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Reversal of Multidrug Resistance by Novel Nitrophenyl Pyrones, SNF4435C and D

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SNF4435C and D, novel immunosuppressants produced by a strain of *Streptomyces spectabilis*, were examined for their reversing effects *in vitro* on various multidrug-resistant (MDR) tumor cells overexpressing P-glycoprotein. These two compounds in the range of 3–10 μM completely reversed the resistance of MDR variant cells, mouse leukemia P388 cells [vincristine (VCR)-resistant P388/VCR and adriamycin (ADM)-resistant P388/ADM], human myelogenous leukemia K562 cells (VCR-resistant K562/VCR and ADM-resistant K562/ADM) and human ovarian cancer A2780 cells (ADM-resistant AD_{10}), against VCR. Both compounds moderately potentiated the sensitivity of the MDR cells to ADM but the reversal was not complete. SNF4435C and D significantly increased the intracellular accumulation of VCR in AD_{10} cells as potently as verapamil, cyclosporin A (CysA) and FK506, whereas the compounds exerted no effect on the accumulation of VCR in the drug-sensitive parent cells. Moreover, SNF4435C improved the chemotherapeutic efficacy of VCR in the treatment of P388/VCR-bearing mice. When 10 mg/kg SNF4435C was administered intraperitoneally to the mice concurrently with 0.2 mg/kg VCR for every 5 days, a treated/control (T/C) value of 143% was obtained. These results suggest that the compounds are useful candidates or tools for MDR modification in cancer chemotherapy.

Key words: Multidrug resistance (MDR) — P-Glycoprotein — SNF4435C — SNF4435D —MDR modifier

The emergence of multidrug resistance (MDR) is one of the major clinical problems in cancer chemotherapy. A well-known mechanism involved in this phenomenon is the overexpression of P-glycoprotein, the product of the *mdr-1* gene in human cells.[1–3] P-Glycoprotein acts as an ATP-dependent, drug-transmembrane transporter that decreases intracellular accumulation of structurally and functionally unrelated anticancer agents.[4,5] Since calcium channel blockers such as verapamil[6] and calmodulin inhibitors[7] were found to overcome MDR *in vivo* as well as *in vitro*, various MDR modifiers have been developed.[8–13] Though some of them are undergoing clinical trials,[8,9] their efficacy is not entirely satisfactory. Accordingly, more potent and less toxic modifiers are indispensable for clinical reversal of MDR.

SNF4435C and D are novel immunosuppressive agents isolated from the culture broth of a strain of *Streptomyces spectabilis*.[14,15] The compounds are nitrophenyl pyrones having an intriguing tricyclic ring system and are diastereoisomers. Interestingly, SNF4435C and D selectively suppressed B-cell proliferation induced by lipopolysaccharide (LPS) versus T-cell proliferation induced by Con A *in vitro*. Several immunosuppressive agents[16,17] reverse MDR by inhibiting the function of P-glycoprotein. We were therefore interested in the effect of these compounds on MDR tumor cells, and we found that the compounds are effective modifiers, overcoming MDR. In this report, we describe the potency of SNF4435C and D in conquering MDR tumor cells.

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MATERIALS AND METHODS

Chemicals SNF4435C and D were isolated from the culture broth of a strain of *Streptomyces spectabilis* SNF4435 in our laboratory, as described previously.[14] The structures of those compounds are illustrated in Fig. 1. The following drugs were purchased: vincristine (VCR) from Shionogi Co., Ltd. (Osaka), adriamycin (ADM) from Kyowa Hakko Co., Ltd. (Tokyo), verapamil from Eisai Co., Ltd. (Tokyo), FK506 from Fujisawa Pharm. Co., Ltd. (Osaka), cyclosporin A (CysA) from Novartis Pharm. Co., Ltd. (Tokyo) and [^{3}H]VCR (389 GBq/mmol) from Amersham Japan, Ltd. (Tokyo).

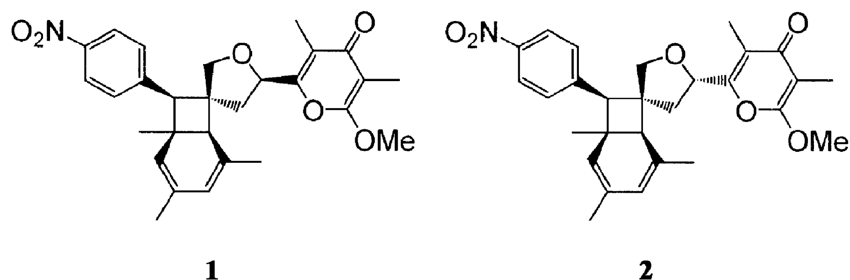

Fig. 1. Structures of SNF4435C (**1**) and D (**2**).

