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Multidrug Resistant Cancer Cells Susceptibility to Cytotoxic Taxane Diterpenes from *Taxus yunnanensis* and *Taxus chinensis*

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Abstract—Twelve taxane diterpenes (**1**–**12**), which were isolated previously from the EtOH extract of the aerial parts of *Taxus yunnanensis* or *Taxus chinensis*, were evaluated for cytotoxicity against the multidrug resistant cancer cells KB-VIN and KB-7d. Compounds **10** and **11** showed significant cytotoxicity in these cell lines. Compounds **3** and **12** also demonstrated significant activity against KB-7d. The biflavonoid **13** isolated from *T. yunnanensis* was only marginally cytotoxic against the A549 (lung) cell line, but a simple methoxylated analogue (**14**) was inactive.

Paclitaxel (Taxol®), which was isolated from *Taxus brevifolia*, has a unique mode of action and is used as an antitumor agent for treating breast and ovarian cancer.[1,2] Consequently, since the discovery of paclitaxel, much effort has been devoted to isolating new taxane diterpenes from various *Taxus* species.[3–8] Recently, several nontaxol-type taxane diterpenes were found to increase cellular accumulation of vincristine in multidrug resistant tumor cells.[9,10] However, few isolated non-paclitaxel-type compounds have been examined as potential antitumor agents. Many flavonoids and biflavonids have also been isolated from various plants and examined as cytotoxic agents.[11,12] In our continuing efforts to discover new antitumor agents from higher plants, we have investigated the taxane diterpene and flavonoid constituents of two *Taxus* species (*T. yunnanensis* Cheng et L. K. Fu and *T. chinensis* (Pilgre) Rehd. var. mairei). Previously, we reported the isolation of taxuchin A, 19-acetoxy-taxagifine, and dantaxusins A, B, C, and D, and the evaluation of seven isolated taxane diterpenes for cytotoxicity against nine human cell lines, including a β-tubulin mutant resistant to paclitaxel.[13–16]

We report herein the cytotoxic activity of previously isolated taxane diterpenes **1**–**12** (Fig. 1) against multidrug resistant cell lines (KB-VIN and KB-7d) and cytotoxic activity of flavonoids **13** and **14** (Fig. 2) against a multidrug resistant cell line (KB-VIN) and a panel of eight human cell lines.

The previously isolated taxane diterpenes were examined as inhibitors of human tumor cell line replication using the nasopharyngeal line, KB, and two multi-drug resistant sublines, KB-VIN, and KB-7d. KB-VIN is a P-glycoprotein over-expressing derivative, and KB-7d exhibits pleotrophic drug resistance to 4′-(9-acridinylamino)methanesulfon-maniside, doxorubicin, vincristine, and methotrexate, in part due to over-expression of the MRP transporter. Table 1 shows the resulting data against the KB cell line panel. Among the tested compounds, **10** had significant ($ED_{50} \leq 4$ μg/mL) cytotoxic activity against the drug-resistant derivatives KB-VIN and KB-7d with ED_{50} values of 2.4 and 3.5 μg/mL, respectively. Interestingly, **10** was twice as active against KB-VIN cell replication than the parent KB cell line. Compound **11** showed significant and comparable cytotoxic activity against all three cell lines with a mean ED_{50} value of 3.9 μg/mL. Compound **12** appeared to be a substrate for P-glycoprotein because the KB-VIN cell line

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Table 1. Cytotoxic activities of taxoids **1**–**12** against KB and drug-resistant sublines

Compd	Cell line/ED_{50} (μg/mL)[a]		
	KB	KB-VIN	KB-7d
1	16.0	16.2	14.2
2	12.1	8.7	7.8
3	9.8	8.6	4.3
4	NA[b]	>20 (5)[c]	>20 (22)[c]
5	12.5	11.0	9.4
6	7.7	6.4	6.1
7	>20 (45)[c]	17.3	9.6
8	16.6	13.3	10.0
9	>20 (10)[c]	>20 (21)[c]	>20 (28)[c]
10	4.8	2.4	3.5
11	3.9	3.6	4.1
12	6.2	17.0	4.6

[a]Cell line ED_{50} in μg/mL (replicates varied no more than 5%). For significant activity of pure compounds, an ED_{50} <4.0 μg/mL is required.
[b]NA = Not active at 20 μg/mL (<5% inhibition).
[c]If inhibition <50% at 20 μg/mL, then the inhibition observed is given in parentheses.

exhibited significant cross-resistance to this compound. Compounds **3**, **5**, and **7** had marginal activity against the KB parent cell line but, for all three, the MRP over-expressing subline was more susceptible. From a structure–activity perspective, the ether linkage (C12 to C17) and C13 carbonyl group of **12** resulted in reduced cytotoxic activity against KB and KB-VIN cell lines compared with **10** and **11**. Compounds **1** and **5** were much less active than **10** although their structures varied only at the C-2 and C-7 substituents. Comparing the structures and activities of **3** and **7** indicated that an ester group at C-14 afforded increased cytotoxic activity. Abeotaxane-type compounds (**4** and **9**) were inactive in the experiments. Based on the activity profile in Table 1, compounds **3** and **10**–**12** may be useful leads for discovery of drugs active against some common types of multi drug-resistant tumors.

