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Reversal of multidrug resistance by new dihydropyridines with lower calcium antagonistic activity

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Summary. BS compounds, a series of new dihydropyridines, successfully overcame multidrug resistance in P388/ADR cells in vitro. These agents synergistically potentiated the cytotoxicity of Adriamycin to P388/ADR cells at a concentration of 1–2 μ*M*, whereas they showed hardly any synergistic effect in the parental cell line (P388/S) at the same concentration. They inhibited the active drug efflux in P388/ADR cells as well as the binding of [G-^{3}H]-vinblastine to membrane vesicles from P388/ADR, which was increased in resistant P388 cells as compared with parental cells. Besides, unlike the activity of clinically used calcium antagonists, the calcium antagonistic activity associated with BS compounds was very weak: their arterial relaxation activity was <21% of that of verapamil. These data suggest that BS compounds specifically overcome multidrug resistance without the serious hypotensive side effects that accompany the use of verapamil or other calcium antagonists.

Introduction

Multidrug resistance (MDR) in tumor cells is a major problem in cancer chemotherapy. Inaba et al. [8] have revealed that the active efflux of anticancer agents such as anthracyclines or vinca alkaloids is enhanced in some MDR cells. Agents normalizing the enhanced active efflux are expected to affect the MDR tumor cells specifically. In fact, several calcium antagonists such as verapamil, niludipine, and diltiazem have been reported to be MDR blockers. They increased the antitumor drug concentration in MDR cells and consequently restored the drug sensitivity of those cells [16–19].

Recently, verapamil attracted much attention as a chemosensitizer. Several combination therapies using verapamil plus anthracyclines or vinca alkaloids have been tried [1, 3, 12], but verapamil causes serious cardiovascular side effects due to its calcium antagonistic activity [1, 13]. Therefore, an MDR blocker with fewer side effects was needed. Nakagawa et al. [11] synthesized *N*-solanesyl-*N,N'*-bis(3,4-dimethoxybenzyl)-ethylenediamine, a verapamil derivative, in response to this demand. We sought a potent chemosensitizer with lower calcium antagonistic activity among the dihydropyridine derivatives and obtained BS compounds. This paper describes the restorative effects of BS compounds in the MDR phenotype in vitro and their low calcium antagonistic activities.

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Materials and methods

BS compounds and other reagents. BS-300, -303, -304, -309, and niludipine were synthesized according to the method of Hatzsch [2]. The structures of BS compounds are shown in Fig. 1. The other chemicals used in this work included: doxorubicin hydrochloride (Adriamycin, ADR), vinblastine sulfate (VBL), and verapamil hydrochloride from Sigma Chemical Co. (Missouri USA); vincristine sulfate (VCR) from Sionogi Co. (Osaka, Japan); and [G-^{3}H]-vinblastine sulfate ([^{3}H]-VBL, 11.5 Ci/mmol) and [G-^{3}H]-vincristine sulfate ([^{3}H]-VCR, 6.1 Ci/mmol) from Amersham Japan Co. (Tokyo, Japan).

P388 cells. ADR-resistant murine leukemia cells (P388/ADR), VCR-resistant cells (P388/VCR), and their parental (P388/S) cells were kindly provided by Dr. T. Tsuruo of the Japanese Foundation for Cancer Research. Both P388/ADR and P388/VCR cells are resistant to various anticancer agents, including anthracycline antibiotics and vinca alkaloids [16]. These three cell lines were maintained in DBA/2 mice and subcultured for at least 1 week before each experiment [17].

Determination of ADR cytotoxicity. P388/S and P388/ADR cells were cultured in graded concentrations of ADR in the presence or absence of test samples at 37° C. After 65 h incubation, the concentration of ADR inhibiting the cell growth by 50% (IC_{50}) was determined by counting the cell number with a Coulter Counter.