Animals Female CDF_1 mice were purchased from Charles River Japan, Inc. (Kanagawa). They were maintained under specific pathogen-free conditions at 23±2°C and 50±10% relative humidity. Lighting was automatic under a 12 h light/dark cycle. The mice were allowed free access to commercially available standard diet and water. All mice were 6 weeks old at the start of experiments.

Tumor cells P388, VCR-resistant P388 (P388/VCR) and ADM-resistant P388 (P388/ADM) cell lines were supplied by the National Cancer Institute (NIH, Bethesda, MD). The K562 cell line was provided by Dr. Ezaki, Cancer Chemotherapy Center (Tokyo), and its VCR-resistant (K562/VCR) and ADM-resistant (K562/ADM) sublines were established in the Cancer Chemotherapy Center, Japanese Foundation for Cancer Research.[18, 19] The human ovarian cancer line A2780 and its ADM-resistant subline (AD_{10}) were provided by Drs. R. Ozols and T. Hamilton, Medicine Branch, NCI, NIH.[20]

Cell culture and drug treatment The tumor cells were cultured in RPMI-1640 medium (Life Technologies, Inc., Rockville, MD) supplemented with 10% fetal bovine serum (Life Technologies, Inc.), 100 μg/ml streptomycin sulfate (Meiji Seika Co., Ltd., Tokyo), 100 U/ml penicillin G (Meiji Seika Co., Ltd.) and 0.2% $NaHCO_3$. For the culture of P388 and its sublines, 20 μM 2-mercaptoethanol was added to the above medium. For the efficacy assessment of the antitumor drugs *in vitro*, tumor cells (P388 and A2780 cells, 2×10^3 cells; K562 cells, 3×10^3 cells; P388/ADM, K562/VCR and AD_{10} cells, 4×10^3 cells; P388/VCR and K562/ADM cells, 6×10^3 cells) in the exponential growth phase were suspended in 100 μl of culture medium and inoculated into 96-well flat-bottomed microplates. The plates were cultured at 37°C for 24 h (A2780 and AD_{10}, which grow on the surface of the plate) or for 6 h (other cell lines, which grow in suspension) in a 5% CO_2 air atmosphere. The cells were then treated with graded concentrations of the indicated agent and reincubated in the CO_2 incubator at 37°C for 72 h. Three individual experiments were conducted at each agent concentration to confirm reproducibility. The colorimetric assay system (Cell Proliferation Kit I, Boehringer Mannheim GmbH, Mannheim, Germany) using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) was used for the quantification of *in vitro* chemosensitivity of tumor cells.[21] Cell growth was determined from the percentage ratio of optical density in agent-treated wells to that in non-treated wells. The median concentration of agent necessary to inhibit the growth of tumor cells by 50% (IC_{50}) was calculated from the dose-response curve.

Intracellular accumulation of [^{3}H]VCR A2780 and AD_{10} cells (10^6 cells) in the growth medium were seeded into 24-well plates and incubated at 37°C for 24 h in a 5% CO_2 air atmosphere. The medium in each well was aspirated and 0.5 ml of fresh culture medium containing 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid was added. Then [^{3}H]VCR (7.5 pmol) and the indicated concentrations of the agent were added to each well. After incubation at 37°C for 2 h, the intracellular accumulation of VCR was determined as described previously.[6, 7] In brief, the cells were washed twice with phosphate-buffered saline (PBS) and then treated with 0.1% sodium dodecyl sulfate (SDS)-0.2 N NaOH (0.15 ml/well) at 56°C for 60 min to solubilize the cells. The solution was transferred into a scintillant, ACSII (Amersham), and the radioactivity was counted by a liquid scintillation system (Beckman, LS9800, Fullerton, CA). Results are given for three independent experiments.

