Notes

Novel Inhibitors for Multidrug Resistance: 1,3,5-Triazacycloheptanes

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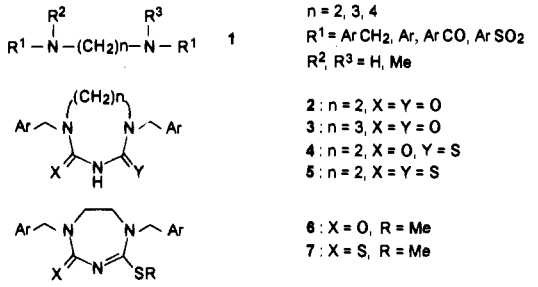
1,3,5-Triazacycloheptanes were synthesized and examined for reversal of the multidrug resistance dependent on P-glycoprotein. Most of these compounds increased the intracellular uptake of vinblastine in multidrug-resistant mouse leukemia P388/ADR cells without influence upon the vinblastine accumulation in P388/S cells. The efficacy of 1,5-dibenzyl-1,3,5-triazacycloheptanes in increasing the vinblastine accumulation was in the order of 2,4-dithioxo (**5**) > 2-oxo-4-thioxo (**4**) ≈ 4-(methylthio)-2-oxo (**6**) > 2,4-dioxo (**2**). The efficacy was further increased when the benzyl group was converted to a chlorobenzyl group. Among these compounds, **6c** [1,5-bis(4-chlorobenzyl)-1,5,6,7-terahydro-4-(methylthio)-2*H*-1,3,5-triazepin-2-one] potentiated the *in vitro* cell growth-inhibitory effect of vinblastine, adriamycin, and mitomycin C on P388/ADR cells and prolonged the life span of P388/ADR-bearing mice in combined therapy with vinblastine more than vinblastine alone.

Introduction

Multidrug resistance (MDR) is a major obstacle in cancer chemotherapy.[1,2] Multidrug-resistant cells generally show resistance to antitumor agents of natural products such as Vinca alkaloids and anthracyclines. Classical multidrug-resistant cells express an outward drug transporter, P-glycoprotein, in the plasma membrane. This protein increases the efflux of antitumor drugs, and the intracellular concentration of these drugs is kept at a low level.

Therefore, the reversal of MDR is a problem that must be overcome for the progress of cancer chemotherapy. There are several efforts to reverse MDR by nonantitumor agents.[3–9] Most of these drugs share a common structural feature of aromatic rings and amino groups located between the rings. These structural features seemed to be involved in counteracting the action of P-glycoprotein. In a previous study,[10] we synthesized a series of diamines, dicarboxamides, and disulfonamides with various aryl groups at both termini (**1**) and showed that *N*-methylethylenediamine and *N*-methylethylenedisulfonamides with terminal methyl- or chloro-substituted benzene rings showed potent efficacy on vinblastine (VBL) uptake in multidrug-resistant P388/ADR cells that overexpress P-glycoprotein.[11] Moreover, these compounds as well as reported MDR inhibitors have inadequate *in vivo* effects for clinical use. On the basis of these observations, we designed medium-sized azacycloalkane compounds to reverse MDR. This study deals with synthesis and structure–activity relationships of 1,3,5-triazacycloalkane derivatives **2**–**7** on VBL accumulation of P388 cells and examined the inhibitory effects on P388/ADR cells *in vitro* and *in vivo*.

Chart 1



Chemistry

Hagemann[12] has reported the synthesis of 1,5-dibenzyl-1,3,5-triazacycloheptane-2,4-dione (**2a**) from *N*,*N*′-dibenzylethylenediamine **8a** and dimethyl imidodicarboxylate. We prepared 1,3,5-triazacycloheptane-2,4-diones **2b**–**d** and the new 1,3,5-triazacyclooctane-2,4-diones **3a**–**d** by the reaction of ethylenediamine **8** or trimethylenediamine **9** with diethyl imidodicarboxylate by the procedure of Hagemann (Scheme 1). **2** reacted with 2,4-bis(4-methoxyphenyl)-1,3-dithia-2,4-diphosphetane 2,4-disulfide (Lawesson's reagent) to give a mixture of the 2-oxo-4-thioxo **4** and the 2,4-dithioxo **5**. Treatment of **4** or **5a** with methyl iodide in the presence of sodium hydride, respectively, afforded the methylthio oxo **6** or the methylthio thioxo **7a**.

