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Overcoming Tumor Drug Resistance with High-Affinity Taxanes: A SAR Study of C2-Modified 7-Acyl-10-Deacetyl Cephalomannines

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A series of C2-modified 10-deacetyl-7-propionyl cephalomannine derivatives was designed, prepared, and biologically evaluated. Some C2 meta*-substituted benzoate analogues showed potent activity against both drug-sensitive and drug-resistant tumor cells in which resistance is mediated through either P-gp overexpression or β-tubulin mutation mechanisms. The taxoid **15b** and related compounds are of particular interest, as they are much more cytotoxic than paclitaxel, especially against drug-resistant tumor cells; they are able to kill both drug-resistant and drug-sensitive cells (low R/S ratio), and they have high affinity for β-tubulin. Our research results led to an important hypothesis, that is, a taxane with very high binding affinity for β-tubulin is able to counteract drug resistance, which may assist in future taxane-based drug-discovery efforts.*

Introduction

The chemistry and biology of taxanes have been extensively explored since the successful development of paclitaxel (Taxol®, **1a**), a natural taxane originally isolated from *Taxus brevifolia*[1] which is one of the most effective antitumor agents.[2] Although **1a** was found to be effective against tumors resistant to some chemotherapeutic agents, resistance to **1a** itself has also occurred after its use in clinics and has become a serious problem in the failure of chemotherapy with paclitaxel. To overcome this problem, many paclitaxel analogues derived from natural taxanes have been designed and prepared. As a result, several semisynthetic taxanes such as IDN5109 (**2**),[3] DJ-927 (**3**),[4] and BMS-184476 (**4**)[5] (Figure 1) have undergone clinical trials. Most of these compounds were found to exert their activity through counteracting overexpression of the P-glycoprotein (P-gp) multidrug efflux pump and by modulating P-gp functions.[6,7]

Although numerous efforts were made to reveal the structure–activity relationship (SAR) of paclitaxel analogues and related taxoids,[2c] few reports of SARs for taxoids effective against multidrug-resistant (MDR) tumors have appeared. Since the first report of the exceptional efficacy of taxol analogues against MDR tumor cells appeared in 1996,[8] many similar taxoids have been prepared, including those with modifications at C7, C9, and C10 in the northern hemisphere, and C2 in the southern hemisphere of the molecule,[4,5,9–15] as well as in combination with changes at the C13 side chain,[16–18] and compounds with structural modifications at combinations of different sites.[19] Taxoid **5** seems to be one of the most promising agents against MDR tumor cells reported to date because of its high activity (IC_{50} values at nanomolar and sub-nanomolar levels) and equal potency towards both sensitive and resistant tumor cells (R/S ratio derived from IC_{50}(resistant)/IC_{50}(sensitive) is about 1), whereas paclitaxel and docetaxel (Taxotere®, **1c**) are 10- to 1000-fold less potent towards MDR tumor cells.

Whereas most semisynthetic taxoids available to date were found to be effective against MDR cells expressing a high level of P-gp which are derived from continuous treatment with cytotoxic agents, overcoming drug resistance mediated by β-tubulin mutations has not been of primary interest, except for a few reports in which human ovarian carcinoma cell lines 1A9 and 1A9/PTX22 with β-tubulin mutations were employed.[15] Complete effectiveness by C2-modified taxanes was not achieved, although partial sensitivity was realized.

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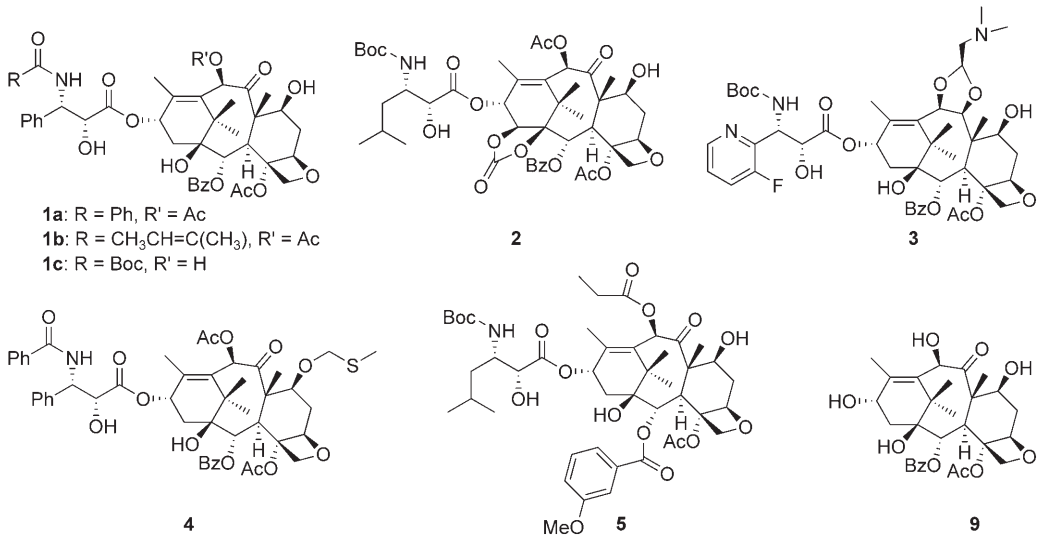


Figure 1. Structures of taxol (**1a**) and its analogues.

Our project aims to design taxoids effective against both drug-sensitive and drug-resistant tumors by counteracting P-gp overexpression and enhancing binding affinity to normal and mutant β-tubulins. We selected cephalomannine (**1b**) as a compound of interest owing to its similarity in both structure and antitumor spectrum to those of **1a** in our primary screen. In addition, it is known that some of the aliphatic 3′-*N*-acyl analogues exhibit slightly greater activity than paclitaxel itself.[2d, 20]

Cephalomannine (**1b**) is a natural congener of paclitaxel, and was isolated in the 1970s from *Taxus wallichiana*, which was erroneously assigned as *Cephalotaxus manii* at the time of its discovery.[21] Its abundance is similar to that of paclitaxel (**1a**) in some *Taxus* spp. Halogenation[22] and epoxidation[23] of the double bond of the 3′-*N*-tigloyl double bond in the C13 side chain of **1b** has led to the preparation of some cephalomannine derivatives with potent cytotoxicity. It was also found that 10-deacetylcephalomannine (**6a**) and 10-deacetoxy-10-oxo-7-*epi*-cephalomannine (**7**) (Figure 2) were less active towards both drug-sensitive MDA-MB 231, and drug-resistant MCF-7/ADR cell lines.[24]

During our initial examination of 7-acylation of cephalomannine, we observed that the antitumor activity of the 7-acyl derivatives decreased with increasing length of the C7 side chain, that is, from acetyl compound **8a** to butyryl compound **8c**, in agreement with previous observations for paclitaxels.[25] Considering that C10 deacetylation in combination with C7 acylation can restore the activity of paclitaxel analogues,[26] we prepared 10-deacetyl-7-acylcephalomannine derivatives and found that 10-deacetyl-7-acetyl and 10-deacetyl-7-propionyl derivatives **6b** and **6c**, respectively, showed improved activity, while 10-deacetyl-7-butyrylcephalomannine (**6d**) exhibited decreased activity. Encouraged by these preliminary results, we set out to prepare C2-modified derivatives of **6c** based on the reasoning that proper structural changes at C7/C10 can counteract P-gp-mediated resistance, and those at C2 may enhance the ability of the compound to bind to wild-type and mutated β-tubulins (because C2 and C4 substitutions are close to the 217–233 and 270–291 sequences of β-tubulin that contain many known mutation sites[27, 28]). The resulting C2-modified 7-propionyl-10-deacetylcephalomannines are expected to exert significant activity against both drug-sensitive and drug-resistant tumors.