Table 2 shows the cytotoxic activity against a human tumor cell line panel of two flavonoids isolated from *Taxus yunnanensis*. Recently, compound **13** isolated from *Selaginella moellendorffii* was reported to inhibit growth of a human ovarian adenocarcinoma (OVCAR-3).[13] In our assays, **13** showed weak activity against KB with an ED_{50} value of 7.5 μg/mL and only marginal activity against A549 with an ED_{50} value of 5.5 μg/mL. Interestingly, compound **13** was inactive against the KB-VIN subline. This cross-resistance profile indicated that that compound **13** is likely a good substrate for P-glycoprotein. Despite the close similarity in structure between **13** and **14**, the latter compound was inactive

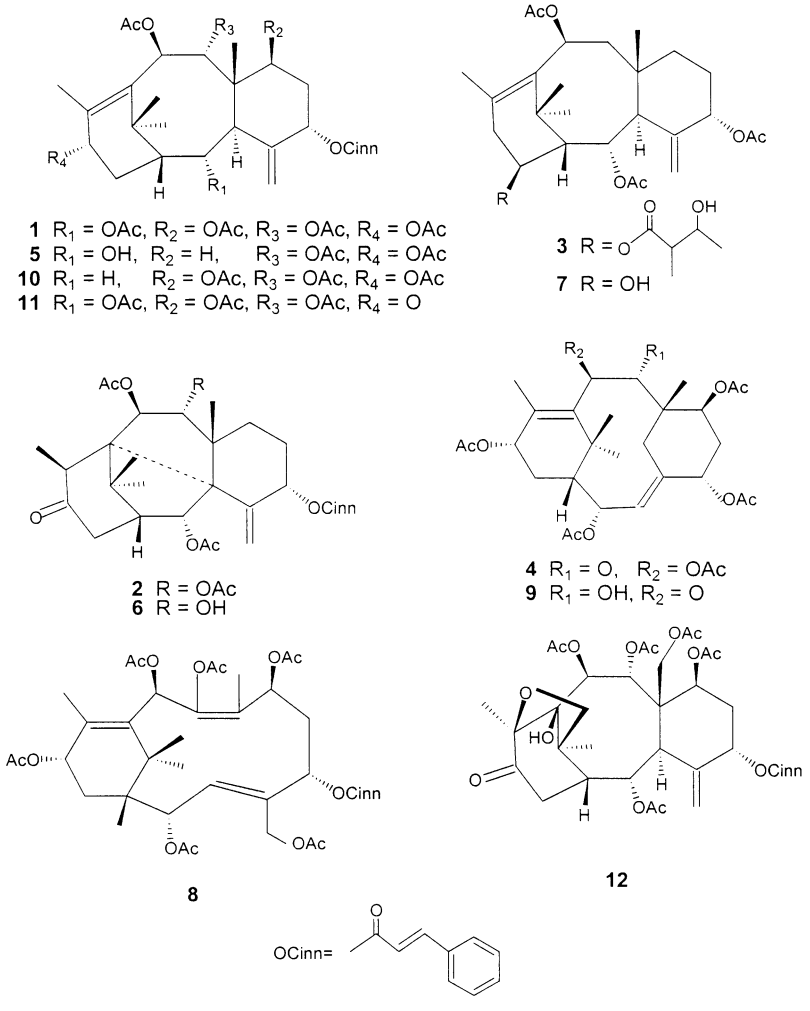

Figure 1. Structures of taxane diterpenes.

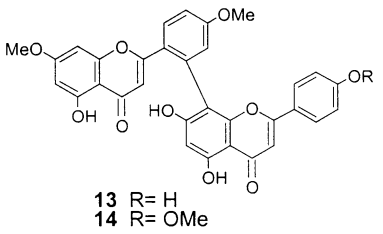

Figure 2. Structure of flavonoids.