Measurement of drug accumulation. P388/ADR cells were suspended in buffer A [Hanks' balanced salt solution (HBSS) containing 0.35 mg/ml $NaHCO_3$ and 25 m*M* HEPES (pH 7.0)] at a density of 1×10^6 cells/ml. The cell suspension (1 ml) was added to 5 μl test sample dissolved in dimethylsulfoxide and 100 μl 100 μg/ml ADR. After incubation at 37° C for 60 min, the cells were washed twice with ice-cold PBS. The washed cells were resuspended in 1 ml PBS and sonicated for 30 s. An aliquot of 0.8 ml lysate was added to 0.2 ml 2.5% bovine serum albumin and

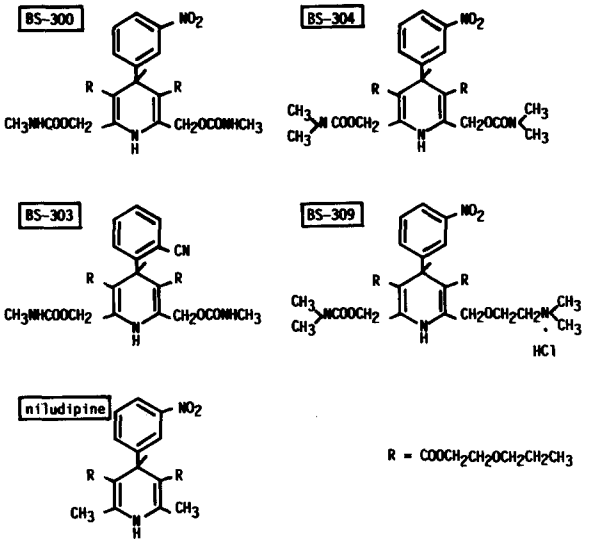

Fig. 1. Chemical structure of BS compounds and niludipine

2 ml 40% (w/v) trichloroacetic acid. Each tube was centrifuged at 1,800 *g* for 10 min, and the ADR concentration in the supernatant was determined with a fluorescence photometer (Shimadzu RF-540: excitation, 500 nm; emission, 590 nm). The net amount of ADR accumulated in the cells was calculated by subtracting the amount of ADR nonspecifically adhering to the cell surface from the gross amount. The nonspecific adhesion of ADR was determined by measuring the fluorescence intensity of a lysate of cells centrifuged immediately after the addition of ADR.

Measurement of active efflux. P388/ADR cells suspended in buffer B [glucose-free HBSS containing 0.35 mg/ml $NaHCO_3$, 25 m*M* HEPES, 10 m*M* NaN_3 (pH 7.0)] at 1×10^6 cells/ml were incubated with 20 μ*M* [^{3}H]-VCR at 37° C for 1 h. After the [^{3}H]-VCR in the medium was removed centrifugally (200 *g*, 10 min), the cells resuspended in prewarmed buffer A were exposed to a test sample at 37° C for 30 or 60 min. To stop the active efflux, the cells were centrifuged on a silicone cushion as described by Inaba et al. [9] and lysed with 0.5 *N* KOH. The radioactivity of the lysate was counted in a liquid scintillation counter (Packard 460-D) after neutralization with 5 *N* HCl.

Preparation of membrane vesicles and drug-binding studies. Membrane vesicles from drug-resistant cells were prepared according to the method of Cornwell et al. [5], except that cells were lysed using a Daunce homogenizer instead of nitrogen cavitation. The protein concentration of the vesicles was determined on 0.1% triton-solubilized samples using the BioRad protein assay.

Drug binding to membrane vesicles was measured as follows. Membrane vesicles (50 μg/assay) were incubated with 0.1 μ*M* [^{3}H]-VBL in the presence or absence of test samples in the assay buffer [0.01 *M* TRIS-HCl (pH 7.5), 0.125 *M* sucrose, and 5 m*M* $MgCl_2$] at 23° C for 20 min. The vesicles were then collected on glass-fiber filters that had first been wetted with 0.1% bovine serum albumin to minimize nonspecific absorption. The radioactivity of each filter was measured after it had been washed with the assay buffer. Nonspecific binding was determined by the addition of 100 μ*M* unlabeled VBL.

Measurement of calcium antagonistic activity. The calcium antagonistic activity of test samples was estimated by the relaxation effect on the isolated superior mesenteric artery of a rabbit, which had been stably contracted with 50 m*M* KCl [15]. The relaxation induced by 10^{-4} *M* papaverine was taken to be 100%, and the concentration of test samples that relaxed the contracted artery by 50% (ED_{50}) was determined.

