

Multidrug Resistance Reversal Activity of Taxoids from *Taxus cuspidata* in KB-C2 and 2780AD Cells

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Some non-taxol-type taxoids having neither an oxetane ring at C-4 and C-5 nor an *N*-acylphenylisoserine group at C-13, such as taxuspine C, 2'-desacetoxyaustrospicatine, and 2-desacetoxytaxinine J, which were isolated from the Japanese yew *Taxus cuspidata*, increased cellular accumulation of vincristine (VCR) in multidrug-resistant 2780AD cells as potently as verapamil, and efficiently inhibited [³H]azidopine photolabeling of P-glycoprotein (P-gp). Taxuspine C, 2'-desacetoxyaustrospicatine, and 2-desacetoxytaxinine J at 10 μ M completely reversed the resistance to colchicine, VCR, and taxol in KB-C2 cells, which overexpress P-gp, while taxinine and taxinine M showed no effect. Taxuspine C, 2'-desacetoxyaustrospicatine, and 2-desacetoxytaxinine J may be candidate pharmaceuticals for reversing multidrug resistance (MDR) and also may be good modifiers of MDR in cancer chemotherapy.

Key words: Multidrug resistance reversal activity — P-glycoprotein — Taxoids — MDR modifiers

Resistance to multiple anticancer agents is a major obstacle to the treatment of many types of tumors by chemotherapy. Multidrug resistance (MDR) in cultured cell lines is known to be conferred by two different membrane glycoproteins, the 170 kDa P-glycoprotein (P-gp)¹ and the 190 kDa multidrug resistance protein (MRP).² Tumor cells carrying the MDR phenotypes are characterized by the overexpression of an energy-dependent drug transport protein, P-gp, resulting in a decreased accumulation of the drug within the cells because of the efficient efflux system. Since the discovery of a calcium channel blocker, verapamil, as an agent for overcoming MDR, various compounds including quinidine, tamoxifen, and cyclosporin A, have been reported to overcome MDR.³ We previously reported that among a number of new and known taxoids isolated from the Japanese yew *Taxus cuspidata*,^{4,5} some non-taxol-type taxoids having neither an oxetane ring at C-4 and C-5 nor an *N*-acylphenylisoserine group at C-13, such as taxuspine C (**1**), 2'-desacetoxyaustrospicatine (**2**), and 2-desacetoxytaxinine J (**3**), increased cellular accumulation of vincristine (VCR) in multidrug-resistant human ovarian cancer 2780AD cells as potently as verapamil, and efficiently inhibited [³H]azidopine photolabeling of P-gp.^{4,5} More recently, we found that taxuspine C (**1**) enhanced the chemotherapeutic effect of VCR in VCR-resistant murine leukemia P388/VCR-bearing mice.⁶ In this paper, we report that the non-taxol-type taxoids **1–3** showed remarkable MDR reversal activity on

colchicine-resistant human epidermoid carcinoma KB-C2 cells, which overexpress P-gp, when co-administered with colchicine, VCR, or taxol.

MATERIALS AND METHODS

Chemicals Taxuspine C (**1**), 2'-desacetoxyaustrospicatine (**2**), 2-desacetoxytaxinine J (**3**), taxinine (**4**), and taxinine M (**5**), and taxol (**6**) were isolated from the Japanese yew *Taxus cuspidata* Sieb. et Zucc. (Fig. 1).⁷ Compounds **7–11** were derived from taxinine (**4**).^{8,9} Verapamil was purchased from Wako Pure Chemical Industries, Ltd. (Osaka). **Cell culture and cell lines** Human epidermoid carcinoma KB cells were subcloned twice; a singly recloned line, KB-3-1, was used as the parental cell line for the present study.¹⁰ The KB-3-1 cells were cultured in RPMI-1640 medium (Nissui Pharmaceutical Co., Ltd., Tokyo) with 0.3 mg/ml glutamine, and 100 units/ml penicillin-streptomycin, supplemented with 10% fetal bovine serum (Life Technologies, Tokyo). Multidrug-resistant KB-C2 cells that overexpress P-gp were originally isolated from KB-3-1 cells exposed to increasing concentrations of colchicine, and maintained in medium containing 2 μ g/ml of colchicine.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay Chemosensitivity *in vitro* was measured by means of the MTT colorimetric assay performed in 96-well plates.¹¹ The assay is dependent on the reduction of MTT by the mitochondrial dehydrogenase of viable cells to a blue formazan product that can be measured spectrophotometrically. Equal numbers of cells