Pharmacological Results and Discussion

The effects of a series of 1,3,5-triazacycloalkanes (**2**–**7a**) on the VBL uptake in drug-sensitive P388 cells (P388/S) and multidrug-resistant P388 cells (P388/ADR) are shown in Table 1. P388/ADR cells intrinsically accumulated VBL about 4-fold less and are resistant to VBL about 40-fold more than P388/S cells. These compounds at 10 μM hardly or only slightly increased the VBL accumulation in P388/S cells, but most of compounds promoted the VBL accumulation in P388/

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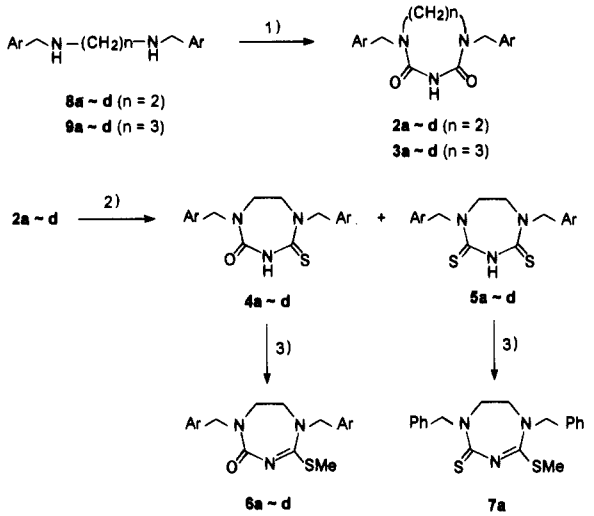
Scheme 1[a]



[a] (1) $HN(CO_2Et)_2$, (2) Lawesson's reagent, (3) NaH, MeI.

Table 1. Effects of Triazacycloalkane Derivatives on Accumulation of VBL in P388/S and P388/ADR Cells

	relative uptake (-fold)[a]			relative uptake (-fold)[a]	
compd	P388/S	P388/ADR	compd	P388/S	P388/ADR
2a	1.09	1.41	**5a**	1.26	4.16
2b	1.20	1.05	**5b**	1.34	5.34
2c	1.22	3.44	**5c**	1.48	6.00
2d	0.93	1.16	**5d**	1.21	5.26
3a	0.93	1.78	**6a**	1.37	2.13
3b	1.12	2.62	**6b**	1.16	6.75
3c	1.17	2.86	**6c**	1.28	6.87
3d	1.06	1.11	**6d**	1.04	1.14
4a	1.20	2.11	**7a**	1.19	3.44
4b	1.26	6.14	**8c**	1.04	2.62
4c	1.30	5.35	verapamil	1.02	5.70
4d	1.14	2.36			

[a] Relative VBL uptake (−fold) in drug (10 μM)−treated cells to the uptake in untreated cells. The uptake of [^{3}H]VBL in untreated P388/S and P388/ADR cells was 30 400 ± 758.2 and 7814 ± 520.4 dpm/10^6 cells, respectively.

