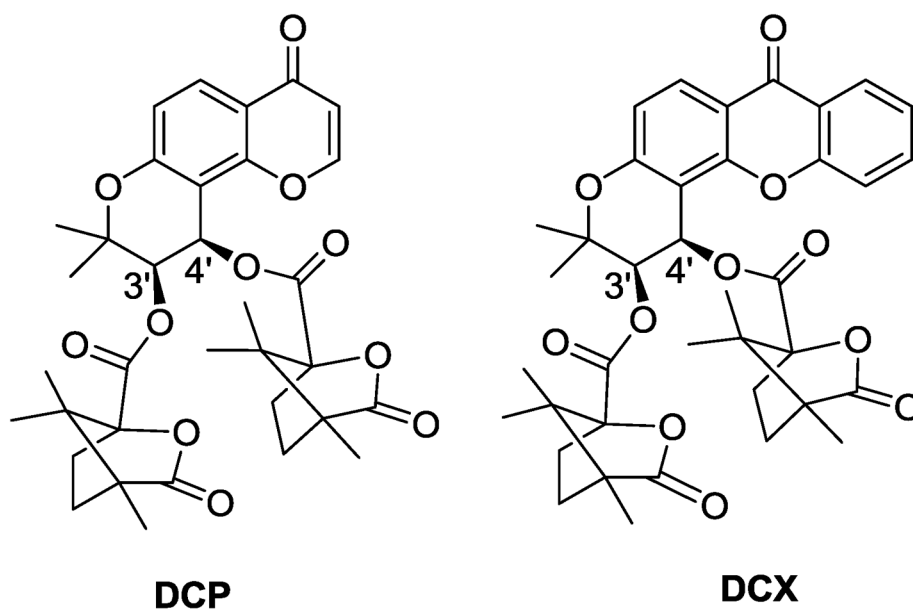
## Acknowledgments

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17. Measurement of the intracellular accumulation of doxorubicin (Dox): KBvin cells ( $5 \times 10^3$  cell/well) were seeded in 96-well plates and pre-incubated for 72 h in a 5% CO<sub>2</sub> incubator at 37 °C. Then, the cells were washed with the culture medium and pretreated with samples or vehicle 1 h before Dox was added at a final concentration of 10 μM. After 3 h incubation, the medium was removed by aspiration, and the cells were washed with ice-cold PBS, then lysed with 1% sodium

dodecyl sulfate (SDS) in PBS. Dox-associated mean fluorescence intensity was measured at Ex = 488 nm and Em = 580 nm with a fluorescence microplate reader (Plate Chameleon Multilabel Detection Platform, Hidex Oy, Turku, Finland) with MikroWin software. Verapamil, as a known P-gp inhibitor/modulator, was used as the positive control. All the data were calculated as the ratio of dox fluorescence with test compound divided by dox fluorescence without test compound after subtraction of the fluorescence of the control.



**Figure 1.**  
Structures of DCP and DCX.

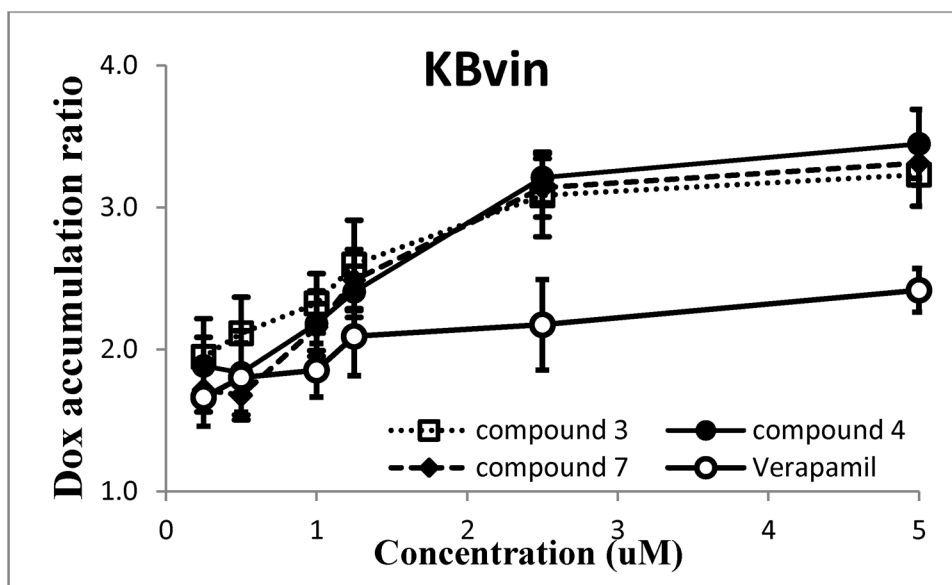
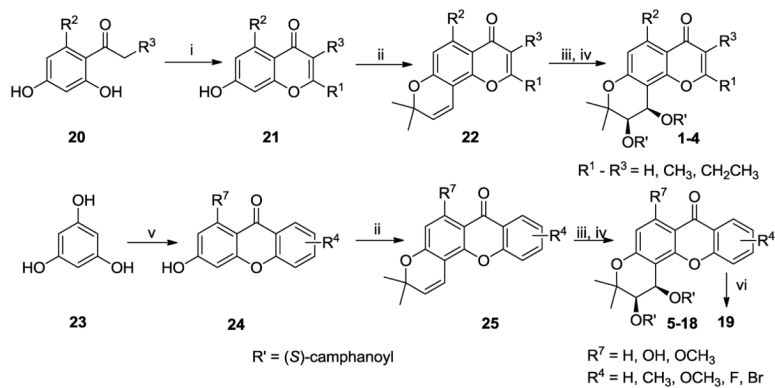