Recent pharmacophore studies suggested that the phenyl ring of the 2-benzoate group is close to His 227/His 229 of β-tubulin and is flanked by a "hydrophobic basin" composed of Phe 272, Ala 273, and Ser 374 (the position of this residue is replaced by Ala in the majority of the human β-tubulin isoform) and other residues, thus leading to strong hydrophobic interactions between paclitaxel and β-tubulin.[29] This is in agreement with previous observations that 2-*ortho*- and 2-*para*-substituted benzoates, especially the latter, exhibit much lower ac-

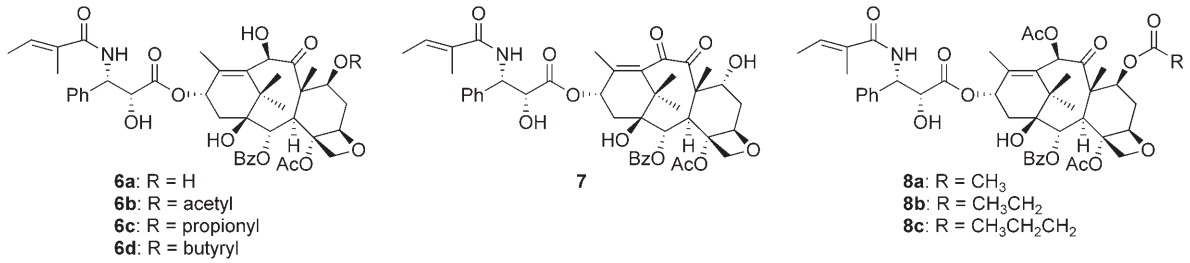


Figure 2. Structures of C7- and C10-modified cephalomannines.

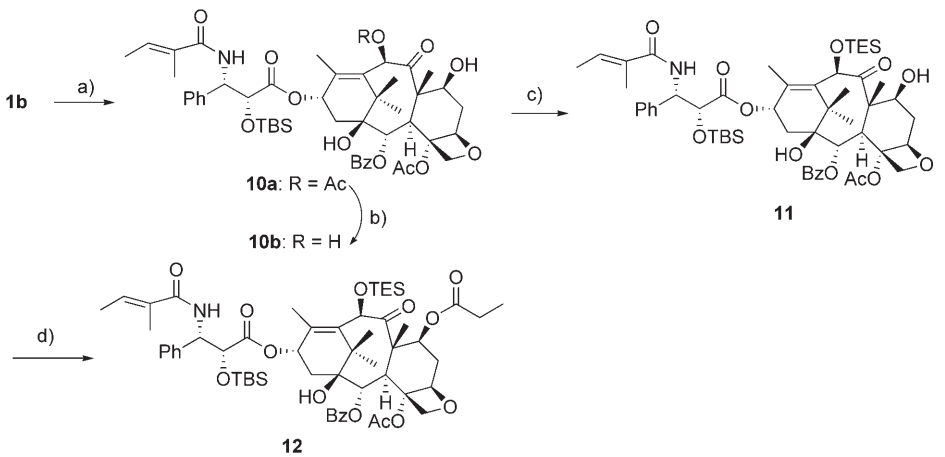
tivity than 2-*meta*-substituted benzoates of paclitaxel.[15,30] The poor activity of 2-*para*-substituted taxanes originates from a steric effect that decreases the hydrophobic interaction between the phenyl ring and adjacent amino acid residues. In addition, some C2-disubstituted benzoates as well as C2 alkanoates or alkenoates also exhibited potent activity.[15,31] Based on this information, we designed and prepared some 2-*meta*-substituted benzoates **15a–j**, heterocyclic carboxylic acid esters **15k–m**, C_{3-4} alkanoates/alkenoates **15n–q**, as well as disubstituted benzoates **15r–t** to examine their SAR at both the cellular (cytotoxicity) and molecular (β-tubulin and P-gp binding ability) levels. Herein we report the syntheses and resulting biological evaluation of these compounds; the SAR of these taxanes and correlations between cellular and binding assays are also discussed.

Results and Discussion

Synthesis

Starting from **1b**, 2′-TBS-10-deacetylcephalomannine **10b** was prepared in >90% yield through protection of the 2′-OH group with *tert*-butyldimethylsilyl chloride (TBSCl) and successive 10-deacetylation by hydrazine hydrate, as previously reported for paclitaxel.[32,33] Selective triethylsilyl (TES) protection of **10b** with *N*,*O*-bis(triethylsilyl)trifluoroacetamide, following a known protocol for selective 10-triethylsilylation of 10-deacetylbaccatin III (**9**),[34] furnished **11**, which was then acylated upon treatment with propanoic anhydride and pyridine (Py) to yield **12** (Scheme 1).

Modification at C2 of **1a** and its derivatives has been realized by several research groups.[30,31,35] Kingston's selective C2 debenzoylation condition (Triton B in CH_2Cl_2) was modified for debenzoylation of **12** to afford taxoid **13** (Scheme 2). To prepare C2-modified taxoids **14** (except **14e**), conjugation of **13** with different acids in the presence of *N*,*N*-dicyclohexylcarbodiimide (DCC) and *N*,*N*-dimethylaminopyridine (DMAP) or pyrrolidinopyridine (PP) was applied, because C2 acylation with 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC)/DMAP or EDC/PP was extremely slow, and the use of acyl chlorides and LHMDS resulted in very poor yields. Unlike others, taxoid **14e** was prepared with $NaBH_4$ as a reduced product of **14d**. All C2-esterified products were obtained as major products, except in the case of esterification with 3-methylbutenoic acid, for which two products (**14n** and **14o**) were found. Taxoid **14o**, showing two terminal olefin signals around $\delta = 4.9$ ppm as broad singlets, corresponds to a rearranged product of 3-methylbutenoyl taxoid **14n**. All taxoids **14**, without purification, underwent desilylation to furnish final products **15a–t** in satisfactory yields (Scheme 2).



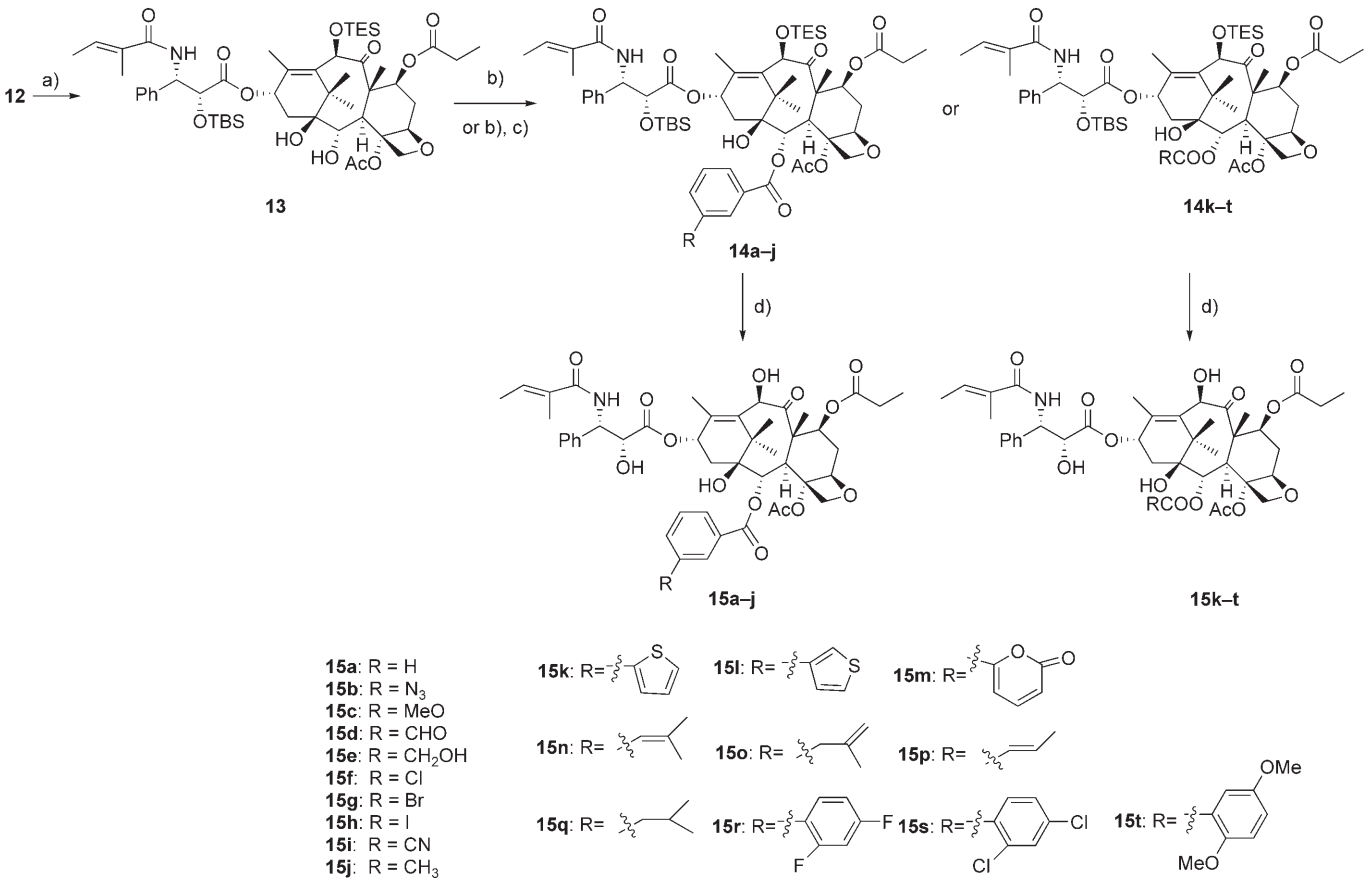
Scheme 1. Reagents and conditions: a) TBSCl, imidazole, DMF, RT, 91.6%; b) 85% $N_2H_4 \cdot H_2O$, ethanol, RT, 91.5%; c) *N*,*O*-bis(triethylsilyl)trifluoroacetamide, LHMDS, THF, −10 °C, 92.9%; d) $(CH_3CH_2CO)_2O$, Py, DMAP, THF, RT, 92.4%.