ADR cells. In P388/ADR cells, comparing the efficacy of the dioxo compounds **2a−d** and **3a−d**, the compounds with same side chain were almost equipotent without distinction of the ring size of $n = 2$ or 3, and **2c** was approximately equipotent to **8c**, a starting material (Scheme 1). In addition, among the compounds with benzyl groups (**2−6a**) or 3,4-dimethoxybenzyl groups (**2−6d**), the efficacy of the compounds with the same triazacycloalkane ring was equipotent, and the efficacy was in the order of dithioxo (**5**) > oxo thioxo (**4**) ≈ methylthio oxo (**6**) > dioxo (**2, 3**). The methylthio thioxo compound **7a** was almost equipotent to the methylthio oxo compound **6a**. On the other hand, substitution on the 3- or 4-chlorobenzyl group increased the efficacy as in **2−6b,c**, and the substitution effect was dramatic in the cases of **4** and **6**. **4b** and **6b,c** increased the VBL accumulation 6-fold in P388/ADR cells. The effects of these compounds were almost equipotent to that of verapamil, and the effect of **6c** was much more potent than that of **8c** (Table 1).

The action of the most effective compound, **6c**, was further investigated as follows. *In vitro* 50% cell growth-inhibitory concentrations of **6c** against P388/S and P388/ADR cells were 13 and 18 μM, respectively. Noncytotoxic concentrations of **6c** potentiated the growth-inhibitory effect of VBL on P388/ADR cells in a dose-dependent manner, while this compound affected the effect of VBL on P388/S cells only a little (Table 2). In P388/ADR cells, most of the potentiation of the effect of VBL by **6c** is probably caused on the increase in VBL uptake, although other unknown mechanisms may be partly involved in the potentiation since smaller potentiation was seen in P388/S cells. When **6c** at 2.5 μM was combined with adriamycin, mitomycin C, or cisplatin, it potentiated the effects of adriamycin and mitomycin C 4- and 2.5-fold, respectively, in P388/ADR cells without influence on the effect of cisplatin. In P388/S cells, **6c** did not affect the effects of these antitumor drugs. Consequently, **6c** selectively potentiated the effects of MDR-related drugs in the resistant cells (Table 3).

Table 2. *In Vitro* Combination Effects of **6c** with VBL in P388/S and P388/ADR Cells[a]

	IC_{50} of VBL (nM)	
6c (μM)	P388/S	P388/ADR
0	1.7 (1.0)	70(1.0)
1.0	0.9 (1.9)	23(3.2)
2.5	0.7 (2.4)	6.2 (11)
5.0	0.4 (4.3)	2.2 (23)

[a] Cells were cultured with or without VBL in the presence or absence of **6c** for 48 h. Numbers in parentheses represent the relative increase in the growth-inhibitory effect of VBL by the combined treatment with **6c**.

Table 3. *In Vitro* Combination Effects of **6c** with Adriamycin, Mitomycin C, and Cisplatin in P388/S and P388/ADR Cells[a]

	IC_{50} of antitumor drugs (nM)			
	P388/S		P388/ADR	
antitumor drug	−	+	−	+
adriamycin	19	13	360	90
mitomycin C	48	44	535	264
cisplatin	48	72	61	51

[a] Cells were cultured in the absence or presence of various concentrations of an antitumor agent and 2.5 μM **6c** for 48 h. The sign of − or + indicates the IC_{50} values of antitumor agent alone or those of combined treatment with **6c** and antitumor agent, respectively.

Table 4. Antitumor Combination Therapy with VBL and **6c** in P388/ADR-Bearing Mice[a]

cell	treatment	survival days (average ± SD)	ILS (%)
P388/ADR	none	9.6 ± 0.5	0
	VBL (0.2 mg/kg)	11.0 ± 0.6	14.6
	VBL + **6c** (10 mg/kg)	11.6 ± 0.5*	20.8
	VBL + **6c** (20 mg/kg)	13.2 ± 1.5*	37.5
	VBL + **6c** (40 mg/kg)	14.0 ± 1.7*	45.8
	6c (40 mg/kg)	10.0 ± 0.9	4.2
P388/S	none	8.2 ± 0.8	0
	VBL (0.2 mg/kg)	15.5 ± 1.4*	89.0

[a] Each mouse was implanted with 1×10^6 cells on day 0 and treated with intraperitoneal injection of VBL and/or oral administration of **6c** on days 1−4 and 6−8. *$P < 0.05$ (Student's t-test or two-sample test with Welch's correction) compared to the no treatment group.