Table 2. Cytotoxic activities of flavonoids **13** and **14** against a panel of human tumor cell lines

Cell line	Compd/ED_{50} (μg/mL)[a]	
	13	**14**
KB	7.5	>20 (12)[c]
KB-VIN	>20 (21)[c]	>20 (9)[c]
A-549	5.5	>20 (58)[b]
HCT-8	8.5	NA[b]
MCF-7	7.5	>20 (22)[c]
SK-MEL-2	7.0	>20 (9)[c]
1A9	6.0	>20 (44)[c]
HOS	8.0	>20 (25)[c]
U-87-MG	7.0	>20 (17)[c]

[a]Cell line ED_{50} in μg/mL (replicates varied no more than 5%). For significant activity of pure compounds, an ED_{50} <4.0 μg/mL is required.
[b]NA = Not active at 20 μg/mL (<5% inhibition).
[c]If inhibition <50% at 20 μg/mL, then the inhibition observed is given in parentheses.

against all tested cell lines. The only structural difference between the two flavanoids is that the methoxy group at C-4‴ in **14** is changed to a hydroxy group in **13**. The overall result suggests that the C-4‴ hydroxy substituent of **13** is a critical structural determinant of cytotoxic activity.

Experimental

General experimental

Cell culture chemicals were purchased from Sigma-Aldrich Chemical Co., Inc. ^1H and ^{13}C NMR spectra were determined on Jeol ALPHA-400 instruments in $CDCl_3$ and C_5D_5N using TMS as an internal standard. Silica gel (Merck, type 60, 70–320 mesh) was used for CC. Precoated Silica gel plates (Merck $60F_{254}$) of 0.25 mm thickness were used for analytical TLC, and plates of 1 and 2 mm thickness were used for prep. TLC. Analytical HPLC was performed on a liquid chromatograph equipped with a UV detector at 254 nm and a reverse-phase column (TSK-gel ODS-80Ts) using a mixed solvent of MeOH/H_2O. Preparative HPLC was carried out on Tosoh liquid chromatograph apparatus equipped with a reverse-phase column (Lichrosorb RP-18) at 254 nm using the same solvents as employed for analytical HPLC.

Plant material

The plant bark, twigs, and leaves of *Taxus yunnanensis* and *chinensis* were collected in August 1993, in Yunnan Province, People's Republic of China and verified by Prof. Daofeng Chen. The voucher specimens were deposited at Shanghai Medical University, Shanghai, and People's Republic of China.

Extraction and isolation

The plant bark, twigs, and leaves of *Taxus yunnanensis* (air dried material, 7.3 Kg) were extracted two times with EtOH. The EtOH solutions were evaporated in vacuo to give two residues (480 g). The extracts were diluted with EtOH and H_2O (3:1), and then extracted with *n*-hexane to give *n*-hexane extracts (42.6 g). The EtOH–H_2O layers then were extracted with CH_2Cl_2 and *n*-BuOH successively, to give CH_2Cl_2 (111 g), *n*-BuOH (152.0 g), and finally, H_2O soluble residues (140.0 g). Silica gel column chromatography of each CH_2Cl_2 extract (111 g) eluting with benzene-EtOAc–*n*-hexane (14:5:6) gave 13 fractions, with EtOAc–Et_2O (1:1, v/v) gave 9 fractions, and with $CHCl_3$–MeOH–H_2O (50:14:3, v/v) gave 4 fractions, respectively. Each fraction was checked by analytical HPLC. From Fraction 2, which was eluted with the mixed solvent of EtOAc–Et_2O (1:1, v/v), MeOH insoluble material was obtained (1.92 g). Purification of this crude substance with repeated preparative TLC $CHCl_3$–MeOH–H_2O (50:14:3, v/v) and HPLC (MeOH–H_2O, 75:25 v/v) provided two flavonoids (**13**) (3.3 mg) and (**14**) (25 mg). Isolation procedures for taxane diterpenes from *Taxus yunnanensis* and *chinensis* have been described in the literature.[14–16]

The spectral analysis (IR, UV, ^1H, ^{13}C, and 2D-NMR) identified the structures of **13** and **14** as ginketin and sciadopitysin.

Cells

The human tumor cell line panel included KB (nasopharyngeal), KB-VIN (P-glycoprotein multidrug resistant), KB-7d (MRP multidrug resistant), A549 (lung carcinoma), HTC-8 (colon tumor), CAKI-1 (renal), MCF-7 (breast), SK-MEL-2 (melanoma), 1A9 (ovarian), HOS (bone), and U-87-MG (glioblastoma) and are described in detail elsewhere.[15,18] Cells were cultured under standard conditions as described below.

Cytotoxicity assay

The assay was performed following the NCI's standard procedure using micro titer plate format and sulforhodamine-B (a cationic protein stain).[17] Human tumor cell lines were continuously exposed to the test compounds for 3 days. Cells were cultured in RPMI-1640 growth medium, supplemented with 25 mM HEPES, 2% (w/v) sodium bicarbonate, 100 μg/mL kanamycin, and 10% (v/v) fetal bovine serum in a humidified 5% CO_2 atmosphere at 37 °C. ED_{50}, the concentration that inhibited cell replication by 50% relative to control under the test conditions, was interpolated from graphed dose-response results (Graphpad Software, San Diego, CA).

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