Cellular and molecular assays

Two groups of tumor cell lines were employed for cytotoxicity assays: human ovarian carcinoma A2780 and A2780/AD10 (MDR overexpressing P-gp), human ovarian carcinoma 1A9 (a subclone of A2780), and mutant lines derived from 1A9, 1A9/PTX10 and 1A9/PTX22. The multidrug-resistant A2780/AD10 tumor cell line is derived from continuous adriamycin treatment of A2780 cells, which induced P-gp overexpression as the major cause of their resistance to cytotoxic agents. The paclitaxel-resistant cell lines 1A9/PTX10 and 1A9/PTX22 are derived from 1A9 by selection with paclitaxel in combination with verapamil.[35] Because P-gp overexpression was inhibited by verapamil, drug resistance in these drug-resistant cells is mediated mainly through β-tubulin mutations. Three taxanes, including the commercially available drugs paclitaxel (**1a**), docetaxel (**1c**), and the starting material cephalomannine (**1b**), were used as reference compounds. From Table 1, it can be observed that **1b** is comparably or slightly less active than paclitaxel (**1a**) in all cellular and molecular assays, whereas docetaxel (**1c**) is usually more active.

Activities against drug-sensitive and drug-resistant tumor cells with P-gp overexpression

All four alkanoates **15n–q** were found to be 2–3 orders of magnitude less active than the three reference taxoids **1a–c** toward drug-sensitive and drug-resistant tumor cell lines. Ojima et al.[31] claimed that C2 benzoate is not an essential group for potent activity of taxoids, as 2-cyclohexanoate or 2-methylbutenoate analogues of docetaxel showed similar cytotoxicity to paclitaxel. They also found that the cytotoxicity of 2-debenzoyl-2-alkyl and 2-alkenyl esters depends on C3′ substitution variations.[31] However, this seems not to be true in our case; all alkyl and alkenyl esters were only weakly active in the parental cell lines 1A9 and A2780, and even the most



Scheme 2. Reagents and conditions: a) Triton B, CH_2Cl_2, −23 °C, 68.1 %; b) RCOOH, $PhCH_3$, DCC, PP, 65 °C; c) $NaBH_4$, CH_3OH/THF (only for **14e**); d) HF, Py, CH_3CN, RT.

active one (compound **15o**) was much less active than its C2 benzoyl counterpart.

In contrast, C2-*meta*-substituted benzoates were much more potent in cytotoxicity assays. For C2-modified 10-deacetyl-7-propionylcephalomannines **15**, a trend in cytotoxicity[15,30] was observed similar to that of C2-modified paclitaxel analogues: 2-*meta*-OMe and 2-*meta*-N_3 were the most active, and other *meta*-substituted and non-substituted benzoates were less active. It is interesting to note that 2-*meta*-N_3 taxoid **15b** exhibited not only potent activity against A2780 and A2780/AD10 cells, but also a low R/S value, a remarkable characteristic of some of the "advanced second-generation taxoids".[19]

Kingston et al. reported earlier[15,30] that the 2-*meta*-N_3-benzoate of paclitaxel was the most active taxane against HL-60 leukemia and HCT-116 human colon carcinoma cell lines, but less active than 2-*meta*-MeO-benzoate against the 1A9 cell line, and both were more active than paclitaxel. We prepared these two taxanes and found their cytotoxicity matched well with published results, showing superior activity to paclitaxel and even docetaxel, and confirming that 2-*meta*-MeO-benzoate analogues are better than 2-*meta*-N_3 analogues (data not shown). However, in our 10-deacetyl-7-propionyl series, the azido analogue **15b** is more active than the methoxy analogue **15c** in all cases.

We wondered whether the highly potent activity of **15b** originates from covalent binding of its reactive azido group to β-tubulin, so we performed experiments in which the amount of reversibly bound ligand was measured (Figure 3). Bound **15b** can be extracted from the pellets and aqueous solutions with an organic solvent, indicating that it is not irreversibly bound.[36,37] A comparison between the amounts of **15b** recovered from the pellet in the presence of microtubules and from the supernatant in the absence of microtubules showed that under the conditions of the assay **15b** is fully soluble and does not covalently bind to microtubules.

Previous studies demonstrated that neither steric nor electronic nature alone is the dominant factor in the activity of 2-*meta*-substituted benzoates. Although it seems that the polar group (in **15b**, **15c**, and **15f**) may make a positive contribution to the activity in comparison with **15a**, the low activity of **15d** and **15e** suggested the impairing effect of a polar group at the *meta* position of C2 benzoate. Furthermore, similar cytotoxicity among compounds with different halogen substituents **15f–h** and their bioisosteres **15i** and **15j** suggested that there is enough room in this region of β-tubulin to accommodate short-length substituents attached to the benzene ring. However, the interchange of C and O atoms from **15c** to **15e** led to a 200-fold loss of cytotoxicity, indicating that a very impor-

Table 1. Cytotoxicity and binding affinity to β-tubulin and P-gp.