Next, we examined the combined effects of **6c** with VBL on P388/ADR-bearing mice. As shown in Table 4, oral administration of **6c** alone did not increase the survival days of the mice, and intraperitoneal injection of VBL at 0.2 mg/kg prolonged the life span only 15%, while it was prolonged 89% in P388/S-bearing mice. When VBL was combined with **6c** in P388/ADR-bearing mice, the life span was dose-dependently elongated by **6c**.

P388/ADR cells were shown to express P-glycoprotein in the plasma membrane and to extrude it in a energy-

dependent manner.[11] Since **6c** and the related compounds showed more potent action in P388/ADR cells than in P388/S cells negative for P-glycoprotein, these compounds are thought to have their antiresistant effect through an inhibitory action on P-glycoprotein.

In this study, the effects of triazacycloalkanes with an aryl side chain were investigated on the anti-MDR effect *in vitro* and *in vivo*. The effects of these compounds on VBL uptake seem to be due to the triazacycloalkane ring structures because the efficacy of the dibenzyl compounds was in the order of dithioxo (**5**) > oxo thioxo (**4**) ≈ methylthio oxo (**6**) > dioxo (**2, 3**). These may indicate that the thioxo or methylthio group containing sulfur atoms could be better for the activity, as indicated in our previous reports that isoquinolinesulfonamides[13] and ethylenedisulfonamides[10] are effective *in vitro* inhibitors of MDR. On the other hand, the efficacy was inversely related to the number of the oxo groups in 1,3,5-triazacycloalkanes. This may be similar to our previous evidence that the activity of dicarboxamide compounds was less than that of diamines.[10] The conversion of benzyl or the 3,4-dimethoxybenzyl group into a 3- or 4-chlorobenzyl group increased the efficacy of the oxo thioxo (**4**) or methylthio oxo (**6**) compounds for VBL accumulation in P388/ADR cells. These effects may be due to a moderate increase in the lipid solubility or in the affinity for a target molecule P-glycoprotein.

In conclusion, from the structure–activity relationships of 1,3,5-triazacyclohexanes and -octanes for VBL accumulation in P388/ADR cells, we found the bis(4-chlorobenzyl) derivative **6c** to be an effective inhibitor of MDR.

Experimental Section

General. All melting points were measured on a Yanagimoto micromelting point hot stage apparatus and are uncorrected. Infrared (IR) spectra were taken with a Hitachi 270-30 spectrometer, and mass spectra (MS) were measured with a JEOL DX-300 instrument. ^{1}H-NMR spectra were recorded on a JEOL PMX-60 spectrometer in $CDCl_3$ using tetramethylsilane as an internal standard; spectral assignments were confirmed by spin-decoupling experiments. Experimental analyses were done with a Perkin-Elmer 240C elemental analysis apparatus on solid samples only; the analytical results (C, H, N) are within ±0.4% of the theoretical values.

Starting Materials. *N,N'*-Bis(4-chlorobenzyl)ethylenediamine (**8**),[14] *N,N'*-bis(3,4-dimethoxybenzyl)ethylenediamine (**8d**),[15] *N,N'*-dibenzyltrimethylenediamine (**9a**),[16] *N,N'*-bis(3-chlorobenzyl)trimethylenediamine (**9b**),[17] *N,N'*-bis(4-chlorobenzyl)trimethylenediamine (**9c**),[18] and *N,N'*-bis(3,4-dimethoxybenzyl)trimethylenediamine (**9d**)[19] were prepared by the reported methods.