Figure 2.  
Dose-dependent Doxorubicin accumulation<sup>17</sup>

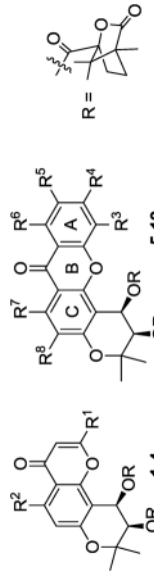
**Scheme 1.**

Reactions and Conditions: (i) ethyl alkanoates, NaH, THF, reflux, amberlyst 15 resin, isopropanol reflux; (ii) 4,4-dimethoxy-2-methyl-2-butanol, pyridine, microwave; (iii)  $\text{K}_3\text{Fe}(\text{CN})_6$ ,  $(\text{DHQ})_2\text{Pyr}$ ,  $\text{K}_2\text{OsO}_2(\text{OH})_4$ ,  $\text{K}_2\text{CO}_3$ , methanesulfonamide, *t*-butanol/ $\text{H}_2\text{O}$ ,  $0^\circ\text{C}$ ; (iv) (*S*)-camphanoyl chloride, DMAP,  $\text{CH}_2\text{Cl}_2$ ; (v) 2-hydroxybenzoic acids, Eaton's reagent, reflux; (vi) NBS,  $\text{CH}_2\text{Cl}_2$ .

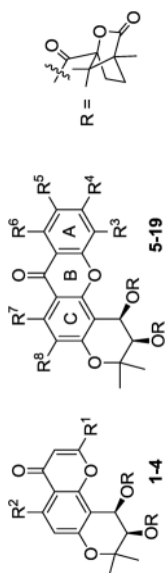


Table 1

Anti-HIV and chemosensitizing activity of selected DCP and DCX analogs.



Compd.	Structure								EC <sub>50</sub> (anti-HIV) (μM) <sup>a</sup>	GI <sub>50</sub> (nM) <sup>b</sup> (cytotoxicity)	Cytotoxicity fold <sup>c</sup>
	R <sup>1</sup>	R <sup>2</sup>	R <sup>3</sup>	R <sup>4</sup>	R <sup>5</sup>	R <sup>6</sup>	R <sup>7</sup>	R <sup>8</sup>			
1	CH <sub>3</sub>	H	---	---	---	---	---	---	0.10	30	88
2	CH <sub>2</sub> CH <sub>3</sub>	H	---	---	---	---	---	---	0.07	20	132
3	CH <sub>3</sub>	CH <sub>3</sub>	---	---	---	---	---	---	0.036	2.9	910
4	CH <sub>2</sub> CH <sub>3</sub>	CH <sub>3</sub>	---	---	---	---	---	---	0.10	3.9	677
5	---	---	H	H	H	H	H	H	0.308	2.1	1257
6	---	---	H	H	H	H	OH	H	N/A	>110	<24
7	---	---	H	H	H	H	OCH <sub>3</sub>	H	0.063	3.4	776
8	---	---	CH <sub>3</sub>	H	H	H	OCH <sub>3</sub>	H	1.52	2.4	1100
9	---	---	H	CH <sub>3</sub>	H	H	OCH <sub>3</sub>	H	0.095	7.5	352
10	---	---	H	H	CH <sub>3</sub>	H	OCH <sub>3</sub>	H	0.065	6.8	388
11	---	---	H	H	H	CH <sub>3</sub>	OCH <sub>3</sub>	H	0.15	1.9	1389
12	---	---	OCH <sub>3</sub>	H	H	H	OCH <sub>3</sub>	H	N/A	2.6	1015
13	---	---	H	OCH <sub>3</sub>	H	H	OCH <sub>3</sub>	H	0.362	27	98
14	---	---	H	H	OCH <sub>3</sub>	H	OCH <sub>3</sub>	H	0.121	7.7	343
15	---	---	H	OCH <sub>3</sub>	H	CH <sub>3</sub>	OCH <sub>3</sub>	H	0.14	5.1	518
16	---	---	F	H	H	H	OCH <sub>3</sub>	H	0.23	3.0	880
17	---	---	H	H	F	H	OCH <sub>3</sub>	H	0.10	2.3	1148
18	---	---	H	Br	H	H	OCH <sub>3</sub>	H	N/A	10	264
19	---	---	H	H	H	H	OCH <sub>3</sub>	Br	N/A	1.6	1650



Compd.	Structure								EC <sub>50</sub> (anti-HIV) (μM) <sup>a</sup>	GI <sub>50</sub> (nM) <sup>b</sup> (cytotoxicity)	Cytotoxicity fold <sup>c</sup>
	R <sup>1</sup>	R <sup>2</sup>	R <sup>3</sup>	R <sup>4</sup>	R <sup>5</sup>	R <sup>6</sup>	R <sup>7</sup>	R <sup>8</sup>			
Verapamil (4μM)									---	44	60
VCR									---	2640	---
VCR in KB									---	2.0	1320

<sup>a</sup>The anti-viral assay was performed using HIV/NL4-3 infected TZM-bl cells; the results were reported.<sup>7-8</sup>

<sup>b</sup>GI<sub>50</sub> values of the compounds on the cytotoxicity of vincristine (VCR) toward vincristine resistant KB (KBvin) and parental KB cells were determined by SRB assay.<sup>11</sup>

<sup>c</sup>The Cytotoxicity fold values, as potency parameter of test compounds, were calculated as: Cytotoxicity fold = GI<sub>50</sub> (anticancer drug alone)/GI<sub>50</sub> (anticancer drug + test compound). All experiments were performed at least three times.