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EFFECTS OF TAXOIDS FROM *TAXUS CUSPIDATA* ON MICROTUBULE DEPOLYMERIZATION AND VINCRISTINE ACCUMULATION IN MDR CELLS

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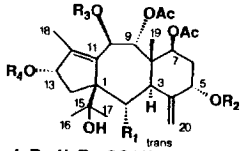
Abstract: Among new taxoids, taxuspines A ~ H and J ~ T (**1** ~ **19**), and known taxoids **20** ~ **56** containing taxol (**56**) and taxol-type compounds **37** ~ **44** with an *N*-acylphenylisoserine group at C-13 and an oxetane ring at C-4 and C-5 isolated from stems and leaves of the Japanese yew *Taxus cuspidata* Sieb. et Zucc., non-taxol-type compounds **4** and **32** remarkably reduced $CaCl_2$-induced depolemerization of microtubules. Furthermore, some non-taxol-type compounds **2**, **3**, **9**, **24**, **25**, **27**, and **28** increased cellular accumulation of vincristine in multidrug-resistant tumor cells as potent as verapamil, while taxol (**36**) and taxol-type compounds did not show such an activity. In addition, the non-taxol-type compounds enhancing vincristine accumulation inhibited competitively binding of azidopine to P-glycoprotein. These results suggest that some non-taxol-type taxoids may be useful for overcoming multidrug resistance in tumor cells.

Taxol exhibits potent antitumor activity against different cancers which have not been effectively treated by existing antitumor drugs. This is due to unique activity of taxol that it inhibits remarkably microtubule depolymerization process. Many researchers have reported that an *N*-acylphenylisoserine group at C-13 and an oxetane ring at C-4 and C-5 should play important roles for this unique activity of taxol. Recent reports on clinical trials of taxol, however, have disclosed that this hightly effective drug is inactive against colon cancer and renal cell carcinoma, which are originated from tissues that express consitutively the MDR1 gene. Thus, the observed lack of activity against these tumors could be partly due to the fact that this drug is subjected to multidrug-resistance (MDR).[1,2] In our continuing search for new bioactive taxoids, we examined extracts of the Japanese yew *Taxus cuspidata* Sieb. et Zucc. and obtained new taxoids, taxuspines A ~ H and J ~ T (**1** ~ **19**)[3~8], together with known taxoids **20** ~ **56** containing taxol (**36**) and taxol-type compounds **37** ~ **44** with an *N*-acylphenylisoserine group at C-13 and an oxetane ring at C-4 and C-5. Among the taxoids, non-taxol-type compounds **4** and **32** were found to reduce $CaCl_2$-induced depolymerization of microtubules remarkably. Furthermore, some non-taxol-type taxoids increased cellular accumulation of vincristine (VCR) in MDR tumor cells, while taxol (**36**) and taxol-type compounds did not show such an activity. In this paper we describe the effects of the taxoids **1** ~ **56** on microtubule depolymerization, vincristine accumulation in MDR cells, and cytotoxicity against tumor cells.

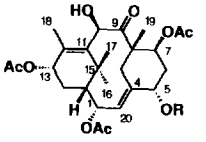
Taxoids from *Taxus cuspidata*.[3~8] The methanolic extract of stems and leaves of the yew collected at Sapporo was partitioned between toluene and water and then aqueous layer was partitioned with

$CHCl_3$. The toluene and $CHCl_3$ soluble portions were subjected to a silica gel column followed by reversed-phase and silica gel column chromatographies to afford taxuspines A ~ H and J ~ T (**1** ~ **19**) together with known taxoids (**20** ~ **56**).[9]

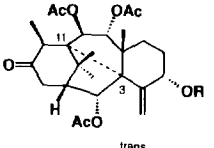
Inhibitory Activity of Ca^{2+}-Induced Microtubule Depolymerization by Taxoids. Taxol is unique among antimitotic drugs in that it promotes the polymerization of tubulin α,β-heterodimers by binding to and stabilizing the resulting microtubule polymer. It differs from antimitotic drugs such as colchicine, podophyllotoxin, and the vinca alkaloids, which inhibit microtubule assembly. Microtubules polymerized in the presence of taxol are resistant to depolymerization by Ca^{2+} ions. The effect of taxoids **1** ~ **56** was examined against the $CaCl_2$-induced depolymerization of microtubules.