***N,N'*-Bis(3-chlorobenzyl)ethylenediamine (8b).** 3-Chlorobenzaldehyde (15.17 g, 108 mmol) was added to a stirred solution of ethylenediamine (3.24 g, 54 mmol) in MeOH (20 mL) at 0–5 °C. The reaction mixture was stirred for 3 h at room temperature, and the imino compound precipitated as a solid was collected by filtration. A mixture of the imino compound, sodium borohydride (6.78 g), and MeOH (150 mL) was refluxed for 3 h. The solvent was then evaporated, and water was added to the residue. The aqueous mixture was extracted with benzene, and the extracts was washed with water, dried, and evaporated *in vacuo*. The residue was chromatographed on silica gel using Et_2O:MeOH (95:5) as eluent to give **8b**: 7.35 g, 95% yield; viscous oil; IR (KBr) 3316 cm^{-1} (NH); ^{1}H-NMR δ 1.59 (2H, br s), 2.73 (4H, s), 3.74 (4H, s), 7.10–7.51 (8H, m); MS *m/z* 308 (M^+); dihydrochloride ($C_{16}H_8N_2Cl_2$·2HCl) mp 300 °C dec. Anal. ($C_{16}H_{10}N_2Cl_4$) C, H, N.

1,5-Bis(3-chlorobenzyl)-1,3,5-triazacycloheptane-2,4-dione (2b). A solution of *N,N'*-bis(3-chlorobenzyl)ethylenediamine (**8b**) (1.55 g, 5 mmol) and diethyl iminodicarboxylate (0.81 g, 5 mmol) in xylene (50 mL) was refluxed for 5 h and then concentrated *in vacuo*. The residue was chromatographed on silica gel using a CH_2Cl_2–AcOEt mixture as an eluent to give **2b** as colorless crystals (0.90 g, 48%). Recrystallization from AcOEt afforded colorless needles: mp 151–152 °C; ^{1}H-NMR ($CDCl_3$) δ 3.35 (4H, s), 4.58 (4H, s), 7.00 (1H, br s), 7.10–7.45 (8H, m); IR (KBr) 3216 (NH), 1688 cm^{-1} (C=O); MS *m/z* 377 (M^+). Anal. ($C_{18}H_{17}N_3O_2Cl_2$) C, H, N.

Other 1,3,5-triazacycloheptane-2,4-diones (**2c,d**) and new 1,3,5-triazacyclooctane-2,4-diones (**3a–d**) were prepared in a manner similar to that described for **2b** from the corresponding ethylenediamine **8** or trimethylenediamine **9**. Physicochemical data are summarized in Table 5.

1,5-Dibenzyl-4-thioxo-1,3,5-triazacycloheptan-2-one (4a) and 1,5-Dibenzyl-1,3,5-triazacyclooctane-2,4-dithione (5a). Lawesson's reagent (6.1 g, 15 mmol) was added to a stirred suspension of **2a** (3.1 g, 10 mmol) in toluene (200 mL) at room temperature under N_2 atmosphere. The reaction mixture was heated at reflux for 3 h and evaporated *in vacuo*. The residue was chromatographed on alumina using a PhH–AcOEt mixture as an eluent to give **4a**[20] and **5a**.

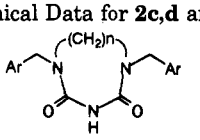
4a: 38% yield; mp 139–140 °C; colorless needles (from AcOEt); ^{1}H-NMR ($CDCl_3$) δ 3.10–3.30 (2H, m), 3.50–3.70 (2H, m), 4.50 (2H, s), 5.15 (2H, s), 7.20–7.40 (10H, br s), 8.20 (1H, br s); IR (KBr) 3232 (NH), 1652 cm^{-1} (C=O); MS *m/z* 325 (M^+). Anal. ($C_{18}H_{19}N_3OS$) C, H, N.

5a: 55% yield; mp 165–166 °C; colorless needles (from PhH); ^{1}H-NMR ($CDCl_3$) δ 3.50 (4H, s), 5.17 (4H, s), 7.33 (10H, m), 9.53 (1H, br s); IR (KBr) 3400 cm^{-1} (NH); MS *m/z* 341 (M^+). Anal. ($C_{18}H_{19}N_3S_2$) C, H, N.