Evaluation of antitumor activity P388 and P388/VCR cells (10^6 cells/mouse) were transplanted intraperitoneally (i.p.) into CDF_1 mice on day 0. VCR was dissolved in 0.9% NaCl solution and SNF4435C was suspended in 0.9% NaCl solution containing 20% HCO-60 (Nikko Chemicals Co., Ltd., Tokyo). The agents were administered i.p. daily for 5 consecutive days from 1 day after tumor inoculation, either independently or concurrently with VCR. In the *in vivo* study, the modifier SNF4435C was given at 6 h before injection of VCR. Six mice were used for each experimental group. Antitumor activity of the agents was evaluated in terms of the median survival of the experimental group and was expressed as the treated/control (T/C) value.

RESULTS

Modulatory effects of SNF4435C and D on the cytotoxicity of VCR The sensitizing effects of SNF4435C and D on the cytotoxicity of VCR were examined with the MDR cells and their drug-sensitive parental cells (Table I). The IC_{50} values of VCR in P388, P388/VCR and P388/ADM cells were 5.2, 37 and 95 n*M*, respectively. When SNF4435C and D were added to the medium at a final concentration of 10 μM, the IC_{50} values of VCR in P388/VCR cells shifted to 0.1 and 0.2 n*M*, respectively. Under the same conditions, the IC_{50} values of VCR in P388/ADM cells shifted to 0.3 and 0.2 n*M*, respectively. In K562/VCR cells, K562/ADM cells and AD_{10} cells, 10 μM SNF4435C and D shifted the IC_{50} values from 44 to 0.4 n*M*, from 650 to 0.7 n*M*, and from 1200 to 2.2 and 2.1 n*M*, respectively. Thus, SNF4435C and D completely reversed the resistance to VCR in the tested MDR cells. Moreover, the compounds also moderately potentiated the sensitivity of their drug-sensitive parental cells to VCR. The IC_{50} values of VCR in P388, K562 and A2780 cells were 5.2, 0.7 and 2.4 n*M*, respectively, while these values shifted to 0.3, 0.2 and 0.4 n*M* upon the addition of either 10 μM SNF4435C or D.

Modulatory effects of SNF4435C and D on the cytotoxicity of ADM The effects of SNF4435C and D on the cytotoxicity of ADM were also examined with the MDR cells and their drug-sensitive parental cells (Table II). The IC_{50} values of ADM in P388, P388/VCR and P388/ADM cells were 73, 170 and 8300 n*M*, respectively. When SNF4435C and D were added to the culture medium at a final concentration of 10 μM, the IC_{50} values of ADM in P388/VCR cells shifted to 100 and 90 n*M*, respectively. Under the same conditions, the IC_{50} values of ADM in

Table I. Potentiation of Cytotoxicity of VCR against MDR Cells and Their Parental Cells[a] by SNF4435C and D

Cells	Modifiers	IC_{50} of VCR (n*M*)				
		Concentration (μM)				
		0	0.3	1	3	10
P388	None	5.2				
	SNF4435C		3.6	1.6	0.8	0.3
	SNF4435D		3.8	2.2	0.7	0.3
P388/VCR	None	37				
	SNF4435C		17	6.8	1.2	0.1
	SNF4435D		15	4.8	1.5	0.2
P388/ADM	None	95				
	SNF4435C		70	36	4.2	0.3
	SNF4435D		72	33	3.8	0.2
K562	None	0.7				
	SNF4435C		0.7	0.6	0.4	0.2
	SNF4435D		0.6	0.6	0.4	0.2
K562/VCR	None	44				
	SNF4435C		46	23	2.4	0.4
	SNF4435D		42	25	3.8	0.4
K562/ADM	None	650				
	SNF4435C		450	180	22	0.7
	SNF4435D		480	150	30	0.7
A2780	None	2.4				
	SNF4435C		1.8	0.8	0.7	0.4
	SNF4435D		1.8	0.7	0.6	0.4
AD_{10}	None	1200				
	SNF4435C		920	900	75	2.2
	SNF4435D		1000	850	60	2.1

a) Cells were treated with graded concentrations of VCR in the absence or presence of the indicated concentrations of modifiers.