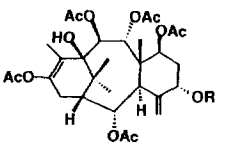
1 R_1=H, R_2=COCH=CHPh (trans), R_3=Bz, R_4=Ac
9 R_1=H, R_2=COCH=CHPh (trans), R_3=R_4=Ac
12 R_1=H, R_2=COCH=CHPh (trans), R_3=H, R_4=Ac
46 R_1=OAc, R_2=R_4=H, R_3=Bz
47 R_1=OAc, R_2=H, R_3=Bz, R_4=Ac



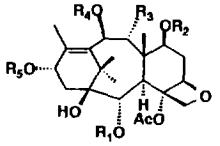
2 R=COCH=CHPh (trans)
52 R=COCH(OH)CH(NMe_2)Ph



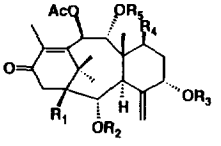
3 R=COCH=CHPh (trans)
8 R=$COCH_2CH(NMe_2)Ph$



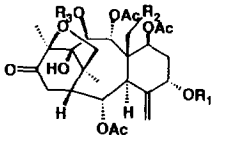
4 R=COCH=CHPh (trans)
15 R=$COCH_2CH(NMe_2)Ph$



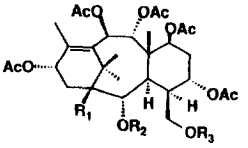
5 R_1=Bz, R_2=Ac, R_4=R_5=H, R_3=OH
13 R_1=R_2=Ac, R_3=OAc, R_4=Bz, R_5=$COCH_2CH(NMe_2)Ph$
53 R_1=Bz, R_2=R_4=R_5=H, R_3==O



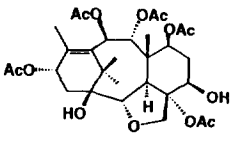
6 R_1=R_3=H, R_2=R_5=Ac, R_4=OAc
7 R_1=R_2=R_3=R_4=H, R_5=Ac
20 R_1=R_4=H, R_2=R_5=Ac, R_3=COCH=CHPh (trans)
21 R_1=R_3=R_4=H, R_2=R_5=Ac
22 R_1=H, R_2=R_5=Ac, R_3=COCH=CHPh (trans), R_4=OAc
23 R_1=OH, R_2=R_5=Ac, R_3=COCH=CHPh (trans), R_4=H
24 R_1=H, R_2=R_5=Ac, R_3=$COCH_2CH(NMe_2)Ph$, R_4=H
48 R_1=OH, R_2=R_5=Ac, R_3=$COCH_2CH(NMe_2)Ph$, R_4=H
55 R_1=OH, R_2=R_5=Ac, R_3=R_4=H
56 R_1=R_4=R_5=H, R_2=Ac, R_3=COCH=CHPh (trans)



18 R_1=COCH=CHPh (trans), R_2=OH, R_3=Ac
19 R_1=COCH=CHPh (trans), R_2=OAc, R_3=H
32 R_1=COCH=CHPh (trans), R_2=H, R_3=Ac
33 R_1=COCH=CHPh (trans), R_2=OBz, R_3=Ac
34 R_1=H, R_2=H, R_3=Ac
35 R_1=H, R_2=OBz, R_3=Ac
49 R_1=H, R_2=OAc, R_3=Ac



11 R_1=R_3=H, R_2=Ac
17 R_1=OH, R_2=H, R_3=Ac



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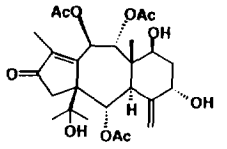

