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Overcoming of Vinblastine Resistance by Isoquinolinesulfonamide Compounds in Adriamycin-Resistant Leukemia Cells

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SUMMARY

We investigated the effects of seven isoquinoline derivatives in overcoming resistance to vinblastine in Adriamycin-resistant mouse leukemia P388/ADR cells and human myelogeneous leukemia K562/ADR cells. *N*-(2-Methylpiperazyl)-5-isoquinolinesulfonamide (H-7), *N*-[2-(methylamino)ethyl]-5-isoquinolinesulfonamide (H-8), and *N*-(2-aminoethyl)-5-isoquinolinesulfonamide (H-9) did not reverse resistance to vinblastine in these resistant cells. *N*-[2-[*N*-[3-(4-Chlorophenyl)-2-propenyl]amino]ethyl]-5-isoquinolinesulfonamide (H-86) and *N*-[2-[*N*-[3-(4-chlorophenyl)-1-methyl-2-propenyl]-amino]ethyl]-5-isoquinolinesulfonamide (H-87) caused significant accumulation of intracellular vinblastine and marked reversal of the resistance to vinblastine in both resistant cell lines. Addition of a formyl group at the terminal amino group of H-86 (H-85) or addition of an aminoethyl group to the nitrogen atom at the sulfonamide group of H-86 (W-66) reduced those activities. The activity on vinblastine accumulation seems to correlate with the hydrophobicity of the compounds. The compounds that effectively reversed resistance to vinblastine inhibited [^{3}H]vinblastine efflux and photoaffinity labeling of P-glycoprotein with a photosensitive analogue of vinblastine, *N*-(*p*-azido-(3-[^{125}I]iodo)-salicyl)-*N'*-β-aminoethylvindesine. Although these isoquinoline derivatives inhibited protein kinase A and protein kinase C with various potencies, these inhibitory activities did not correlate with the reversal of drug resistance. These results indicate that hydrophobic isoquinoline derivatives reverse multidrug resistance due to the suppression of drug binding to P-glycoprotein, without involvement of their activities on protein kinase A and protein kinase C.

Multidrug resistance is a major obstacle in cancer chemotherapy. In multidrug-resistant cells, the efflux of some kinds of antitumor drugs, such as vinblastine and Adriamycin, is enhanced, and the intracellular concentration of these antitumor agents does not increase (1, 2). P-Glycoprotein, which is encoded by the *mdr* gene, is overexpressed in the plasma membrane of multidrug-resistant cells and works as a drug efflux pump (1-5). Overexpression of P-glycoprotein has been recognized in clinical cases (6).

There are several drugs that overcome multidrug resistance *in vitro*, namely, verapamil (7), phenothiazine calmodulin inhibitors like trifluoperazine (8), dihydropyridines (9-11), chloroquine derivatives (12, 13), reserpine (14, 15), and cyclosporin A (16). Some of these drugs inhibit drug efflux from multidrug-resistant cells and [^{125}I]NASV photolabeling of P-glycoprotein in multidrug-resistant cells (17).

We have reported that an isoquinolinesulfonamide compound (H-87), which is a potent inhibitor of cAMP-dependent protein kinase (protein kinase A), overcame multidrug resistance of Adriamycin-resistant mouse leukemia P388/ADR cells *in vitro* (18). In this report, we investigated the effects of several isoquinolinesulfonamide derivatives on the overcoming of resistance to vinblastine in mouse and human Adriamycin-resistant leukemia cells and discussed the mechanism, with respect to the chemical structure.

Experimental Procedures

Materials. [^{3}H]Vinblastine (374 GBq/mmol) and [γ-^{32}P]ATP (110 TBq/mmol) were purchased from Amersham International (Bucking-

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ABBREVIATIONS: [^{125}I]NASV, *N*-(*p*-azido-(3-[^{125}I]iodo)-salicyl)-*N'*-β-aminoethylvindesine; SDS, sodium dodecyl sulfate; H-7, *N*-(2-methylpiperazyl)-5-isoquinolinesulfonamide; H-8, *N*-[2-(methylamino)ethyl]-5-isoquinolinesulfonamide; H-9, *N*-(2-aminoethyl)-5-isoquinolinesulfonamide; H-85, *N*-[2-[*N*-formyl-*N*-[3-(4-chlorophenyl)-2-propenyl]amino]ethyl]-5-isoquinolinesulfonamide; H-86, *N*-[2-[*N*-[3-(4-chlorophenyl)-2-propenyl]amino]ethyl]-5-isoquinolinesulfonamide; H-87, *N*-[2-[*N*-[3-(4-chlorophenyl)-1-methyl-2-propenyl]amino]ethyl]-5-isoquinolinesulfonamide; W-66, *N*-(2-aminoethyl)-*N*-[2-[3-(4-chlorophenyl)-2-propenyl]amino]ethyl-5-isoquinolinesulfonamide; PBS, phosphate-buffered saline; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