Compd	R	IC_{50} [nM][a]		R/S[b]	K_d [nM][c]	ΔG [KJ mol^{-1}][d]	K_d [nM][e]
		A2780	A2780AD				
15a	H	13.2 ± 7	1222 ± 300	92.5	240 ± 40	−39.4 ± 0.56	46 ± 2.8
15b	*m*-N_3	3 ± 1.7	17 ± 4	5.6	1 ± 0.2	−52.6 ± 0.50	66 ± 5.2
15c	*m*-OMe	8.3 ± 2	160 ± 19	19.2	19 ± 4	−45.5 ± 0.59	54 ± 3.0
15d	*m*-CHO	69 ± 77	1500 ± 100	21.7	1449 ± 133	−34.4 ± 0.25	58 ± 6.0
15e	*m*-CH_2OH	1700 ± 120	> 20000		> 10000	> −30	51 ± 5.0
15f	*m*-Cl	13.2 ± 7.6	274 ± 30	20.7	62 ± 8	−42.4 ± 0.35	66 ± 6.4
15g	*m*-Br	28.7 ± 1.9	196 ± 14	6.8	83 ± 36	−41.7 ± 1.47	36 ± 4.0
15h	*m*-I	30 ± 0.5	246 ± 27	8.2	57 ± 19	−42.6 ± 1.07	47 ± 7.0
15i	*m*-CN	15.3 ± 6.1	2100 ± 660	137.2	256 ± 34	−38.8 ± 0.37	88 ± 9.4
15j	*m*-CH_3	15.5 ± 7	596 ± 105	38.4	203 ± 26	−39.4 ± 0.35	58 ± 5.4
15k	2-thiophene	62 ± 17	3200 ± 250	51.6	417 ± 104	−37.6 ± 0.74	58 ± 5.0
15l	3-thiophene	106 ± 4.2	2950 ± 480	27.8	1020 ± 301	−35.3 ± 0.89	53 ± 4.0
15m	2-chromone	14000 ± 2000	> 20000		4762 ± 762	−31.3 ± 0.45	76 ± 11
15n	$CH{=}C(CH_3)_2$	950 ± 80	10200 ± 1900	10.7	2326 ± 686	−33.2 ± 0.89	39 ± 3.9
15o	$CH_2C(CH_3){=}CH_2$	980 ± 70	4000 ± 700	4.1	1389 ± 265	−34.5 ± 0.54	45 ± 5.2
15p	$CH{=}CHCH_3$	3000 ± 800	15000 ± 3500	5	3571 ± 794	−32.1 ± 0.64	44 ± 4.0
15q	$CH_2CH(CH_3)_2$	4900 ± 600	> 20000		2381 ± 381	−33.1 ± 0.45	41 ± 3.2
15r	2,4-difluoro	82 ± 16	1880 ± 200	22.9	571 ± 113	−36.7 ± 0.56	46 ± 5.0
15s	2,4-dichloro	102 ± 8.8	690 ± 60	6.7	402 ± 98	−37.7 ± 0.71	53 ± 5.0
15t	2,5-dimethoxy	25 ± 2	153 ± 39	6.12	114 ± 53	−40.9 ± 1.61	46 ± 5.0
Paclitaxel (**1a**)		1.3 ± 0.4	900 ± 160	692	70 ± 8	−42.1 ± 0.20	35 ± 3.0
Cephalomannine (**1b**)		1.8 ± 0.2	1100 ± 320	611	145 ± 15	−40.3 ± 0.28	37 ± 3.9
Docetaxel (**1c**)		0.3 ± 0.1	290 ± 321	966	25 ± 1	−44.8 ± 0.20	39 ± 4.0

[a] IC_{50} values determined in the parental ovarian carcinoma A2780 line and the MDR P-gp-overexpressing ovarian carcinoma A2780/AD10. IC_{50} values in nM were determined after two days' exposure to drugs using the MTT cell proliferation assay. Data are the mean ± SE of at least four independent experiments. [b] Ratio of IC_{50} (resistant cell line)/IC_{50} (parental cell line). Values are the calculated relative resistance of each mutant cell line obtained by dividing the IC_{50} value of the resistant line by the IC_{50} value of the parental line, A2780. [c] Equilibrium dissociation constants of the ligands to microtubules at 35 °C. [d] Free energy changes derived from equilibrium binding constants of the ligands to microtubules at 35 °C ($-\Delta G_{0app} = RT\ln K_{binding}$). [e] Binding affinity of taxanes for purified P-gp determined by Trp fluorescence quenching.

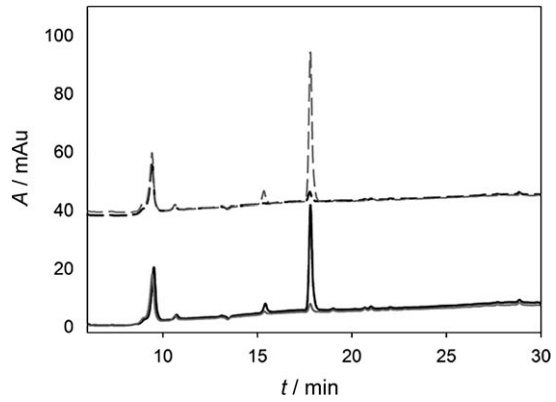


Figure 3. Taxane **15b** is not irreversibly bound to microtubules. Analysis of the **15b** content of microtubule pellets: 5 μM **15b** in GAB was incubated at 37 °C with (——) and without (-----) 10 μM taxoid binding sites in stabilized microtubules. The ligand in the pellets (black line) and supernatants (grey line) was extracted and analyzed as described in the Experimental Section.

tant interaction (such as hydrogen bonding or electrostatic interactions) between β-tubulin and the taxoid was broken in **15e**.

Heterocyclic carboxylic acid esters **15k**–**m**, like their precedent counterparts in the taxol series, were less active than **1a**–**c**. The 2-chromone **15m** is the least active among these analogues. For disubstituted benzoates **15r**–**t**, **15t** was found to show activity similar to that of the *meta*-methoxy taxoid **15c** for both IC_{50} and R/S values, whereas the other two (compounds **15r,s**) were less active.

Binding affinities toward β-tubulin and P-gp

Based on an established protocol,[38,37] we measured the equilibrium binding constants of the different analogues to β-tubulin using a fluorescence-based displacement method, and ΔG was calculated from Gibbs' equation. It was found that ΔG correlated roughly with published cytotoxicity (IC_{50} values) for C2-modified taxol analogues,[15] and also for our C2/C7/C10 taxanes (data not shown). It has been shown that for a group of chemically related taxoid binding site ligands (epothilones) a good correlation can be found between cytotoxicity (IC_{50}) and the free energy of binding to β-tubulin ($r^2 = 0.76$).[38] These findings were apparently unexpected, because it is not possible to know the total equilibrium concentration of the drugs in the cells, the extent of their metabolism, or whether there are any other targets or binding sites for the drugs in intact cells. If −40 to −42 kJ mol^{-1} (for reference taxanes **1a** and **1b**) is set as the standard, we can find only three taxanes that have higher apparent affinity for β-tubulin: docetaxel (**1c**) (−45 kJ mol^{-1}), **15b** (−46 kJ mol^{-1}), and **15c** (< -51 kJ mol^{-1}), all of which have been proven to be more active than **1a** and **1b** in cellular assays. Analogues with lower β-tubulin binding affin-

ity ($\Delta G > -35$ kJ mol^{-1}), including 2-alkanoates and alkenoates (compounds **15n–q**) and 2-chromone (**15m**), were found to be about three orders of magnitude less cytotoxic than reference taxanes **1a–c**.

To examine the influence of these taxanes on the P-gp drug efflux pump, we determined their K_d values for binding to the transporter by quenching the intrinsic Trp fluorescence of P-gp. A correlation was previously noted between the IC_{50} value for the inhibition of P-gp transport function by a drug, and its K_d value for binding to P-gp, which extended over four orders of magnitude.[39] It was found that the K_d values (Table 1) for all our modified taxanes were similar to those of **1a–c** in the assay, suggesting a similar ability of P-gp to pump them out of the cells.

Table 2. Cytotoxicity against parental and drug-resistant tumor cell lines with β-tubulin mutations.

Compd	R	IC_{50} [nM][a]				
		1A9	PTX10	R/S[b]	PTX22	R/S
15a	H	16 ± 2.5	900 ± 70	56.25	435 ± 17	27.1
15b	*m*-N_3	1.9 ± 0.1	11.6 ± 3.7	6.1	1.9 ± 0.4	1
15c	*m*-OMe	5.7 ± 2.6	37 ± 5.43	6.49	7.2 ± 1.1	1.2
15d	*m*-CHO	52.5 ± 8	2340 ± 820	44.5	950 ± 3	18
15e	*m*-CH_2OH	1390 ± 420	> 20000		14000 ± 4000	10
15f	*m*-Cl	6.4 ± 2.9	97 ± 8	15.1	51 ± 9.8	7.9
15g	*m*-Br	25 ± 0.7	212 ± 30	8.5	160 ± 24	6.4
15h	*m*-I	27 ± 2	505 ± 90	18.7	230 ± 2 8	8.5
15i	*m*-CN	12.3 ± 3	645 ± 3.5	52.4	320 ± 14	26
15j	*m*-CH_3	33 ± 4.2	722 ± 2	21.8	435 ± 17	13.1
15k	2-thiophene	56.5 ± 10	4000 ± 420	70.7	2000 ± 260	35.3
15l	3-thiophene	70.5 ± 1	6300 ± 910	89.3	3000 ± 530	42.5
15m	2-chromone	15000 ± 2700	> 20000		> 20000	
15n	$CH{=}C(CH_3)_2$	900 ± 70	15000 ± 2000	16.6	7900 ± 400	8.7
15o	$CH_2C(CH_3){=}CH_2$	1070 ± 50	1950 ± 600	1.8	3800 ± 30	3.5
15p	$CH{=}CHCH_3$	4000 ± 380	> 10000		> 10000	
15q	$CH_2CH(CH_3)_2$	8000 ± 1000	> 20000		> 20000	
15r	2,4-difluoro	92 ± 7	3600 ± 140	39.1	2500 ± 5	27.1
15s	2,4-dichloro	117 ± 5	3500 ± 170	29.9	2600 ± 150	22.2
15t	2,5-dimethoxy	24.2 ± 1.2	240 ± 20	9.9	167 ± 10	6.9
Paclitaxel (**1a**)		0.95 ± 0.5	67 ± 15	70.5	33 ± 4	34.7
Cephalomannine (**1b**)		0.5 ± 0.2	188 ± 10	376	68 ± 3.3	136
Docetaxel (**1c**)		0.7 ± 0.4	10.5 ± 4	15	2.9 ± 0.2	4.1