Results

Potentiation of ADR cytotoxicity by BS compounds

P388/ADR cells used in the present work were 69-fold more resistant to ADR than P388/S cells after continuous exposure to ADR for 65 h (Table 1). BS compounds synergistically potentiated the cytotoxicity of ADR in P388/ADR. The effect of BS-303 equaled that of verapamil, and BS-300, -304, and -309 were approximately twice as effective as verapamil at 1 or 2 μM. Niludipine was less effective than BS compounds or verapamil. BS compounds, nildipine, and verapamil were hardly effective in potentiating the cytotoxicity of ADR to P388/S cells; at the concentrations examined and in the absence of ADR, they showed no cytotoxicity toward any P388 cell. BS compounds circumvented resistance to VCR in P388/VCR as well as P388/ADR cells (data not shown).

Effect of BS compounds on drug accumulation

To explore how BS compounds potentiate the cytotoxicity of ADR, the effects of the former on the accumulation of ADR in P388/ADR cells were tested. As Fig. 2 shows, when BS compounds were added to the cells with ADR, they increased the cellular ADR amount dose-dependently.

To determine the effect of BS compounds on active efflux directly, their effect on the retention of antitumor agents accumulated in P388/ADR cells was investigated using [^{3}H]-VCR instead of ADR (Fig. 3). When the cells that had been exposed to [^{3}H]-VCR were resuspended in

Table 1. Potentiating effect of BS compounds on the cytotoxicity of ADR to P388 cells

Compounds	Dose (μ*M*)	P388/S		P388/ADR	
		IC_{50} (n*M*)	DMF	IC_{50} (n*M*)	DMF
Control		13.4	1	931	1
BS 300	2	5.7	2.4	64	14.5
BS 303	2	6.2	2.2	103	9.0
BS 304	2	5.2	2.6	55	16.9
BS 309	1	6.7	2.0	69	13.5
Niludipine	2	6.7	2.0	155	6.0
Verapamil	1	8.1	1.7	188	5.0
	2	6.6	2.0	103	9.0

IC_{50} indicates the concentration of ADR required for 50% inhibition in cell growth. Dose-modifying factors (DMFs) were calculated in each cell as follows:

$$DMF = \frac{IC_{50}\,(\text{ADR alone})}{IC_{50}\,(\text{ADR} + \text{test compound})}$$

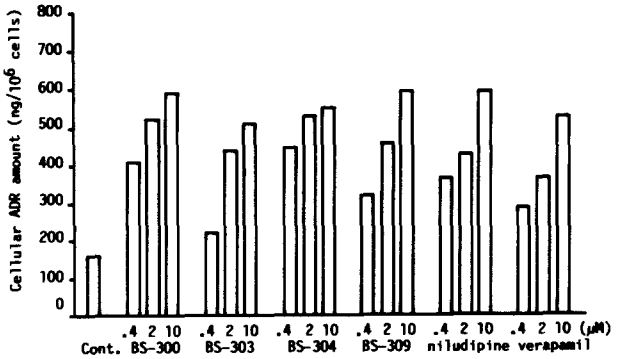

Fig. 2. Effect of BS compounds on drug accumulation in P388/ADR cells. Cells were incubated with 10 μg/ml ADR in the presence or absence of each test compound at 37° C for 1 h. Each *bar* represents the mean of two determinations

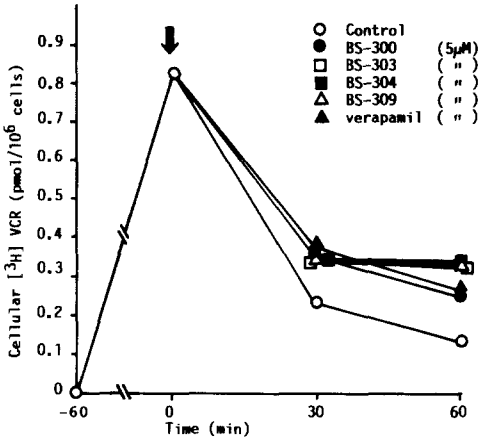

Fig. 3. Effect of BS compounds on the retention of [³H]-VCR in P388/ADr cells. The data are expressed as the mean of two determinations. (↓), initiation of active transport