hamshire, UK). Vinblastine was purchased from Shionogi Co. (Osaka, Japan). [^{125}I]NASV was synthesized by the methods of Safa *et al.* (19, 20). Isoquinoline derivatives H-7, H-8, H-9, H-85, H-86, H-87, and W-66 were synthesized by methods described before (21).

Cells and culture. Parent mouse leukemia P388 cells (P388/S), Adriamycin-resistant P388 cells (P388/ADR), human myelogeneous leukemia K562 cells (K562/S), and Adriamycin-resistant K562 cells (K562/ADR) were used in this study. These cells were kindly provided by Dr. T. Tsuruo of the Cancer Chemotherapy Center, Japanese Foundation for Cancer Research (Tokyo, Japan). P388 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 of isoquinoline compounds on leukemia cells *in vitro*, cells were suspended in RPMI 1640 medium supplemented with 10% fetal calf serum, 20 μM β-mercaptoethanol, and 100 μM kanamycin (G-medium), and 10^5 cells were seeded in 24-well plastic dishes. The effects of drugs on cell growth were evaluated after consecutive culture for 48 hr.

Accumulation and efflux of vinblastine. In the accumulation experiment, cells (10^6) were suspended in 1 ml of 20 mM HEPES-buffered G-medium (pH 7.4) and incubated in the presence of 37 kBq of [^{3}H]vinblastine, at 37°. After the incubation, the cells were chilled on ice and collected by centrifugation (2000 rpm × 5 min), at 2°. The cells were washed twice with chilled PBS (pH 7.4). Vinblastine accumulated in the cells was measured as the radioactivity after solubilization with NaOH and neutralization with acetic acid. In the efflux experiment, cells were loaded with 20 nM [^{3}H]vinblastine (74 kBq) by incubation in glucose-free Hanks' solution (pH 7.4) containing 10 mM NaN_3. The cells were washed once with chilled PBS and incubated, without or with a test compound, in 20 mM HEPES-buffered G-medium (pH 7.4). After incubation of the cells for designated periods at 30°, the radioactivity remaining in the cells was measured as described above. The results were expressed as the percentage of retained vinblastine in the cells, relative to the initially loaded intracellular vinblastine.

Photoaffinity labeling with [^{125}I]NASV. The plasma membrane of the cells was prepared by the Percoll sedimentation method described before (15, 22). The plasma membrane (100 μg) was incubated in 100 μl of 40 mM phosphate buffer (pH 7.4), 4% dimethylsulfoxide, with 7.4 kBq of [^{125}I]NASV, with or without a test compound, for 20 min at room temperature in the dark. The mixture was irradiated at 365 nm for 15 min on ice and was centrifuged for 5 min at 13,500 × *g*. The resultant pellet was solubilized by the addition of SDS sample buffer containing 8.0 M urea and was used for SDS-polyacrylamide gel electrophoresis (7.5% gel). After they were fixed and dried, gels were autoradiographed onto Kodak X-Omat R film, with intensifying screens (DuPont Cronex Lightening-Plus), for 24–48 hr at −70°. Protein was measured by the method of Lowry *et al.* (23), with bovine serum albumin as the standard.

Immunoblotting. Fractionated membrane proteins on gels were transferred onto nitrocellulose membrane filters (Schleicher & Schuell, Dassel, Germany). Each filter was then incubated in 3% gelatin in PBS for 2 hr at room temperature, for blocking, and incubated overnight with 1 μg/ml P-glycoprotein monoclonal antibody C219 (Centocor, Inc., Malvern, PA) (24). The filter was washed and incubated with horseradish peroxidase-conjugated anti-mouse IgG (Organon Teknika Corp., West Chester, PA) for 1 hr. After extensive washing with PBS containing 0.05% Tween 20, the immunopositive band was made visible in a solution containing 0.5 mg/ml diaminobenzidine and 0.03% H_2O_2.