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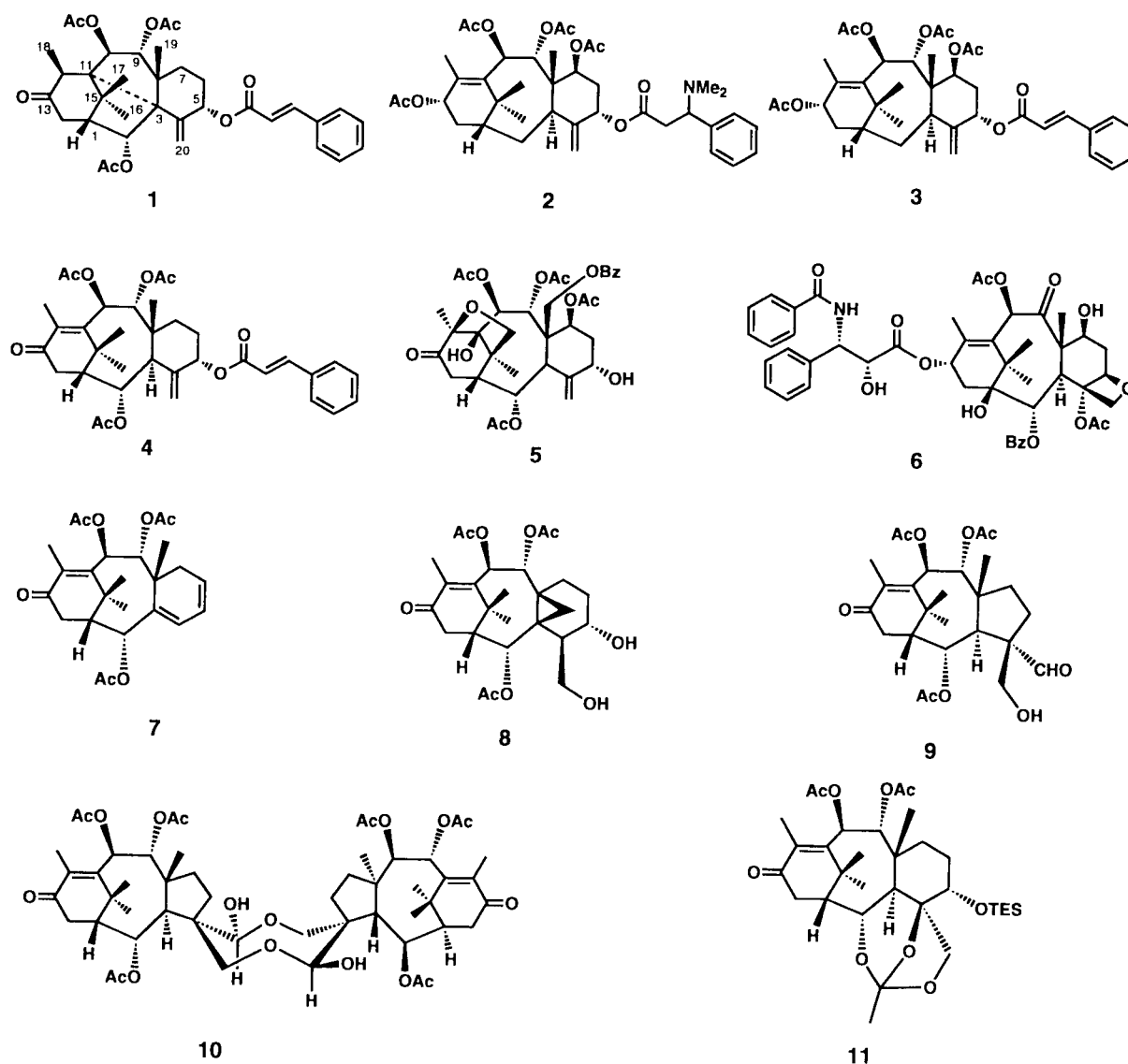