Table II. Potentiation of Cytotoxicity of ADM against MDR Cells and Their Parental Cells[a] by SNF4435C and D

Cells	Modifiers	IC$_{50}$ of ADM (nM) Concentration (μM) 0	0.3	1	3	10
P388	None	73				
	SNF4435C		77	60	54	24
	SNF4435D		67	48	35	28
P388/VCR	None	170				
	SNF4435C		190	160	150	100
	SNF4435D		180	160	120	90
P388/ADM	None	8300				
	SNF4435C		8500	6200	4700	480
	SNF4435D		8200	5900	4500	580
K562	None	65				
	SNF4435C		57	32	34	60
	SNF4435D		68	50	53	48
K562/VCR	None	430				
	SNF4435C		320	310	180	75
	SNF4435D		400	290	190	85
K562/ADM	None	4200				
	SNF4435C		4300	4500	1200	850
	SNF4435D		4500	4800	2200	700
A2780	None	6.3				
	SNF4435C		7.6	7.8	4.4	4.5
	SNF4435D		6.5	7.1	5.8	4.9
AD$_{10}$	None	4300				
	SNF4435C		3300	3800	760	230
	SNF4435D		4800	4200	1000	180

a) Cells were treated with graded concentrations of ADM in the absence or presence of the indicated concentrations of modifiers.

P388/ADM cells shifted to 480 and 580 nM, respectively. In K562/VCR cells, K562/ADM cells and AD$_{10}$ cells, 10 μM SNF4435C and D shifted the IC$_{50}$ values from 430 to 75 and 85 nM, from 4200 to 850 and 700 nM, and from 4300 to 230 and 180 nM, respectively. These two compounds moderately potentiated the sensitivity of the tested MDR cells to ADM, but complete reversal was not observed. In the drug-sensitive parental cells, SNF4435C and D were little effective in modulating the sensitivity to ADM.

Cytotoxicity of SNF4435C and D To confirm that the enhanced efficacy of the antitumor drugs (Tables I and II) was due to reversal of MDR, the cytotoxicity of SNF4435C and D on the tumor cells was tested (Fig. 2). Although the dose-dependent inhibition curves varied among the unrelated cells, the dose-response curve of a MDR cell line was quite similar to that of its drug-sensitive parent cell line. In the presence of 30 μM SNF4435C and D, the growth rates of P388 and its variants, K562 and its variants, and A2780 and its variant were 46–55%, 35–41% and 10–18%, respectively. By contrast, SNF4435C and D at less than 10 μM had little influence on the cytotoxicity. Consequently, we considered that the evaluation of reversal of MDR was little affected by the cytotoxicity of SNF4435C and D.

Effects of SNF4435C and D on the accumulation of VCR The intracellular accumulation of [^{3}H]VCR in tumor cells was examined in the presence or absence of the modifier. It is reported that the MDR modifiers increase the reduced accumulation of antitumor agents in MDR cells.[7, 17] Compared with parental A2780 cells, the accumulation of [^{3}H]VCR was reduced by approximately 20% in the resistant AD$_{10}$ cells (Fig. 3). In A2780 cells, the amount of accumulated [^{3}H]VCR was slightly