Enzyme assay. The catalytic subunit of protein kinase A was purified from bovine heart by the method of Beavo *et al.* (25). Protein kinase C was prepared from rabbit brain as described previously (26). The activities of protein kinases were measured by [^{32}P]phosphate incorporation into protein substrates, as described (21), in the presence of various concentrations of isoquinoline compounds.

Octanol-PBS partition coefficient. An isoquinoline compound was dissolved in PBS or *n*-octyl alcohol at the concentration of 200 μM, and 5 ml of each solution were mixed. The mixture was vigorously shaken for 30 min and equilibrated. Concentrations of each compound in the PBS phase and *n*-octyl alcohol phase were measured by spectrophotometry at 280 nm. The logarithmic apparent partition coefficient was estimated as the logarithm of the concentration in the *n*-octyl alcohol phase, relative to that in the PBS phase.

Results

We examined the effects on overcoming of resistance to vinblastine and the cytotoxicity of seven isoquinoline derivatives in mouse leukemia cells and human leukemia cell lines (Table 1). Overcoming of resistance was evaluated by combination experiments using a nontoxic concentration of isoquinoline compound. In P388/ADR and K562/ADR cells, H-7, H-8, and H-9 did not reverse resistance to vinblastine, even at 10 μM. H-85, H-86, H-87, and W-66 effectively reversed resistance in P388/ADR cells. H-86 and H-87 also effectively reversed resistance in K562/ADR cells, but H-85 and W-66 were less effective. Thus, H-86 and H-87 were effective compounds in human cells. These isoquinoline compounds did not affect the vinblastine sensitivity in the parent cells as much.

We then studied the effects of isoquinoline derivatives on intracellular accumulation of vinblastine in P388/ADR cells (Fig. 1). H-8 and H-9 barely increased vinblastine in the cells, even at 30 μM. H-87 potently increased the accumulation of vinblastine, as reported previously (18). H-85, H-86, and W-66 also increased vinblastine in a dose-dependent manner, but H-85 and W-66 were less effective than H-86. In P388/S cells, H-85 also increased the accumulation of vinblastine in a dose-dependent manner, although its efficacy was lower than in P388/ADR cells. As shown in Fig. 2, the efflux of vinblastine from P388/ADR cells was inhibited by H-85, H-87, and W-66 but not by H-8, even at 30 μM. The inhibitory effect of H-87 was the most potent, and H-85 and W-66 were less effective. These results indicate that vinblastine accumulation produced by isoquinoline derivatives depends on the inhibition of efflux.

[^{3}H]Vinblastine was accumulated in the cells and reached a plateau within 20 min; the amount of vinblastine in P388/ADR and K562/ADR cells was then about one fifth and one fortieth of that in the respective parent cells. Table 2 compares the effects of these isoquinolinesulfonamide compounds, at 3 μM, on vinblastine accumulation in P388/ADR cells and K562/ADR cells. H-86 and H-87 effectively increased the accumulation of vinblastine in K562/ADR cells, as well as P388/ADR cells, to near the level in the sensitive cells. The effects of H-85 and W-66 on vinblastine accumulation were moderate. In both cell lines, H-7, H-8, and H-9 did not affect vinblastine accumulation. Consequently, the overcoming effect on drug resistance (Table 1) seems to be closely related to the potency of these compounds for drug accumulation in these leukemia cells.

Table 2 also shows the inhibitory activities on protein kinase A and protein kinase C and partition coefficients of the compounds. H-7, H-8, and H-9, which have low hydrophobicity, inhibited protein kinase A and protein kinase C with various potencies. H-86 and H-87, which have high hydrophobicity, were potent inhibitors of protein kinase A. The hydrophobicity of H-85 and W-66 was lower than that of H-86 and H-87, but it was much higher than that of H-8 and H-9. H-85 was inactive and W-66 was moderately active on the protein kinases.

Next, we investigated the effects of isoquinoline compounds on [^{125}I]NASV photolabeling in membrane vesicles from P388/

TABLE 1

Combined effects of isoquinoline derivatives with vinblastine in parent and Adriamycin-resistant cell lines of P388 and K562 cells and cytotoxicity of the compounds in resistant cell lines

IC_{50} values are the mean of two experiments done in triplicate. Numbers in parentheses represent the relative resistance to vinblastine. Relative resistance was calculated by dividing each IC_{50} value by the IC_{50} value in each parent cell line.