Other 2-oxo-4-thioxo (**4b–d**) and 2,4-dithione (**5b–d**) compounds were prepared in a manner similar to that described for **4a** and **5a** from the corresponding **2b–d**. Physicochemical data are summarized in Tables 6 and 7.

1,5-Dibenzyl-4-(methylthio)-1,5,6,7-tetrahydro-2*H*-1,3,5-triazepin-2-one (6a). A mixture of **4a** (0.65 g, 2.0 mmol) in DMF (10 mL) and 60% NaH (0.06 g, 2.5 mmol) was stirred for 30 min at room temperature; then methyl iodide (0.28 g, 2.0 mmol) was added to the mixture. The reaction mixture was heated for 2 h at 50 °C and diluted with ice-cold water (15 mL), and the aqueous mixture was extracted with Et_2O. The extracts was washed with water, dried, and evaporated *in vacuo*. The residue was chromatographed on alumina using a PhH–CH_2Cl_2 mixture as an eluent to give **6a**: 82% yield; mp 152–153 °C; colorless needles (from isopropyl ether); ^{1}H-NMR ($CDCl_3$) δ 2.50 (3H, s), 3.15–3.50 (4H, m), 4.58 (2H, s), 4.60 (2H, s), 7.10–7.40 (10H, m); IR (KBr) 1630 cm^{-1} (C=O). Anal. ($C_{19}H_{19}N_3OS$) C, H, N.

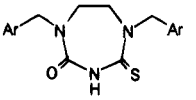
Table 5. Physicochemical Data for **2c,d** and **3a–d**



compd	*n*	Ar	yield (%)	mp (°C)[a]	formula[b]
2c	2	4-ClC_6H_4	53	158–159	$C_{18}H_{17}N_3O_2Cl_2$
2d	2	3,4-$(MeO)_2C_6H_3$	60	136–137	$C_{23}H_{27}N_3O_6$
3a	3	C_6H_5	66	177–179	$C_{19}H_{21}N_3O_2$
3b	3	3-ClC_6H_4	46	148–149	$C_{19}H_{19}N_3O_2Cl_2$
3c	3	4-ClC_6H_4	30	197–198	$C_{19}H_{19}N_3O_2Cl_2$
3d	3	3,4-$(MeO)_2C_6H_3$	48	141–143	$C_{23}H_{29}N_3O_6$

[a] All compounds were recrystallized from ethyl acetate. [b] C, H, and N analyses are within ±0.4% of theoretical values.

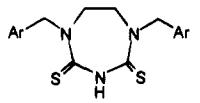
Table 6. Physicochemical Data for **4b–d**



compd	Ar	yield (%)	mp (°C)[a]	formula[b]
4b	3-ClC_6H_4	47	146–147	$C_{18}H_{17}N_3Cl_2OS$
4c	4-ClC_6H_4	45	151–152	$C_{18}H_{17}N_3Cl_2OS$
4d	3,4-$(MeO)_2C_6H_3$	35	150–151	$C_{22}H_{27}N_3O_5S$

[a] All compounds were recrystallized from ethyl acetate. [b] C, H, and N analyses are within ±0.4% of theoretical values.

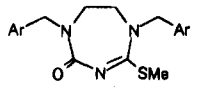
Table 7. Physicochemical Data for **5b–d**



compd	Ar	yield (%)	mp (°C)[a]	formula[b]
5b	3-ClC_6H_4	45	146–147	$C_{18}H_{17}N_3Cl_2S_2$
5c	4-ClC_6H_4	35	148–149	$C_{18}H_{17}N_3Cl_2S_2$
5d	3,4-$(MeO)_2C_6H_3$	60	165–166	$C_{22}H_{27}N_3O_4S_2$

[a] All compounds were crystallized from benzene. [b] C, H, and N analyses are within ±0.4% of theoretical values.