Fig. 1. Structures of taxoids (1–11). 1, taxuspine C; 2, 2'-desacetoxyaustrospicatine; 3, 2-desacetoxytaxinine J; 4, taxinine; 5, taxinine M; 6, taxol; 7, 3,5-diene taxoid; 8, 3,8-cyclopropane taxoid; 9, 6(5→4)*abeo*-taxoid; 10, 6(5→4)*abeo*-taxoid dimer; 11, 2,4,20-ortho-ester taxoid.

(5000 KB-3-1 or KB-C2 cells) were inoculated into each well with 180 μ l of culture medium. After an overnight incubation (37°C, 5% CO₂), 10 μ l of colchicine, VCR, or taxol and 10 μ l of taxoids 1–5 were added to the cultures, and incubation was continued for 3 days. Thereafter, 25 μ l of MTT (2 mg/ml phosphate-buffered saline (PBS)) was added to each well and the plates were incubated for 4 h. The resulting formazan was dissolved in 250 μ l of dimethyl sulfoxide after aspiration of the culture medium. Plates were shaken for 2 min on a plate shaker and read immediately at 570 nm using a Micro Plate Reader.

Cellular accumulation of VCR Multidrug-resistant human ovarian cancer 2780AD cells were provided by Dr. R. Ozols (National Cancer Institute, NIH),¹² and maintained in RPMI-1640 medium supplemented with 5% fetal bovine serum and 100 μ g/ml kanamycin. One million 2780AD cells were plated in Corning six-well tissue-culture clusters and incubated for 24 h at 37°C. The medium in each well was aspirated and 0.5 ml fresh growth medium containing 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid and [³H]VCR (7.5 pmol, specific activity: 259 GBq/mmol) was added. Then the indicated

concentrations of drugs dissolved or suspended in PBS were added. After incubation at 37°C for 2 h, the intracellular VCR accumulation was determined as described previously.¹³⁻¹⁶

RESULTS

Effects of taxoids on the sensitivity to colchicine in colchicine-resistant KB-C2 cells The IC₅₀ values of colchicine against KB-3-1 and KB-C2 cells were 0.0054 and 0.28 μg/ml, respectively. KB-C2 cells were about 52-fold more resistant to colchicine than KB-3-1 cells. The IC₅₀ values of colchicine against KB-C2 cells with taxoids 1-5 and verapamil at 10 μM were 0.0054, 0.0045, 0.0030, 0.28, 0.26, and 0.011 μg/ml, respectively (Fig. 2). Taxuspine C (1), 2'-desacetoxyaustrospicatine (2), and 2-desacetoxytaxinine J (3) at 10 μM increased the sensitivity to colchicine of KB-C2 cells by 52, 62, and 93-fold, respectively, while taxinine (4) and taxinine M (5) showed no effect (Fig. 2).

Effects of taxoids on the sensitivity to VCR in colchicine-resistant KB-C2 cells The IC₅₀ value of VCR against KB-C2 cells was 0.017 μg/ml. The IC₅₀ values of VCR against KB-C2 cells with taxoids 1-5 at 10 μM were 0.00062, 0.00056, 0.00082, 0.0048, and 0.0040 μg/ml, respectively (Fig. 3). Compounds 1, 2, and 3 at 10 μM increased the sensitivity to VCR of KB-C2 cells by 27, 30, and 21-fold, respectively, while compounds 4 and 5 showed weak effects (Fig. 3).