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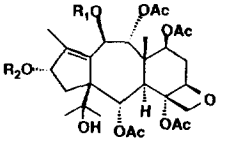
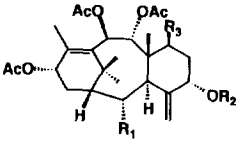
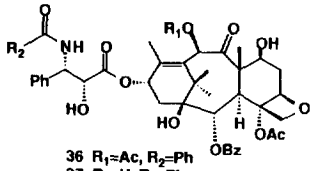


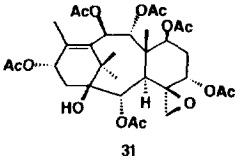
16 R_1=tigloyl, R_2=H
45 R_1=Bz, R_2=COCH=CHPh (trans)
50 R_1=Ac, R_2=H
51 R_1=Bz, R_2=H



25 R_1=H, R_2=$COCH_2CH(NMe_2)Ph$, R_3=OAc
26 R_1=H, R_2=COCH=CHPh (trans), R_3=H
27 R_1=H, R_2=COCH=CHPh (trans), R_3=OAc
28 R_1=R_3=H, R_2=$COCH_2CH(NMe_2)Ph$
29 R_1=OAc, R_2=Ac, R_3=H
30 R_1=R_3=OAc, R_2=H
54 R_1=OH, R_2=H, R_3=OAc



36 R_1=Ac, R_2=Ph
37 R_1=H, R_2=Ph
38 R_1=Ac, R_2=C_4H_7
39 R_1=H, R_2=C_4H_7
40 R_1=Ac, R_2=*n*-C_5H_{11}
41 R_1=H, R_2=*n*-C_5H_{11}
42 R_1=Ac, R_2=*n*-C_3H_7
43 R_1=H, R_2=Ph, C-7 epimer
44 R_1=Ac, R_2=Ph, C-7 epimer



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Microtubule proteins were polymerized under normal polymerization condition in the absence and the presence of taxol (**36**) or taxoids **1** ~ **35** and **37** ~ **56**, and, after 30 minutes incubation, $CaCl_2$ was added. Microtubule polymerization and depolymerization were monitored by the increase and the decrease in turbidity. The results were summarized in Fig. 1 as the changes in the relative absorbance at 400 nm.

The $CaCl_2$-induced depolymerization of microtubules (shown as control) was completely inhibited by 10 μM of taxol (**36**). Among the tested taxoids, taxuspine D (**4**) and taxagifine (**32**) remarkably reduced the depolymerization process, suggesting that these compounds have taxol-like activity to microtubule systems. The potency of **4** and **32** in inhibition of the depolymerization process corresponded to half to one third of that of taxol (**36**). Compounds **2**, **22**, and **45** exhibited moderate activity, while the other compounds showed little or no such effect. On the other hand, taxol-type compounds **37** ~ **44** inhibited the depolymerization process as potent as taxol. It is noted that compounds **4** and **32** lacking both an oxetane ring and an *N*-acylphenylisoserine moiety exhibited potent activity. Since these active compounds **2**, **4**, **22**, and **32** possessed a cinnamoyl group at C-5, the cinnamoyl group may play an important role for binding to microtubules like the acyl group at C-13 in taxol (**36**). In addition, model consideration suggests that the enol acetate group in **4** or the tetrahydrofuran ring in **32** may contribute to the inhibition of microtubule depolymerization like the oxetane moiety in taxol (**36**).

Increased Cellular Accumulation of Vincristine in Multidrug-Resistant Cells by Taxoids. The cellular accumulation of vincristine (VCR) is reduced in multidrug-resistant (MDR) tumor cells as compared with the parental cells. MDR-reversing agents such as verapamil increase the reduced accumulation of antitumor agents in MDR cells and overcome multidrug resistance. The effect of taxoids **1** ~ **56** on the cellular accumulation of VCR in multidrug-resistant human ovarian cancer 2780AD cells was examined and the