Compounds	Concentration	IC_{50} of vinblastine				IC_{50} for resistant cell lines	
		P388 cell lines		K562 cell lines		P388/ADR	K562/ADR
		P388/S	P388/ADR	K562/S	K562/ADR		
	μM	nM				μM	
None		5.8 (1.0)	50.0 (8.62)	0.6 (1.0)	150 (250)		
H-7	10	5.7 (0.98)	49.6 (8.55)	0.6 (1.0)	150 (250)	30	60
H-8	10	5.8 (1.0)	50.0 (8.62)	0.6 (1.0)	150 (250)	>100	38
H-9	10	5.8 (1.0)	50.0 (8.62)	0.6 (1.0)	150 (250)		38
H-85	1	5.5 (0.95)	17.5 (3.02)	0.6 (1.0)	150 (250)	26	25
	3	2.4 (0.41)	11.9 (2.05)	0.6 (1.0)	122 (203)		
H-86	1	5.6 (0.97)	13.5 (2.33)	0.6 (1.0)	45 (75)	5.2	14
	3			0.6 (1.0)	17 (28)		
H-87	1	5.8 (1.0)	12.5 (2.16)	0.6 (1.0)	20 (33)	6.1	12
	3			0.4 (0.7)	3 (5)		
W-66	1	4.0 (0.69)	16.0 (2.76)	0.6 (1.0)	120 (200)	5.3	15
	3			0.6 (1.0)	105 (175)		

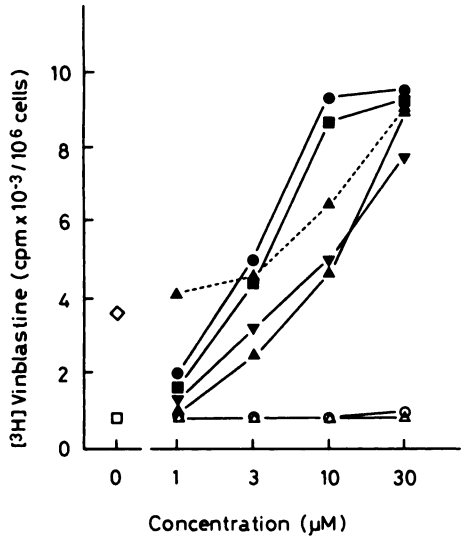

Fig. 1. Effects of isoquinolinesulfonamide compounds on intracellular accumulation of [^{3}H]vinblastine. Cells were incubated with [^{3}H]vinblastine for 20 min in the absence (◇, P388/S; □, P388/ADR) or presence (○, H-8; △, H-9; ▲, H-85; ■, H-86; ●, H-87; ▼, W-66) of isoquinolinesulfonamide compounds. – – –, P388/S; ——, P388/ADR cells. Each *point* represents the mean of two experiments done in triplicate.

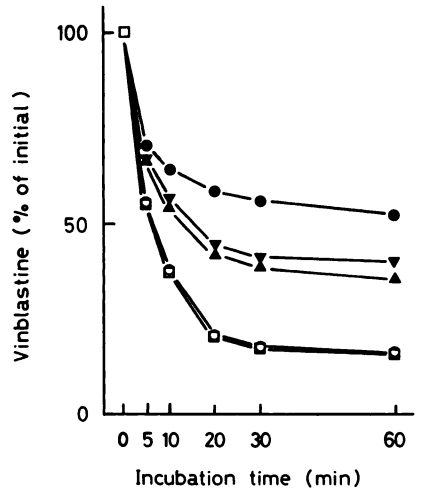

Fig. 2. Inhibitory effects of isoquinoline compounds on the efflux of vinblastine from P388/ADR cells. □, Control; ○, 30 μM H-8; ▲, 10 μM H-85; ●, 10 μM H-87; ▼, 10 μM W-66. Each *point* represents the mean of two experiments done in triplicate.

S and P388/ADR cells (Fig. 3). The 140-kDa membrane protein of P388/ADR cells was photolabeled with [^{125}I]NASV and selectively inhibited by vinblastine (Fig. 3A), and the protein was immunopositive with C219 monoclonal antibody against P-glycoprotein (Fig. 3B). This protein was barely detectable in sensitive cells (P388/S). H-87 selectively inhibited [^{125}I]NASV photolabeling of this protein, in a dose-dependent manner (Figs. 4 and 5). H-86, H-87, and W-66 also inhibited the photolabeling in a dose-dependent manner (Fig. 5). The inhibitory effect of H-8 was much less than that of other compounds (Figs. 4 and 5). The effects of these compounds on the photolabeling appeared to correlate with the effects on vinblastine accumulation (Fig. 1).