Effects of taxoids on the sensitivity to taxol in colchicine-resistant KB-C2 cells IC₅₀ values of taxol against KB-C2 cells was 0.012 μg/ml. IC₅₀ values of taxol against

KB-C2 cells with taxoids 1-5 at 10 μM were 0.00074, 0.00084, 0.0017, 0.0078, and 0.010 μg/ml, respectively (Fig. 4). Compounds 1, 2, and 3 at 10 μM increased the sensitivity to taxol of KB-C2 cells by 16, 14, and 7-fold, respectively, while compounds 4 and 5 showed no effect (Fig. 4).

Increased cellular accumulation of VCR in multidrug-resistant 2780AD cells in the presence of taxoids The cellular accumulation of VCR is reduced in multidrug-resistant tumor cells as compared with the parental

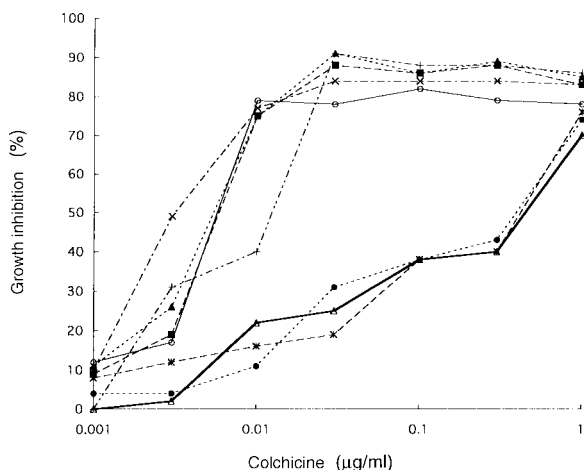


Fig. 2. Effects of taxoids (1-5) on the sensitivity of KB-3-1 cells and P-gp-overexpressing KB-C2 cells to colchicine. ■ KB-C2+1, ▲ KB-C2+2, × KB-C2+3, * KB-C2+4, ● KB-C2+5, + KB-C2+verapamil, ○ KB-3-1, Δ KB-C2.

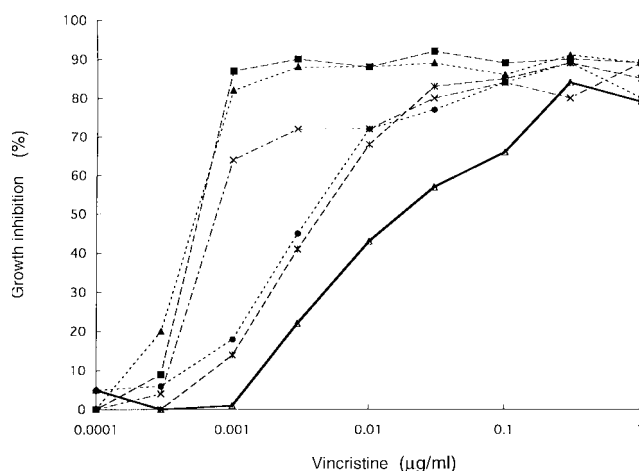


Fig. 3. Effects of taxoids (1-5) on the sensitivity of the P-gp-overexpressing KB-C2 cells to vincristine. ■ KB-C2+1, ▲ KB-C2+2, × KB-C2+3, * KB-C2+4, ● KB-C2+5, Δ KB-C2.