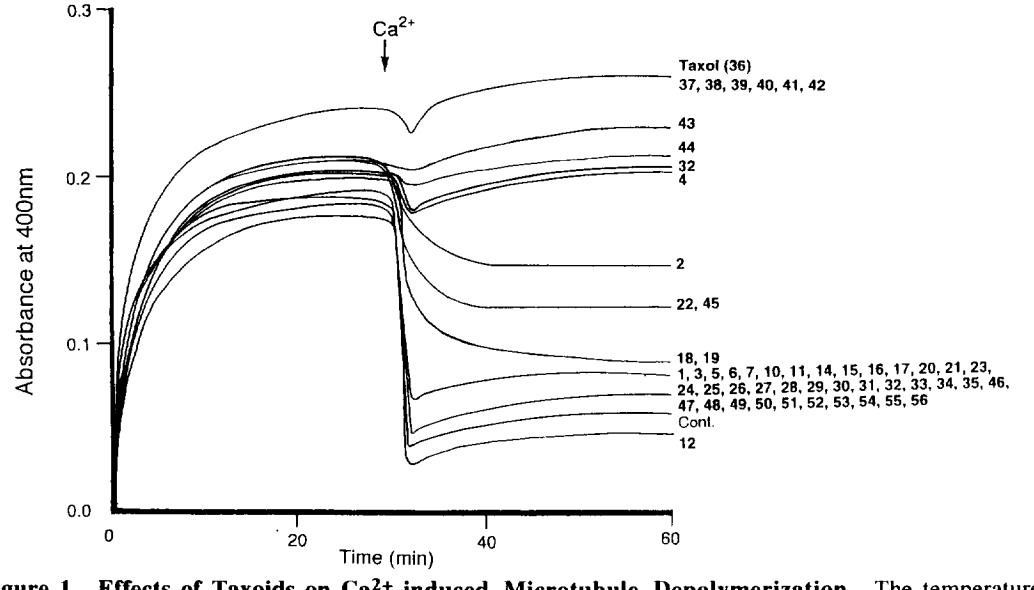

Figure 1. Effects of Taxoids on Ca^{2+}-induced Microtubule Depolymerization. The temperature was held at 37 °C and changes in turbidity were monitored at 400 nm. For the drug-protein studies, 10 μM of drug dissolved in DMSO was added to 1 mL buffer solution containing 2 mg microtubule protein. The final DMSO concentration was less than 1%. After 30 minutes incubation of the test mixture, 4 mM $CaCl_2$ was added, and the mixture was further incubated for another 30 minutes. The turbidity changes were monitored throughout the incubation time.

results were shown in Table 1. Verapamil at 1 and 10 μg/mL increased the VCR accumulation in a dose dependent manner. Compounds **2**, **3**, **9**, **24**, **25**, **27**, and **28** increased the VCR accumulation as potent as verapamil. Compounds **12**, **20**~**23**, **26**, **33**, **45**, **48**, and **54** increased moderately the accumulation, while taxol (**36**) and cephalomannine (**38**) decreased the VCR accumulation in 2780AD cells. It is noted that the potent compounds **2**, **3**, **9**, **24**, **25**, **27**, and **28** possessed a cinnamoyl or 3-*N*,*N*-dimethylamino-3-phenypropanoyl group at C-5. These results suggested that many taxoids could be substrates of P-glycoprotein and some of them might be useful for overcoming multidrug resistance.

Table 1. Effects of Taxoids on Accumulation of Vincristine (VCR) in Multidrug-Resistant 2780AD Cells.

Compound	VCR accumulation (% of control) with a taxoid concentration of		Compound	VCR accumulation (% of control) with a taxoid concentration of	
	1 μg/mL	10 μg/mL		1 μg/mL	10 μg/mL
1	142[a]	246	**28**	250	882
2	219	713	**30**	207	363
3	246	768	**31**	119	135
4	133	325	**32**	194	242
6	141	257	**33**	169	537
7	139	271	**34**	225	213
8	174	567	**35**	115	169
9	258	842	**36 (taxol)**	83	56
10	167	234	**38**	88	93
11	177	264	**43**	192	218
12	176	584	**44**	132	138
14	151	191	**45**	219	639
15	133	358	**46**	143	192
16	145	206	**47**	158	313
17	147	208	**48**	175	466
18	136	190	**49**	142	198
19	196	308	**50**	147	235
20	195	571	**51**	171	240
21	153	461	**52**	119	298
22	116	438	**53**	153	271
23	167	436	**54**	205	439
24	177	704	**55**	152	216
25	233	841	**56**	173	372
26	102	517	**Verapamil**	254	739
27	266	798			

a) The amounts of VCR accumulated in multidrug-resistant human ovarian cancer 2780AD cells were determined in the presence of 1 and 10 μg/mL of taxoids. 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.