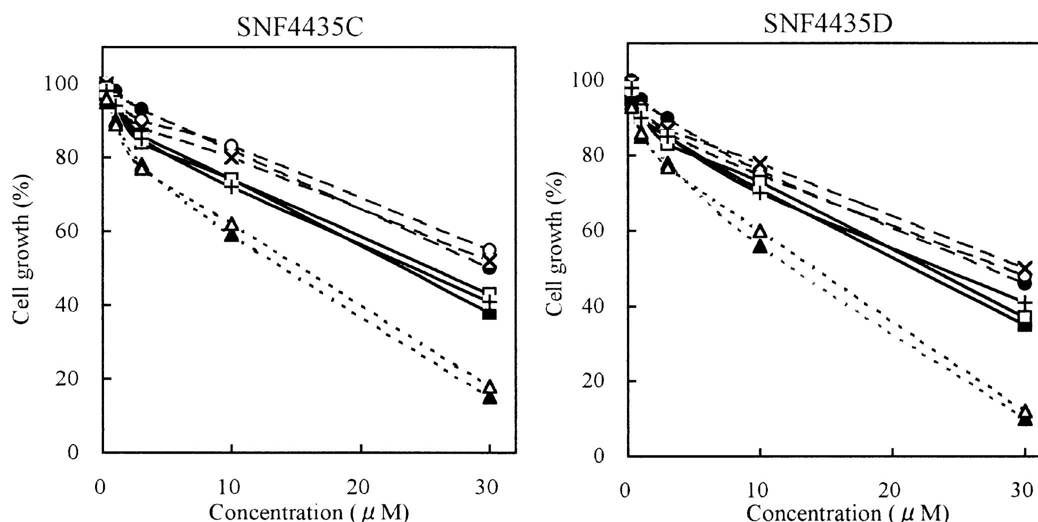

Fig. 2. Inhibitory effects of SNF4435C and D on the growth of MDR cells and the parental cells. Cell growth was determined from the percentage ratio of optical density in agent-treated wells to that in non-treated wells. ● P388, ○ P388/VCR, × P388/ADM, ■ K562, □ K562/VCR, + K562/ADM, ▲ A2780, △ AD_{10}.

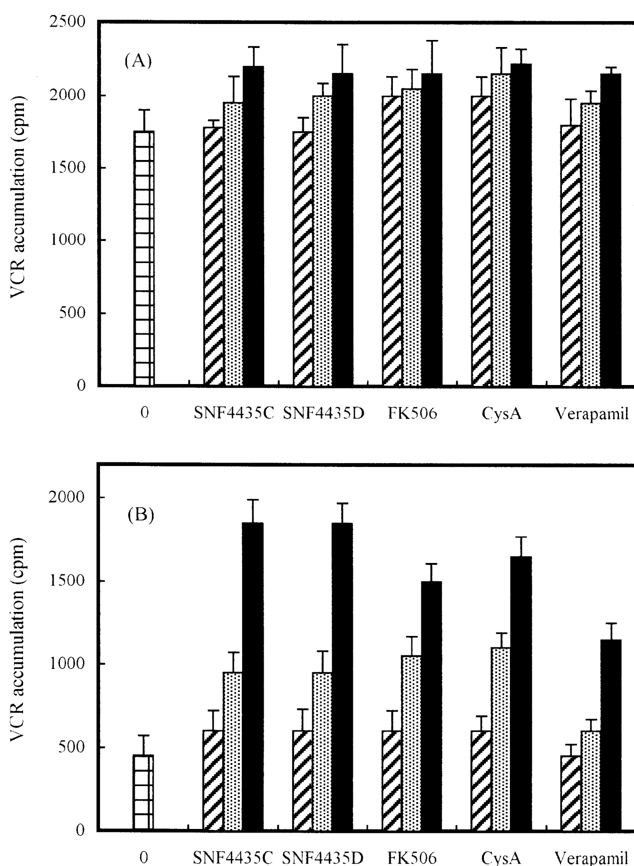

Fig. 3. Effects of SNF4435C and D on the accumulation of VCR in A2780 (A) and multidrug-resistant AD_{10} (B) cells. Each value represents the mean±SD of three independent experiments. ▨ 1 *μM*, ▩ 3 *μM*, ■ 10 *μM*.

increased by the addition of SNF4435C, D, FK506, CysA and verapamil. When FK506, CysA and verapamil were added to the assay solution, the amount of accumulated VCR in AD_{10} cells significantly increased in a dose-dependent manner. Ten micromolar FK506, CysA and verapamil increased the accumulated VCR by more than 2-fold. Similar results were obtained by the addition of SNF4435C and D, and at 10 *μM*, the compounds enhanced the accumulation of VCR in AD_{10} cells more potently than FK506, CysA and verapamil.

Antitumor effect of SNF4435C in P388-bearing mice The antitumor efficacy of SNF4435C was examined with the sensitive P388 leukemia model (Table III). The admin-

Table III. Antitumor Activity of SNF4435C in P388-bearing Mice

Drug	Dosage (mg/kg)	Survival days		Body weight change[a)] (g)
		Mean±SD	T/C (%)	
Control		10.8±1.1	100	1.4
SNF4435C	2.5	10.4±0.5	96	1.6
	5	10.6±1.0	98	1.7
	10	11.6±1.0	107	0.7
VCR	0.1	13.4±1.0*	124	0.8
	0.2	20.6±1.5*	191	0.1

a) Body weight change in the period from day 1 to 5.
SNF4435C and VCR were given i.p. daily from day 1 to 5.
* Significantly different from values for controls by Student's *t* test: $P<0.001$.