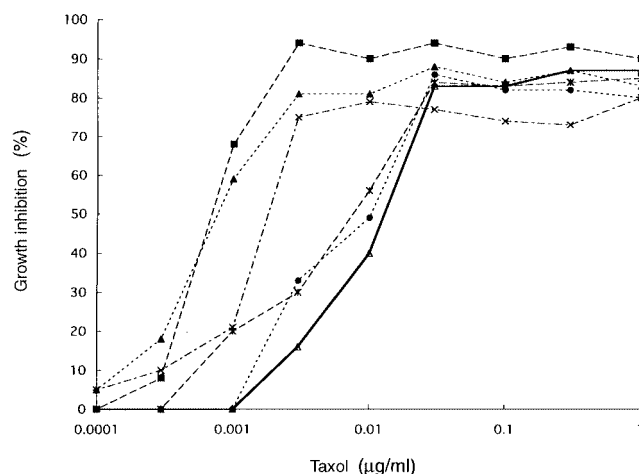


Fig. 4. Effects of taxoids (1-5) on the sensitivity of the P-gp-overexpressing KB-C2 cells to taxol. ■ KB-C2+1, ▲ KB-C2+2, × KB-C2+3, * KB-C2+4, ● KB-C2+5, Δ KB-C2.

Table I. Effects of Taxoids (**1–11**) on the Accumulation of VCR in Multidrug-resistant 2780AD Cells

Compound	VCR accumulation (% of control) ^{a)} with a taxoid concentration of	
	1 $\mu\text{g/ml}$	10 $\mu\text{g/ml}$
1	246 ^{a)}	768
2	233	841
3	266	798
4	195	571
5	115	169
6 (taxol)	83	56
7	149	310
8	143	184
9	129	190
10	161	292
11	203	308
Verapamil	254	739

a) The amounts of VCR accumulated in multidrug-resistant 2780AD cells were determined in the presence of 1 and 10 $\mu\text{g/ml}$ of taxoids as described in "Materials and Methods." The values represent means of triplicate determinations, and are expressed as the relative amounts of VCR accumulated in the cells as compared with the control experiment. The accumulation of VCR in 2780AD cells without taxoids (control values) ranged from 27.4 to 44.3 fmol/10⁶ cells.

cells.^{17–19}) MDR-reversing agents such as verapamil increase the reduced accumulation of antitumor agents in MDR cells and overcome drug resistance.^{13–16, 20}) The effect of taxoids (**1–11**) on the cellular accumulation of VCR in multidrug-resistant 2780AD cells is shown in Table I. Verapamil at 1 and 10 $\mu\text{g/ml}$ increased the VCR accumulation in a dose-dependent manner. Compounds **1**, **2**, and **3** increased the VCR accumulation as well as verapamil. Compounds **4** and **5** increased the accumulation moderately and compounds **7–11** showed weak activity, while taxol (**6**) decreased the VCR accumulation in 2780AD cells.

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DISCUSSION

Taxuspine C (**1**), 2'-desacetoxyaustrospicatin (**2**), and 2-desacetoxytaxinine J (**3**) at 10 μM completely reversed the resistance to colchicine, VCR, and taxol in KB-C2 cells, which overexpress P-gp, while taxinine (**4**) and taxinine M (**5**) showed weak or no effect. These results indicate that the presence of a cinnamoyl or 3-*N,N*-dimethylamino-3-phenylpropanoyl group at C-5 is required for effective binding to P-gp. Compounds **1**, **2**, and **3** showed weak or no cytotoxicity against human epidermoid carcinoma KB cells. Compounds **1**, **2**, and **3** increased the VCR accumulation in multidrug-resistant 2780AD cells as well as verapamil, while compounds **4–11** showed weak activity. On the other hand, compounds **1**, **2**, and **3** reduced the binding activity of P-gp with azidopine in multidrug-resistant 2780AD cells as well as verapamil, while compound **4** and taxol (**6**) were inactive.⁵⁾ These results suggest that taxoids (**1**, **2**, and **3**) bind to the same site as that of azidopine to P-gp. Recently, we found that taxuspine C (**1**) given i.p. enhanced the chemotherapeutic effect of VCR in P388/VCR-bearing mice.⁶⁾ These taxoids **1–3** may be useful for the treatment of tumors when they are used in combination with anticancer agents, since they increase the sensitivity to anticancer agents in P-gp-overexpressing cells by reversing the drug resistance. Taxuspine C (**1**) interacts directly with P-gp and inhibits the active efflux of antitumor agents, thus overcoming MDR *in vivo*, like verapamil.

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