Competitive Binding of [^{3}H]Azidopine and Taxoids to P-glycoprotein. Many calcium channel blockers reverse the multidrug-resistant phenotype and inhibit active transport of cytotoxic drugs out of multidrug-resistant cells. Azidopine is a photoaffinity analog of the dihydropyridine class of calcium channel blockers that specially labels P-glycoprotein. The ability to inhibit photoaffinity labeling of P-glycoprotein by azidopine has frequently been used as an indicator of whether a particular compound is a P-glycoprotein "substrate".[10,11] In particular, it is believed that compounds which compete with azidopine for a common binding site on the multidrug transporter will be able to block photolabeling. As shown in Fig. 2, [^{3}H]azidopine labeled P-glycoprotein (170 kDa) that was present in adriamycin-resistant human leukemia K562/ADM cells (lanes 1 and 10), and binding of [^{3}H]azidopine to P-glycoprotein was significantly reduced by the presence of verapamil (lanes 17 and 18). Compounds **2**, **3**, **24**, **25**, and **27** reduced remakably binding of [^{3}H]azidopine to P-glycoprotein more potent than verapamil (lanes 2 ~ 5, 8, 9, and 11 ~ 14), while taxinine (**20**) did not show

such activity (lanes 6 and 7) and taxol (**36**) showed quite weak reduction (lanes 15 and 16). Since compounds **2**, **3**, **24**, **25**, **27**, and **28** increased the VCR accumulation as potent as verapamil as described above, it is suggested that these taxoids may bind to the same binding site on P-glycoprotein as that of azidopine.

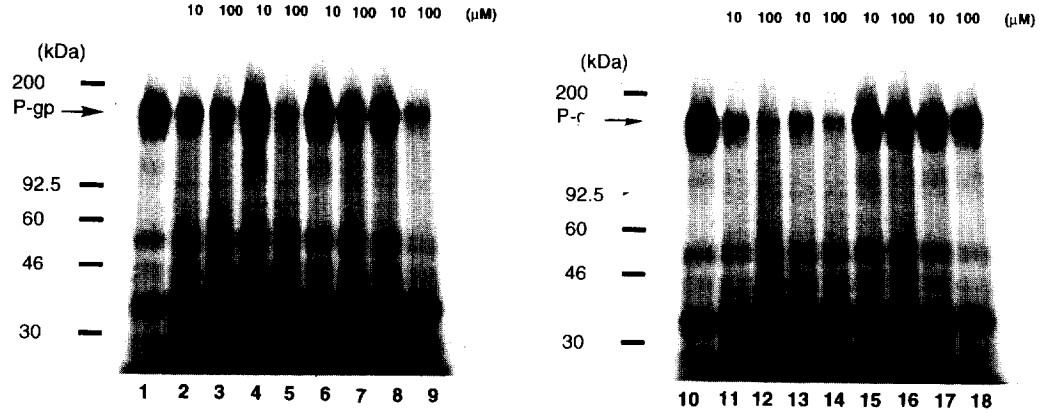

Figure 2. Effect of Taxoids on [^{3}H]Azidopine Photoaffinity Labeling of P-glycoprotein. Adriamycin-resistant human leukemia K562/ADM cell membrane vesicles (50 μg of protein) were incubated with [^{3}H]azidopine (200 nM) in the presence of taxoids (10 μM and 100 μM), taxuspine B (**2**, lanes 2 and 3), taxuspine C (**3**, lanes 4 and 5), taxinine (**20**, lanes 6 and 7), 2-desacetoxyaustrospicatine (**25**, lanes 8 and 9), 2-desacetoxytaxinine J (**27**, lanes 11 and 12), taxine II (**24**, lanes 13 and 14), and taxol (**36**, lanes 15 and 16), and verapamil (lanes 17 and 18). After 15 min at room temperature, the samples were subsequently irradiated with UV light for 10 min. After separation by SDS-polyacrylamide gel electrophoresis, the intensity of P-glycoprotein photolabeling was detected by fluorography; the band with a molecular mass of 170 kDa is due to P-glycoprotein. The position of molecular mass standards in kDa are indicated at the left of gel.