[a] IC_{50} values determined in parental ovarian carcinoma 1A9 (a clone of A2780), paclitaxel-resistant PTX10, and PTX22 with β-tubulin mutations. IC_{50} values in nM were determined after two days' exposure to drugs using the MTT cell proliferation assay. Data are the mean ± SE of at least four independent experiments. [b] Values are the calculated relative resistance of each mutant cell line obtained by dividing the IC_{50} value of the resistant line by the IC_{50} value of the parental line 1A9.

Activities against drug-sensitive and drug-resistant tumor cells with β-tubulin mutations

PTX10 and PTX22 cells have mutations in the β-tubulin gene at two different positions (Phe 270 → Val in PTX10 and Ala 364 → Thr in PTX22). These two amino acids lie on the floor of the deep hydrophobic pocket surrounding paclitaxel, and changes to them confer paclitaxel resistance.[29] Although these residues do not contact the C2 side chain in the T-Taxol binding conformation model,[29] Phe 270 is in direct contact with the methyl group of C4 of paclitaxel, while Ala 364 forms part of the hydrophobic pocket, but is not in direct contact with the drug molecule. It was previously reported that modifications in the C2 side chain of paclitaxel (*meta*-N_3-paclitaxel)[35] restore at least partially (R/S value of 3) the sensitivity of these cell lines to paclitaxel cytotoxicity. This suggests an interaction between C2 and these hydrophobic pocket residues, prompting us to investigate the effect of the modifications in this region of the molecule on cytotoxicity against cell lines containing β-tubulin mutations.

Similar to the results with P-gp-overexpressing MDR cells, all C2 alkyl analogues were less active than C2 *meta*-substituted benzoyl analogues (Table 2). While **15b** and **15c**/**15t** are the most active compounds, **15m** is still the least active against 1A9 and its drug-resistant counterparts bearing β-tubulin mutations. Full restoration of cytotoxicity was obtained only for compounds **15b** and **15c** with PTX22 cells (R/S values for **15b** and **15c** in 1A9/PTX22 are 1 and 1.2, respectively), and quite good restoration of sensitivity was observed for PTX10 cells (R/S value in 1A9/PTX10 is 6.1 and 6.49, respectively).

Those taxoids with high association constants for binding to β-tubulin, **15b** (-N_3) and **15c** (-OMe), also showed a great improvement in cytotoxicity against cell lines with mutant β-tubulins when compared with paclitaxel. While they were effective in killing the parental 1A9 cell lines (IC_{50} ratios to paclitaxel of 2 and 6, respectively), they were more cytotoxic than paclitaxel in both mutated cell lines (IC_{50} ratios for PTX10 of 0.17 and 0.55, IC_{50} ratios for PTX22 of 0.05 and 0.21, respectively). The fact that the IC_{50} ratios are better in the PTX22 than in the PTX10 cell lines may arise from the fact that a polar residue (Thr) is introduced into β-tubulin in place of a nonpolar residue (Ala), which may facilitate the accommodation of the newly introduced polar groups.

Discussion

Overexpression of P-gp is one of the major reasons for the MDR phenotype in tumors. P-gp is able to pump many hydrophobic molecules, including taxoids, out of the cell. From the high correlation between IC_{50} and ΔG for β-tubulin binding in drug-sensitive cells, it can be deduced that the binding affinity for β-tubulin is the main driving force for entry of taxoids into the cell. In this way, a competition model between P-gp and β-tubulin for the taxoids can be used to explain the results. For our analogues, only small differences are found between the

K_d values for binding of the taxoids to P-gp, whereas differences of 2–3 orders of magnitude are found in the dissociation constants for binding to β-tubulin. The activity of taxoids in cells overexpressing P-gp largely depends on the balance between cell uptake of the drug and pumping of the drug out of the cell. It was shown some time ago that P-gp intercepts drugs at the level of the plasma membrane in intact cells. If P-gp is present and can keep up with the rate of entry of the taxane (in other words, if the taxane concentration is kept well below the IC_{50} value), then the cells are likely not killed. The potent cytotoxic activity of taxanes such as **15b** is probably due to their enhanced β-tubulin binding ability in comparison with paclitaxel, even in P-gp-overexpressing tumor cells. Compound **15b**, which has a very low IC_{50} value (1–2 orders of magnitude lower than paclitaxel in MDR tumor cells), interacts with the P-gp pump in a similar fashion to paclitaxel, but must be retained in cells at a concentration above its IC_{50} value, and is thus able to initiate biochemical cascades leading to cell-cycle arrest and tumor cell killing.

It is logical to suppose that a ligand with a binding affinity for β-tubulin that is orders of magnitude higher than that for binding to P-gp will accumulate inside the cells despite the overexpression of P-gp, and little or no resistance should be observed in this case. Thus an alternative way to combat MDR due to P-gp overexpression is to increase the binding affinity of the drug for its β-tubulin target. According to our data, the affinity of taxoids for binding to P-gp is of the same order of magnitude as that for binding to β-tubulin. This means that a 10-fold increase in P-gp expression can be counteracted by a 10-fold increase in the affinity of the taxoid for β-tubulin, as long as the other parameters that control the flux of taxoids through the membrane and the affinity for P-gp remain constant. To check for the first parameter, the compounds were assayed against drug-sensitive cells, and their cytotoxicity correlated with their binding affinity. Because the two parameters correlated very well, it can be inferred that the modifications made to the taxoid molecules do not affect the parameters (other than β-tubulin binding affinity) that control their entry into cells. The affinity of the ligands for binding to P-gp was measured, and as no significant differences were found between the taxoids as a group, it can be proposed that compounds with very high affinity for binding to β-tubulin will be handled by P-gp in a similar fashion to those with lower affinity. A low resistance index can also be observed for the low-affinity taxanes; low-affinity taxanes should work as taxane-based reversal agents for themselves.[40,41] Owing to the high concentration required to bind tubulin and to be cytotoxic, they should fill all the P-gp sites with a small percentage of the compound available in solution, thus allowing the rest of the compound to enter the cells and kill them.

Although taxoid **15b** exhibits better activity than paclitaxel in A549 xenograft mice (ip doses of 10 and 30 mg kg^{-1}) and shows a C_{max} value of 1.7 mg L^{-1} after ip 30 mg kg^{-1}, it cannot be detected in plasma after oral administration at the same dose (30 mg kg^{-1}). This observation can be explained by the inability of **15b** to effectively penetrate P-gp-expressing cellular barriers in the gastrointestinal tract (in a similar fashion to paclitaxel[42]), also supporting the proposal that the potent activity of **15b** originates from its high β-tubulin binding affinity.

It is important to point out that those taxoids which display high-affinity β-tubulin binding, **15b** and **15c**/**15t**, also show improved activity against cells containing mutant tubulins (PTX10 and PTX22[35]). Our initial thought on mutation-mediated resistance is that it may depend on the position of mutation, that is, whether or not the altered amino acid residues on β-tubulin are close to the site of small-molecule binding. One can postulate that if the mutation in the protein is at a location distant from those regions interacting with the modified groups in the taxoids, a very similar effect would be observed for all ligands. On the other hand, if the mutation is close to those regions interacting with the modified groups in the taxoids, differential effects would be noticed. For our analogues, the differential effects of these taxoids suggests that the C2 modifications are located close to the sites of mutation in 1A9/PTX, such as F270V and A364T, two of six mutants as revealed earlier.[27,28]

In a recent report,[28] the mutation Q292E was identified as being located in helix 9 of β-tubulin, outside the taxoid binding region. The high degree of resistance to epothilones and taxoids exhibited by cells carrying this mutation is mediated through a change in the conformation of β-tubulin so as to hamper the entry of small molecules into the binding region. The high potency of **15b** and **15c** against drug-sensitive 1A9 and drug-resistant 1A9/PTX cell lines may reflect small conformational changes in β-tubulin induced by these mutations, in comparison with wild-type tubulin. However, more studies will be needed to explore this possibility, including assays for binding of **15b** and **15c** to mutant tubulins, as well as computer simulations of their conformations and docking of these molecules into the binding sites of mutant β-tubulin.