Table 8. Physicochemical Data for **6b–d**



compd	Ar	yield (%)	mp (°C)[a]	HRMS (m/z)[b]
6b	3-ClC_6H_4	82	oil	407.0626 (407.0612)
6c	4-ClC_6H_4	95	oil	407.0626 (407.0652)
6d	3,4-$(MeO)_2C_6H_3$	88	oil	459.1828 (459.1762)

[a] Compounds were purified by column chromatography on alumina. [b] Calcd (found).

Other 4-(methylthio)-2-ones (**6b–d**) were prepared in a manner similar to that described for **6a** from the corresponding **4b–d**. Physicochemical data are summarized in Table 8.

1,5-Dibenzyl-4-(methylthio)-1,5,6,7-tetrahydro-2*H*-1,3,5-triazepine-2-thione (7a). A mixture of **5a** (1.2 g, 3.5 mmol) in DMF (8 mL) and 60% NaH (0.2 g, 5.0 mmol) was stirred for 20 min at 50 °C; then methyl iodide (0.75 g, 5.3 mmol) was added to the mixture. The reaction mixture was stirred for 3 h at room temperature and diluted with ice-cold water, and the aqueous mixture was extracted with CH_2Cl_2. The extracts was washed with water, dried, and evaporated *in vacuo*. The residue was chromatographed on silica gel using an AcOEt–*n*-hexane mixture as an eluent to give **7a**: 70% yield; mp 121–122 °C; yellow needles (from diethyl ether); ^{1}H-NMR ($CDCl_3$) δ 2.58 (3H, s), 3.28 (2H, m), 3.61 (2H, m), 4.54 (2H, s), 5.26 (2H, s), 7.10–7.60 (10H, m); IR (KBr) 1566 cm^{-1} (C=N). Anal. ($C_{19}H_{21}N_3S_2$) C, H, N.

Evaluation of Cytotoxicity. Parent mouse leukemia P388 cells (P388/S) and adriamycin-resistant P388 cells (P388/ADR) were generously provided by the Cancer Chemotherapy Center, Japanese Foundation for Cancer Research, Tokyo, Japan. Cells were passaged weekly in the abdominal cavities of female BALB/c × DAB/2 (CDF_1) mice (Nippon SLC, Hamamatsu, Japan). To assess the effects on P388/S and P388/ADR cells *in vitro*, cells were suspended at a density of 10^5 cells/mL in RPMI-1640 medium with 10% fetal calf serum, 20 μM β-mercaptoethanol, and 100 μg/mL kanamycin (G-medium). Two hundred microliters of the cell suspension was seeded in each well of 96-well plastic dishes. A test compound was dissolved in dimethyl sulfoxide (DMSO), and the final concentration of DMSO was 0.25% (v/v). The effects of drugs on cell growth were evaluated after consecutive culture for 48 h by MTT assay.[21]

Evaluation of VBL Accumulation. Cells (10^6) were suspended in 1 mL of 20 mM Hepes-buffered G-medium (pH 7.4) and incubated with 4.6 kBq of [^{3}H]VBL in the presence or absence of a test compound at 37 °C for 30 min. The final concentration of DMSO was 1%. After the incubation, the cells were chilled on ice and collected by centrifugation (2000 rpm, 5 min) at 2 °C. The cells were washed twice with chilled phosphate-buffered saline (PBS; pH 7.4), and their VBL content was measured by the radioactivity after solubilization with NaOH and neutralization with acetic acid.

***In Vivo* Combined Chemotherapy.** Female $CD2F_1$ mice (5/group) were inoculated intraperitoneally with 1.0×10^6 P388/ADR cells/head on day 0 and treated with drugs on days 1–4 and 5–8. Treated drugs were **6c** and VBL: **6c** was given orally, and VBL was given intraperitoneally 2 h after **6c**.

Materials for Pharmacological Evaluations. [^{3}H]VBL (333 GBq/mmol) was purchased from Moravek Biochemicals. The antitumor drugs used were VBL (Shionogi, Osaka), adriamycin, mitomycin C (Kyowa Hakko Kogyo, Tokyo), and cisplatin (Nippon Kayaku, Tokyo).

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