Table IV. Effect of SNF4435C on Antitumor Activity of VCR in P388/VCR-bearing Mice

Drug and dosage	Survival days		Body weight change[a] (g)
	Mean±SD	T/C (%)	
Control	11.2±0.6	100	1.1
SNF4435C (2.5 mg/kg)	11.3±0.8	101	1.5
SNF4435C (5 mg/kg)	10.9±0.5	97	0.9
SNF4435C (10 mg/kg)	12.2±1.1	109	0.4
VCR (0.2 mg/kg)	12.4±0.5*	111	0.3
VCR (0.2 mg/kg)+SNF4435C (2.5 mg/kg)	13.2±0.8*	118	1
VCR (0.2 mg/kg)+SNF4435C (5 mg/kg)	14.1±0.6*	126	0.4
VCR (0.2 mg/kg)+SNF4435C (10 mg/kg)	16.0±1.0*	143	0

a) Body weight change in the period from day 1 to 5.
SNF4435C and VCR were given i.p. daily from day 1 to 5.
* Significantly different from values for controls by Student's *t* test: $P<0.001$.

istration of SNF4435C (2.5–10 mg/kg) had no antitumoral effect in P388/sensitive-bearing mice (T/C values 96–107%). By contrast, administration of 0.2 mg/kg VCR showed good antitumoral activity (T/C=191%).

Synergistic effect of VCR and SNF4435C on P388/VCR-bearing mice The ability of SNF4435C to potentiate the antitumor activity of VCR in the drug-resistant P388/VCR leukemia model was investigated (Table IV). The administration of SNF4435C alone at a dose range of 2.5–10 mg/kg scarcely altered the survival time as compared to the control group (T/C values 97–109%). The administration of 0.2 mg/kg VCR alone also exhibited only a modest chemotherapeutic effect (T/C=111%). However, the combination of SNF4435C and VCR increased the life span of P388/VCR-bearing mice in a dose-dependent manner. When 10 mg/kg SNF4435C was administered concurrently with 0.2 mg/kg VCR, the antitumor efficacy was significantly potentiated (T/C=143%) in comparison with the administration of either SNF4435C or VCR alone. No severe losses of body weight were observed with this combination.

DISCUSSION

SNF4435C and D are novel immunosuppressants having an intriguing tricyclic ring system.[15] The compounds potently suppress B-cell proliferation induced by LPS versus T-cell proliferation induced by Con A.[14] The mechanism involved in the immunosuppressive action of SNF4435C and D is clearly different from that of CysA and FK506, which suppress the immune system by blocking T-cell activation. We found that SNF4435C and D were also effective in overcoming MDR, like CysA and FK506. The resistance to VCR in P388/VCR, P388/ADM, K562/VCR, K562/ADM and AD_{10} cells *in vitro* was completely reversed by SNF4435C and D, in an almost nontoxic dose range. Furthermore, SNF4435C and D moderately enhanced the sensitivity of the MDR cells to ADM, though the reversal was not complete. The accumulation of VCR in drug-resistant AD_{10} cells was increased by SNF4435C and D as effectively as by verapamil, CysA and FK506, while these compounds had no effect on the accumulation of VCR in the drug-sensitive parental A2780 cells. From these results, it is likely that the mechanism involved in the action of SNF4435C and D in overcoming MDR is similar to that of verapamil, CysA and FK506. It was found that those drugs are modulators of cellular P-glycoprotein via direct binding, leading to inhibition of the pump activity.[22, 23] Alternatively, other unknown mechanisms may be involved in the action, because SNF4435C and D significanlly potentiated the cytotoxicity of VCR towards drug-sensitive parental P388 cells. *In vivo*, SNF4435C was also effective in overcoming VCR resistance. When 10 mg/kg SNF4435C was concomitantly administered with 0.2 mg/kg VCR, the chemotherapeutic effect of VCR in P388/VCR-bearing mice was potentiated by 143% (T/C). The dosage of SNF4435C for overcoming the resistance in P388/VCR-bearing mice was almost the same as that exhibiting suppression of graft rejection (data not shown). The immunosuppressive activity might limit the clinical use of SNF4435C and D for conquering MDR. Considering the possible direct interaction between SNF4435C or D and P-glycoprotein, the reversing activity is not likely to be related to the immunosuppressive activity. Through studies of cyclosporins, non-immunosuppressive analogues of CysA that exhibit MDR modifying activity have been developed.[8, 24] Accordingly, exploration and development of non-immunosuppressive SNF4435 derivatives might provide promising agents for cancer chemotherapy.

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