Discussion

It was reported that mouse leukemia P388/ADR and human myelogenous leukemia K562/ADR cells show multidrug resistance because of their increased capacity for active drug efflux (27, 28). There were large differences in the degree of vinblastine resistance and the drug accumulation between P388/ADR and K562/ADR cells. This may be caused by the following two reasons. (i) These cells express P-glycoproteins with different properties, as a drug efflux pump. Indeed, K562/ADR cells are well characterized multidrug-resistant cells that overexpress a 170-kDa P-glycoprotein (28), and P388/ADR cells express a 140-kDa P-glycoprotein in the plasma membrane (Ref. 29 and this study). It has also been reported that two independently selected resistant cell lines showed different drug-binding characteristics of P-glycoproteins and different transport properties (30). (ii) These cells express different amounts of P-glycoprotein, although this has not been confirmed. In either case, in both Adriamycin-resistant cell lines isoquinolinesulfonamides increased the drug accumulation and reversed the drug resistance, with a close correlation with their own hydrophobicity.

These compounds inhibited photolabeling, with a vinblastine analogue, of the 140-kDa membrane protein. We have recently

TABLE 2

Effects on vinblastine accumulation, inhibitory activities on protein kinase A and protein kinase C, and the partition coefficients of isoquinoline compounds

IC_{50} values for protein kinase A and protein kinase C are the mean of two experiments done in triplicate. The effects on vinblastine accumulation were estimated as the increase (fold) of vinblastine accumulation produced by 3 μM isoquinoline compounds. The partition coefficients (log PC) were calculated as described in Experimental Procedures.

Compounds	Increase of vinblastine accumulation by 3 μM compound		IC_{50} value for		log PC
	P388/ADR	K562/ADR	Protein kinase A	Protein kinase C	
	fold		*μM*		
H-7	1.0	1.0	2.3	8	
H-8	1.0	1.0	2.7	15	−0.67
H-9	1.0	1.0	3.1	18	−0.73
H-85	2.9	1.8	62	>100	1.87
H-86	5.3	21.4	0.06	18	2.32
H-87	6.0	25.8	0.04	>100	2.35
W-66	3.8	3.2	1.4	37	1.70

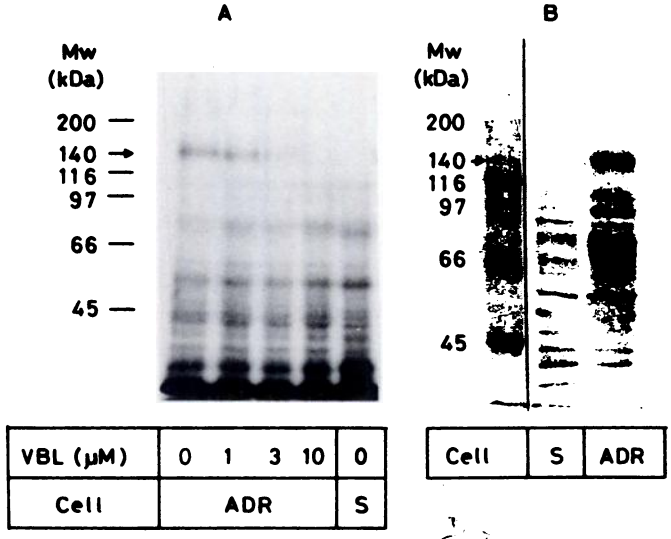

Fig. 3. Photoaffinity labeling with [^{125}I]NASV (A) and immunostaining with C219 antibody (B) of membrane vesicles from P388/S and P388/ADR cells. *S* and *ADR*, P388/S and P388/ADR cells, respectively. Positions of molecular weight standards are indicated at the *left*. *VBL*, vinblastine.