The SAR of the taxoids in this study might have been expected to show different patterns of cytotoxicity in drug-sensitive tumor cell lines relative to P-gp-overexpressing cell lines and cell lines with mutated tubulins. To our surprise, a particular compound exhibited a similar potency in all the cell lines. This may be explained by the fact that the ability of the taxoids to bind to β-tubulin varies significantly, whereas their physical properties and their interactions with P-gp are similar. Thus, their cytotoxicities against both drug-sensitive and P-gp-expressing drug-resistant cells are determined mainly by their abilities to bind to β-tubulin. Regarding the activity of the taxoids in the drug-resistant cells with β-tubulin mutations, the similar ability of any taxoid in the C2-modified 10-deacetyl-7-acyl series to bind to mutated tubulins and wild-type β-tubulin provides an explanation for their behavior.

However, it is especially noticeable that the IC_{50} values for many of the compounds are much lower than their dissociation constants (for paclitaxel, $IC_{50} = 1$ nM for 1A9 cells, with $K_d = 100$ nM at 37 °C), which would imply that unless the local drug concentration inside the cell is higher, only a very small fraction of the β-tubulin molecules would contain bound paclitaxel. A possible reason for the cytotoxic effect observed at such a low total drug concentration would be drug accumulation inside the cell due to the high local β-tubulin concentra-

tion, which would make the total intracellular concentration of drug much higher than that found extracellularly. In the case of compounds with similar physicochemical properties, the β-tubulin binding affinity would be the driving force for them to pass through the membrane and the parameter that maximizes the internal concentration of taxoid.

Although a correlation was found between β-tubulin mutations and paclitaxel resistance in non-small-cell lung cancer patients in a clinical report,[43] further work did not support the results from this study. Because all genes obtained in this study were pseudogenes, it has been proposed in a recent review[44] that β-tubulin mutations are unlikely to be induced during chemotherapy. Although there is still a debate on the role of these pseudogenes, high-affinity taxanes are useful in probing ligand–β-tubulin interactions even if their involvement is of lesser clinical relevance.

Conclusions

We have prepared a series of C2-modified 10-deacetyl-7-propionylcephalomannine derivatives, some of which showed potent activity against both drug-sensitive tumor cells and tumor cells with drug resistance arising from both P-gp overexpression and β-tubulin mutation mechanisms. The high potency of some taxoids in this series, such as **15b**, **15c**, and **15t**, is of interest because the superior antitumor activity of **15b** over that of paclitaxel has been proven not only at molecular and cellular levels, but also in tumor-bearing nude mice. Although we speculated from the above data that the cytotoxicity of these compounds against drug-resistant tumor cells is probably due to their high affinity for binding to β-tubulin, the details of their interactions with P-gp and β-tubulin merit further investigations. Overall, our research results led to an important hypothesis, that is, a taxane with very high affinity for binding to β-tubulin ($\Delta G < -50$ kJ mol^{-1}) is able to effectively counteract drug resistance mediated by both P-glycoprotein overexpression and β-tubulin mutations. It was also found recently[45] that some taxanes that are highly active against P-gp-overexpressing tumor cells are not P-gp inhibitors. Thus, we wonder if enhancement of binding affinity to the appropriate receptor could be a generally applicable strategy for the design of MDR-fighting cytotoxic agents.

Experimental Section

General experimental procedures: All chemicals other than anhydrous solvents were obtained from Aldrich and Acros and used without further purification. All anhydrous reactions were performed under N_2, and anhydrous THF was dried over sodium (benzophenone as indicator). All reactions were monitored by TLC (silica gel, GF_{254}) with UV light and H_2SO_4–anisaldehyde spray visualization. All compounds were homogeneous as judged by TLC and were >95% pure by NMR spectroscopy.

2′-*O*-(*tert*-butyldimethylsilyl)cephalomannine (10a): Imidazole (367 mg, 5.34 mmol) and *tert*-butyldimethylsilyl chloride (541 mg, 3.59 mmol) were added successively to a stirred solution of cephalomannine (**1b**; 600 mg, 0.72 mmol) in dried *N*,*N*-dimethylformamide (DMF; 5 mL). The reaction mixture was stirred for 3 h at room temperature and worked up with methanol (2 mL). The crude material was separated over silica gel using EtOAc/petroleum ether (1:3) as eluent to obtain 2′-*O*-(*tert*-butyldimethylsilyl)cephalomannine (**10a**) as a white solid (625 mg, 91.6%); ^{1}H NMR (300 MHz, $CDCl_3$): δ = 8.13 (2H, d, J = 7.2 Hz), 7.60 (1H, t, J = 7.2 Hz), 7.52 (2H, t, J = 7.2 Hz), 7.25–7.50 (5H, m), 6.65 (1H, d, J = 9.0 Hz), 6.45 (1H, q, J = 6.9 Hz), 6.30 (1H, s), 6.29 (1H, overlapped), 5.70 (1H, d, J = 7.2 Hz), 5.59 (1H, d, J = 9.0 Hz), 4.98 (1H, d, J = 7.8 Hz), 4.58 (1H, d, J = 1.8 Hz), 4.44 (1H, dd, J = 6.9, 10.9 Hz), 4.32 (1H, d, J = 8.7 Hz), 4.21 (1H, d, J = 8.4 Hz), 3.82 (1H, d, J = 6.9 Hz), 2.50–2.62 (1H, m, overlapped), 2.54 (3H, s), 2.38 (1H, dd, J = 9.6, 12.3 Hz), 2.24 (3H, s), 2.15 (1H, dd, J = 8.7, 15.3 Hz), 1.85–1.95 (1H, m, overlapped), 1.90 (3H, s), 1.81 (3H, s), 1.72 (3H, d, J = 6.9 Hz), 1.69 (3H, s), 1.29 (3H, s), 1.15 (3H, s), 0.79 (9H, s), −0.07(3H, s), −0.33 ppm (3H, s); ESIMS: m/z $[M+Na]^+$ 968.5, $[M+K]^+$ 989.4.

2′-*O*-(*tert*-butyldimethylsilyl)-10-deacetylcephalomannine (10b): Hydrazine hydrate (2.5 mL, 85% *v*/*v*) was added to a stirred solution of 2′-*O*-(*tert*-butyldimethylsilyl)cephalomannine (**10a**) (380 mg, 0.40 mmol) in anhydrous ethanol (25 mL). The reaction mixture was stirred for 2 h at room temperature and worked up by dilution with EtOAc (500 mL). The solution was washed sequentially with NH_4Cl (aq, 2×200 mL) and brine (2×100 mL), and the organic layer was separated and dried over Na_2SO_4, concentrated in vacuo to yield a crude residue, which was separated over silica gel using EtOAc/petroleum ether (2:3) as eluent to furnish **10b** as a white solid (332 mg, 91.5%); ^{1}H NMR (500 MHz, $CDCl_3$): δ = 8.13 (2H, d, J = 7.5 Hz), 7.60 (1H, t, J = 6.5 Hz), 7.52 (2H, t, J = 7.5 Hz), 7.37 (2H, t, J = 7.5 Hz), 7.24–7.31 (3H, m), 6.68 (1H, d, J = 9.0 Hz), 6.45 (1H, q, J = 7.0 Hz), 6.30 (1H, t, J = 9.0), 5.71 (1H, d, J = 7.5 Hz), 5.58 (1H, d, J = 8.0 Hz), 5.19 (1H, s), 4.97 (1H, d, J = 9.0 Hz), 4.58 (1H, d, J = 1.5 Hz), 4.33 (1H, d, J = 8.5 Hz), 4.26 (1H, overlapped), 4.23 (1H, d, J = 8.5 Hz), 3.94 (1H, d, J = 7.0 Hz), 2.57–2.63 (1H, m), 2.54 (3H, s), 2.36 (1H, dd, J = 10.0, 15.0 Hz), 2.13 (1H, dd, J = 9.0, 15.3 Hz), 1.93 (3H, s), 1.83–1.89 (1H, m), 1.83 (3H, s), 1.82 (3H, s), 1.72 (3H, d, J = 7.5 Hz), 1.26 (3H, s), 1.13 (3H, s), 0.79 (9H, s), −0.05 (3H, s), −0.32 ppm (3H, s); ESIMS: m/z $[M+Na]^+$ 926.6.