Cytotoxicity Studies. Cytotoxic activity of all the taxoids **1** ~ **56** against murine lymphoma L1210 cells and human epidermoid carcinoma KB cells was shown in Table 2. Taxol (**36**) and taxol-type compounds **37** ~ **44** exhibited very potent cytotoxicity against KB cells (IC_{50} 0.0015 ~ 0.086 μg/mL). Compounds **5**, **16**, **18**, **19**, **32**, **46**, **55**, and **56** also showed potent cytotoxicity against KB cells (IC_{50} 0.08 ~ 0.86 μg/mL), and compound **5** possessing an oxetane ring but no *N*-acylphenylisoserine group was most potent (IC_{50} 0.08 μg/mL) among these taxoids. It is interesting that compounds **16**, **18**, **19**, **32**, **46**, **55**, and **56** without an oxetane ring and an *N*-acylphenylisoserine moiety exhibited such cytotoxicity.

Extensive studies on syntheses of taxol analogs and its structure-activity relationships (SAR) have been carried out so far, while SAR of natural taxoids including non-taxol type compounds have been rarely reported. Taxol (**36**) and taxol-type compounds **37** ~ **44** exhibited quite potent inhibition of the microtubule depolymerization and extremely potent cytotoxicity, while non-taxol-type compounds **4** and **32** inhibiting microtubule depolymerization showed modest cytotoxicity. Many kinds of drugs with cationic and hydrophobic groups in their molecules have been reported to bind to P-glycoprotein, competing with the antitumor drugs and reversing the drug resistance. Some taxoids **2**, **3**, **24**, **25**, and **27** inhibiting binding of azidopine to P-glycoprotein in adriamycine-resistant K562/ADM cells increased the cellular accumulation of vincristine in multidrug-resistant 2780AD cells as potent as verapamil. In addition these taxoids exhibited weak or no cytotoxicity. These results suggests that some taxoids may be a good modifier of multidrug resistance in cancer chemotherapy.

Table 2. Cytotoxicity of Taxoids 1 ~ 56 against Murine Leukemia L1210 Cells and Human Epidermoid Carcinoma KB Cells

compound	L1210 IC_{50} (μg/mL)	KB IC_{50} (μg/mL)	compound	L1210 IC_{50} (μg/mL)	KB IC_{50} (μg/mL)
1	4.2	>10	**29**	>10	>10
2	>10	>10	**30**	>10	>10
3	5.8	>10	**31**	>10	>10
4	3.0	1.8	**32**	1.3	0.86
5	0.27	0.08	**33**	>10	>10
6	>10	>10	**34**	>10	>10
7	>10	>10	**35**	>10	9.4
8	>10	1.6	**36** (taxol)	0.33	0.0088
9	>10	>10	**37**	0.88	0.015
10	4.5	8.8	**38**	0.25	0.086
11	10.0	4.5	**39**	0.95	0.0048
12	1.2	5.8	**40**	0.21	0.0053
13	7.8	>10	**41**	0.24	0.0017
14	>10	>10	**42**	0.21	0.0016
15	2.5	2.6	**43**	0.71	0.013
16	4.0	0.3	**44**	0.026	0.0015
17	4.6	>10	**45**	3.8	>10
18	3.8	0.26	**46**	>10	0.4
19	1.0	0.06	**47**	>10	>10
20	>10	>10	**48**	2.7	>10
21	8.9	>10	**49**	>10	>10
22	>10	>10	**50**	>10	>10
23	4.6	6.9	**51**	>10	>10
24	>10	>10	**52**	7.1	>10
25	7.2	>10	**53**	>10	>10
26	9.5	8.2	**54**	10.0	7.4
27	4.9	>10	**55**	6.2	0.60
28	>10	>10	**56**	1.1	0.17

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