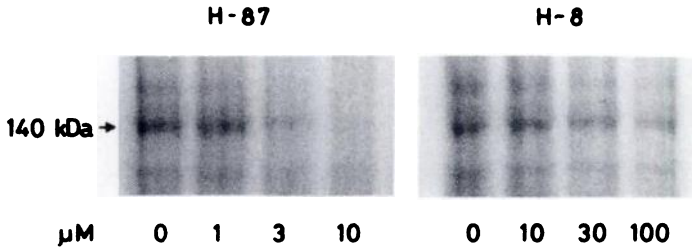

Fig. 4. Effects of H-8 and H-87 on [^{125}I]NASV labeling of the 140-kDa P-glycoprotein of membrane vesicles from P388/ADR cells.

indicated that the 140-kDa protein in the plasma membrane from P388/ADR cells could be purified by affinity chromatography, using W-66, and proved to be a P-glycoprotein (29). Consequently, it is confirmative that isoquinoline derivatives interact competitively with vinblastine on P-glycoprotein of P388/ADR cells and inhibit the efflux of vinblastine from the cells. The inhibition of P-glycoprotein as a drug efflux pump

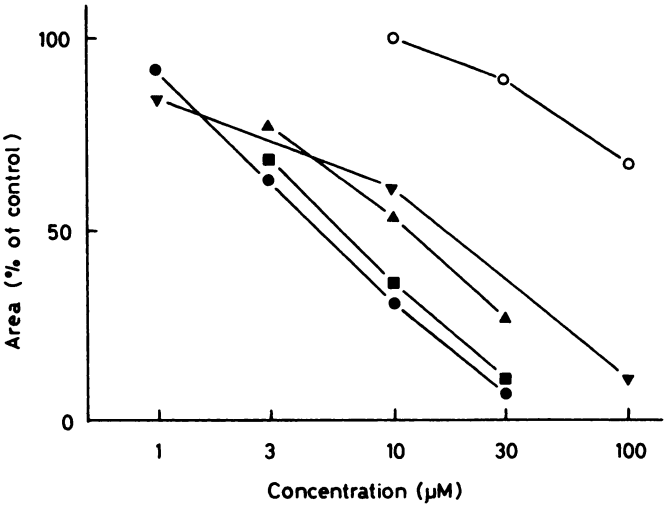

Fig. 5. Dose-dependent inhibition of [^{125}I]NASV labeling of 140-kDa P-glycoprotein in P388/ADR membrane vesicles by H-8, H-85, H-86, H-87, and W-66. *Abscissa*, concentration of compounds. *Ordinate*, percentage of the integral value obtained by the densitometric analysis of autoradiograms. Each *point* represents the mean of two experiments. ○, H-8; ▲, H-85; ■, H-86; ●, H-87; ▼, W-66.

seems to provide the reversal of resistance in P388/ADR cells *in vitro*.

Considering the structures of the compounds that reversed vinblastine resistance of P388/ADR cells, we see that the compounds have a chlorinated benzene ring at the end of the side chain of isoquinolinesulfonamide. The ring seems to introduce high hydrophobicity. Both isoquinoline ring and terminal ring structures may be necessary to interact with the hydrophobic region of P-glycoprotein by van der Waals' forces. H-7, H-8, and H-9, which have only an isoquinoline ring as the hydrophobic moiety, barely interacted with P-glycoprotein and did not reverse the drug resistance. H-87 has three sites that may be able to form hydrogen bonds, the nitrogen atom in the isoquinoline ring, the terminal amino group, and the sulfonamide moiety. The introduction of a formyl group at the terminal amino group (H-85), to reduce the basicity, reduced the binding activity for P-glycoprotein and the overcoming of drug resistance. This indicates that these secondary amino groups also act in binding to P-glycoprotein. W-66, which has an aminoethyl group at the nitro atom of the sulfonamide group of H-86, showed lower activity on drug resistance than did the parent compound, but activity was high enough for affinity purification of P-glycoprotein (29).

P-Glycoprotein has been shown to be a phosphorylated protein (31). Recent studies showed that P-glycoprotein was phosphorylated by stimulation of protein kinase A and protein kinase C and suggested that the function of the protein was regulated by phosphorylation (32, 33). In this study, isoquinolinesulfonamide derivatives, which have different magnitudes of inhibitory activities on protein kinase A and protein kinase C, reversed the drug resistance under unstimulated conditions, and there was no correlation between the inhibitory activities on these protein kinases and the effects on drug resistance of these compounds (Table 2). This indicated that, regarding the overcoming effects of isoquinolinesulfonamide derivatives on drug resistance, the inhibition of binding of antitumor agents to the P-glycoprotein is more important than the inhibition of

phosphorylation of the protein by protein kinases, under physiological conditions.

In conclusion, isoquinolinesulfonamide compounds that have terminal hydrophobic ring structures and amino groups in the internal chain reverse multidrug resistance by inhibition of drug binding to P-glycoprotein, without involvement of their inhibitory activities on protein kinases.

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