2′-*O*-(*tert*-butyldimethylsilyl)-10-*O*-triethylsilyl-10-deacetylcephalomannine (11): *N*,*O*-Bis(triethylsilyl)trifluoroacetamide (907 mg, 2.68 mmol) was added dropwise to a solution of taxane **10b** (300 mg, 0.332 mmol) in freshly distilled THF (10 mL) under N_2 at −10 °C, followed by the addition of lithium bis(trimethylsilyl)amide (3 μL, 0.003 mmol). The reaction mixture was stirred for 10 min at −10 °C and worked up with methanol (1 mL). The crude material was separated over silica gel using EtOAc/petroleum ether (1:2) as eluent to obtain **11** as a white solid (314 mg, 92.9%); ^{1}H NMR (300 MHz, $CDCl_3$): δ = 8.12 (2H, d, J = 6.9 Hz), 7.60 (1H, t, J = 7.2 Hz), 7.51 (2H, t, J = 7.8 Hz), 7.25–7.40 (5H, m), 6.76 (1H, d, J = 8.7 Hz), 6.46 (1H, q, J = 6.3 Hz), 6.22 (1H, t, J = 8.4 Hz), 5.71 (1H, d, J = 7.2 Hz), 5.55 (1H, d, J = 7.8 Hz), 5.21 (1H, s), 4.96 (1H, d, J = 8.4 Hz), 4.75 (1H, d, J = 3.6 Hz), 4.32 (1H, d, J = 8.7 Hz), 4.21 (1H, d, J = 8.4 Hz), 4.20 (1H, overlapped), 3.93 (1H, d, J = 7.2 Hz), 2.58–2.63 (1H, m), 2.52 (3H, s), 2.37 (1H, dd, J = 9.3, 15.6 Hz), 2.16 (1H, dd, J = 9.3, 15.0 Hz), 1.91 (1H, m, overlapped), 1.85 (3H, s), 1.83 (3H, s), 1.73 (3H, d, J = 6.9 Hz), 1.69 (3H, s), 1.21 (6H, s), 0.98 (9H, t, J = 8.4 Hz), 0.80 (9H, s), 0.58–0.68 (6H, m), −0.04 (3H, s), −0.33 ppm (3H, s); ESIMS: m/z $[M+Na]^+$ 1040.5.

2′-*O*-(*tert*-butyldimethylsilyl)-7-propionyl-10-*O*-triethylsilyl-10-deacetylcephalomannine (12): Taxane **11** (322 mg, 0.316 mmol) was dissolved in 5 mL dried THF, followed by the addition of propanoic anhydride (0.40 mL, 3.120 mmol), DMAP (20 mg, 0.164 mmol), and Et_3N (0.8 mL). The mixture was stirred for 20 h at room tempera-

ture, and was worked up by diluting with EtOAc (300 mL). The solution was washed sequentially with $NaHCO_3$ (aq, 2×50 mL) and brine (50 mL), and the aqueous layers were combined and extracted by EtOAc (150 mL). The organic layers were dried over Na_2SO_4 and concentrated under reduced pressure to yield a crude residue, which was separated over silica gel using EtOAc/petroleum ether (1:2) as eluent to obtain **12** (314 mg, 92.4%); ^{1}H NMR (300 MHz, $CDCl_3$): δ = 8.12 (2H, d, J = 7.8 Hz), 7.60 (1H, t, J = 7.8 Hz), 7.51 (2H, t, J = 7.8 Hz), 7.28–7.40 (5H, m), 6.70 (1H, d, J = 8.7 Hz), 6.48 (1H, q, J = 6.9 Hz), 6.20 (1H, t, J = 9.3 Hz), 5.73 (1H, d, J = 6.3 Hz), 5.55 (1H, d, J = 9.3 Hz), 5.47 (1H, dd, J = 7.2, 10.3 Hz), 5.26 (1H, s), 4.94 (1H, d, J = 8.4 Hz), 4.58 (1H, d, J = 2.1 Hz), 4.33 (1H, d, J = 8.4 Hz), 4.22 (1H, d, J = 8.7 Hz), 4.00 (1H, d, J = 7.2 Hz), 2.48–2.56 (1H, m, overlapped), 2.52 (3H, s), 2.37 (1H, dd, J = 10.2, 16.5 Hz), 2.29 (2H, q, J = 7.8 Hz), 2.16 (1H, dd, J = 10.2, 16.5 Hz), 1.92 (3H, s), 1.84 (3H, s), 1.83 (1H, overlapped), 1.81 (3H, s), 1.78 (3H, d, J = 6.9 Hz), 1.21 (6H, s), 1.10 (3H, t, J = 7.2 Hz), 0.97 (9H, t, J = 7.8 Hz), 0.81 (9H, s), 0.53–0.62 (6H, m), −0.03 (3H, s), −0.33 ppm (3H, s); ESIMS: *m/z* $[M+Na]^+$ 1096.5.

2′-*O*-(*tert*-butyldimethylsilyl)-2-debenzoyl-7-propionyl-10-*O*-triethylsilyl-10-deacetyl-cephalomannine (13): Benzyltrimethylammonium hydroxide (40% *w/w* in MeOH, 190.9 μL) was added to a solution of taxane **12** (222.2 mg, 0.21 mmol) in anhydrous CH_2Cl_2 (5 mL) at −23 °C. The reaction mixture was stirred for 10 min and quenched with saturated NH_4Cl (aq, 30 mL). The aqueous layer was extracted with CH_2Cl_2 (3×50 mL), the organic layers were combined, dried over Na_2SO_4, and concentrated under reduced pressure. Silica gel chromatography of the residue (hexane/EtOAc/acetone = 8:3:1) afforded starting material (56.6 mg, 28.2%) and **13** (136.8 mg, 68.1%; 94.9% based on unrecovered starting material); ^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.35 (2H, t, J = 7.0 Hz), 7.29 (1H, d, J = 7.0 Hz), 7.23 (2H, d, J = 7.5 Hz), 6.69 (1H, d, J = 9.0 Hz), 6.47 (1H, q, J = 8.0 Hz), 6.13 (1H, t, J = 8.5 Hz), 5.51 (1H, d, J = 9.5 Hz), 5.44 (1H, dd, J = 7.0, 10.0 Hz), 5.19 (1H, s), 4.94 (1H, d, J = 8.5 Hz), 4.66 (2H, br s), 4.51 (1H, d, J = 1.5 Hz), 3.95 (1H, d, J = 7.0 Hz), 3.64 (1H, d, J = 6.5 Hz), 2.47–2.53 (1H, m), 2.37 (3H, s), 2.27 (2H, q, J = 7.0 Hz), 2.14–2.17 (2H, m), 1.90 (1H, overlapped), 1.87 (3H, s), 1.86 (3H, s), 1.81 (3H, s), 1.77 (3H, d, J = 7.0 Hz), 1.18 (3H, s), 1.08 (3H, t, J = 7.5 Hz), 1.06 (3H, s), 0.95 (9H, t, J = 7.5 Hz), 0.80 (9H, s), 0.51–0.62 (6H, m), −0.07 (3H, s), −0.31 ppm (3H, s); ESIMS: *m/z* $[M+Na]^+$ 992.5.

General procedure for the preparation of 2′-*O*-(*tert*-butyldimethylsilyl)-2-debenzoyl-2-acyl-7-propionyl-10-*O*-triethylsilyl-10-deacetylcephalomannine (14): An appropriate carboxylic acid (0.1 mmol) was dissolved in dried toluene (0.2 mL), and then DCC (20.6 mg, 0.1 mmol) and PP (1.0 mg, 0.007 mmol) were added to the solution. The mixture was stirred at room temperature for 5 min, taxane **13** (9.7 mg, 0.01 mmol) was added, the mixture was stirred at 65 °C until compound **13** was consumed (TLC analysis). The reaction mixture was diluted with EtOAc (10 mL), filtered through a pad of celite, and the celite was washed with EtOAc (10 mL). The filtrate was concentrated in vacuo to dryness. The residue was purified using PTLC (silica gel, hexane/EtOAc/acetone = 8:3:1) to afford **14**. Although it was found that the products were contaminated with dicyclohexylurea by ^{1}H NMR, they were subjected to desilylation without further purification.

General procedure for desilylation of 14: Pyridine (0.533 mL, 6.60 mmol) and HF (aq, 0.293 mL, 6.60 mmol) were added dropwise to a solution of taxane **14** (0.022 mmol) in acetonitrile (1.06 mL) in a 5-mL plastic bottle. The mixture was stirred at room temperature until the starting material was consumed as determined by TLC. The reaction mixture was diluted with EtOAc, washed with saturated $NaHCO_3$ and brine, and the aqueous layers were combined and extracted three times with EtOAc. The organic layer was dried over Na_2SO_4 and concentrated to dryness. Purification of the residue was carried out using PTLC (1:2 EtOAc/petroleum ether) to give homogeneous products.

Analytical data for compounds **15a–t** are given in the Supporting Information.

Tubulin and ligands: Purified calf brain tubulin and chemicals were used as described.[36] Paclitaxel (Taxol®) was provided by the late M. Suffness from the National Cancer Institute (Bethesda, MD, USA). Docetaxel (Taxotere) was kindly provided by Rhône Poulenc Rorer, Aventis (Schiltigheim, France). Taxoids were synthesized by us and characterized on the basis of spectroscopic methods. All the compounds were diluted in dimethyl sulfoxide (DMSO) to a final concentration of 10 mM and stored at −70 °C.

Preparation of stabilized microtubules: Stabilized cross-linked microtubules were prepared by mild cross-linking with 0.2% glutaraldehyde. Excess glutaraldehyde was quenched with $NaBH_4$ (Fluka), and the solution was dialyzed overnight using Slide-A-Lyzer 10K dialysis cassettes (Pierce) against the desired buffer and drop-frozen in liquid nitrogen.[46,47] After this treatment, 90% of the tubulin was found to have incorporated into microtubules, which are stable against dilution and low temperatures. The concentration of paclitaxel binding sites in these cross-linked microtubules was determined as described.[47] 100% of the assembled tubulin dimers were found to bind taxoids immediately after dialysis.

Equilibrium constants for binding of taxoid ligands to microtubules: The equilibrium binding constants of the different ligands to the paclitaxel binding site of assembled microtubules were measured as described.[38,37] Binding constants for compounds reversibly displacing Flutax-2 were calculated using a PC program written by us (Equigra v 5, J. F. Diaz, unpublished software). This program fits the experimental data by least squares to the equilibrium binding constant of the ligand investigated, employing the known values of the reference ligand Flutax-2. The apparent ΔG_0 was calculated from the binding constants using the equation: $-\Delta G_{0app} = RT \ln K_{binding}$

Binding of ligands to microtubules: Samples containing 5 μM compound **15b** and 10 μM taxoid binding sites in stabilized cross-linked microtubules (prepared as described above), were incubated for 30 min at 25 °C in polycarbonate centrifuge tubes (Beckman) in GAB (glycerol assembly buffer; 3.4 M glycerol, 10 mM sodium phosphate, 6 mM magnesium chloride, and 1 mM glycol-bis(2-aminoethylether)-*N,N,N′,N′*-tetraacetic acid (EGTA), pH 6.7) with 0.1 mM GTP (DMSO concentration was always kept under 2%). The samples were then centrifuged at 90000 *g* for 10 min at 25 °C in a Beckman Optima TLX ultracentrifuge with a TLA100 rotor. The supernatants were collected by pipetting, and the pellets were resuspended in 10 mM phosphate (pH 7.0). Both the pellets and supernatants were extracted three times with an equal volume of dichloromethane, dried in vacuo, and dissolved in 60 mL methanol/water (75:25 *v/v*). Ligands reversibly bound to pelleted polymers and free in the supernatant were determined by HPLC. HPLC analysis of the samples was performed in an Agilent 1100 series instrument employing a C18 column (Supercosil, LC18 DB, 250×4.6 mm, 5 μm bead diameter) developed in a gradient from 50–80% (*v/v*) acetonitrile in water at a flow rate of 1 mL min^{-1}, following the absorbance at λ = 220 nm.

Determination of dissociation constants for binding to P-gp by quenching of intrinsic Trp fluorescence: Purified P-gp was isolat-

ed from MDR cells according to established methods.[39] Fluorescence quenching was carried out using 50 μg mL^{-1} purified P-gp in buffer consisting of 2 mM 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS), 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), 100 mM NaCl, 5 mM $MgCl_2$, pH 7.5, as described previously.[48] Modified taxanes were dissolved in DMSO at 10 mg mL^{-1} as stock solutions, and working solutions (from 1.25–40 μg mL^{-1}) were made by diluting the stock solution with the above buffer. Quenching experiments were performed by successively adding 5-μL aliquots of taxane working solutions to 500 μL P-gp as described previously.[39] The final concentrations of the added taxanes were in the range 0.0125–1.5 μg mL^{-1}. After each addition, the steady-state fluorescence was measured at λ_{ex} = 290 nm and λ_{em} = 330 nm with 2-nm slits. Because the taxanes do not absorb light at the excitation and emission wavelengths, the recorded fluorescence intensities were corrected only for dilution using the following equation:

$$F_{icor} = (F_i - B)(V_i/V_0) \quad (1)$$

for which F_{icor} is the corrected value of the fluorescence intensity at a given point in the titration, F_i is the experimentally measured fluorescence intensity, B is the background fluorescence of the buffer, V_0 is the initial volume of the sample, and V_i is the volume of the sample at a given point in the titration. The data were computer fitted to the following equation:

$$\left(\frac{\Delta F}{F_0} \times 100\right) = \frac{\left(\frac{\Delta F_{max}}{F_0} \times 100\right) \times [S]}{K_d + [S]} \quad (2)$$

in which ($\Delta F/F_0 \times 100$) is the percent quenching (relative to the initial value) following addition of taxane at a concentration [*S*], and the values of dissociation constant (K_d) and maximal percent quenching ($\Delta F_{max}/F_0 \times 100$) were extracted using the Regression Wizard of Sigma Plot (Systat Software, Point Richmond, CA, USA).

Cell culture: Human ovarian carcinoma cell lines 1A9, A2780, A2780/AD (multidrug-resistant, overexpressing P-gp), PTX10, and PTX22 (a gift of P. Giannakakou) were cultured in RPMI-1640 supplemented with 10% fetal calf serum, 2 mM L-glutamine, 40 μg mL^{-1} gentamycin, 100 IU mL^{-1} penicillin, and 100 μg mL^{-1} streptomycin. A2780 and A2780/AD media were supplemented with 0.25 U mL^{-1} bovine insulin.

Cell cytotoxicity assay: Cells were seeded in 96-well plates at a density of 12000 cells in 0.08 mL per well. The next day, cells were exposed to 0.02-mL serial dilutions (0.005 nM to 40 μM) of ligands for 48 h, at which time an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay to determine viable cells was performed with some modifications.[49] Briefly, 20 μL of 2.5 mg mL^{-1} MTT was added to each well, incubated for 4 h at 37 °C, then treated with 0.1 mL MTT solubilizer (10% SDS, 45% DMF, pH 5.5). Plates were again incubated overnight at 37 °C to solubilize the blue formazan precipitate before measuring the absorbance at 595/690 nm in an automated Multiscan MC microplate reader. Control wells containing medium without cells were used as blanks. The MTT response is expressed as a percentage of the control (untreated) cells. IC_{50} was calculated from the log dose–response curves and expressed as the mean of at least four independent experiments with standard errors.

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