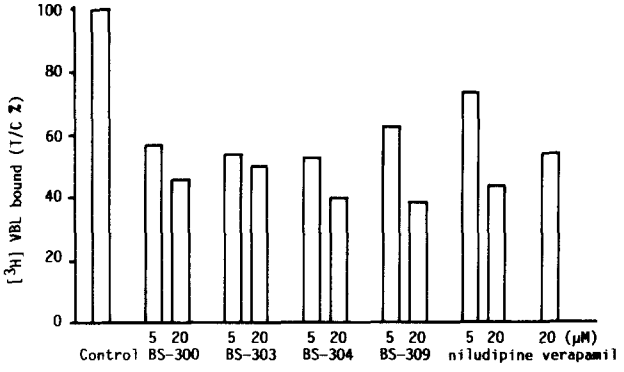

Fig. 4. Effect of BS compounds on drug binding to P388/ADR membrane vesicles. Data are expressed as the percentage of the specific binding of [³H]-VBL

[³H]-VCR-free buffer, a large part of the [³H]-VCR accumulated in the control cells was released to the medium. BS compounds inhibited this release and maintained the cellular [³H]-VCR at higher levels.

Inhibition of [³H]-VBL binding to membrane vesicles

The effect of the binding of [³H]-VBL to membrane vesicles from P388/ADR cells was examined. BS compounds, as well as verapamil and niludipine, inhibited the specific binding of [³H]-VBL to vesicles from the resistant cells by about 50% at 20 μ*M* (Fig. 4). Nifedipine, which is known to be less effective as an MDR blocker, was also tested; it hardly inhibited the binding at the same concentration (data not shown).

Table 2. Effect of BS compounds on the relaxation of isolated mesenteric arteries

Compounds	ED_{50} (μ*M*)	Ratio
BS-300	0.26 ±0.06	0.21
BS-303	5.1 ±0.2	0.011
BS-304	0.27 ±0.01	0.20
BS-309	0.37 ±0.18	0.15
Niludipine	0.00033 ±0.00018	167
Verapamil	0.055 ±0.005	1

ED_{50} indicates the concentration at which the compound produces a 50% relaxation of sustained contraction induced by 50 m*M* KCl. Each value represents the mean ± SE of triplicate trials.

$$\text{Ratio} = \frac{ED_{50} \text{ on verapamil}}{ED_{50} \text{ on test compounds}}$$

Effect of BS compounds on the relaxation of isolated arteries

The calcium antagonistic activities of BS compounds were examined using the method of KCl-induced contractions in isolated rabbit mesenteric arteries. The relaxation activities of BS-300, -304, and -309 were <21% of that of verapamil, and that of BS-303 amounted to only 1% of verapamil's activity at the same concentration. While, niludipine strongly relaxed the arteries, with an activity of 167 times that of verapamil (Table 2).

Discussion

Several calcium antagonists have previously been reported to potentiate sensitivity to antitumor vinca alkaloids and anthracycline antibiotics in experimental tumor cell lines with the MDR phenotype [19]. One of the promising chemosensitizing substances is verapamil, a diphenylalkylamine calcium antagonist. Tsuruo et al. [16, 17] reported that verapamil restored the cytotoxicity of VCR and ADR to multidrug-resistant P388 leukemia cells in vitro and in vivo. Similar results have been reported in other cell lines with acquired drug resistance [6, 7, 14, 16].

In spite of these affirmative observations in experimental models, however, the effect of verapamil in clinical trials has been controversial [3, 12]. The cause for the discrepancy between the effect observed in experiments and that observed in clinical trials involves many factors, e.g., tumor type, prior therapy, and administration protocol. Insufficient plasma concentrations of verapamil may be responsible for its limited effectiveness in clinical studies. However, the achievement of elevated plasma levels of this drug is limited by its cardiovascular effects, atrioventricular block and arterial hypotension, due to its calcium antagonistic activity [1, 13]. Therefore, a more effective chemosensitizer with lower calcium antagonistic activity has been desired.

Kessel and Wilberding [10] suggested that calcium-influx blockage was not involved in the reversal of resistance

in P388 leukemia cells, and Tsuruo et al. [18] have reported that the expression of circumvention activity has no relation to calcium antagonistic activity. BS compounds can successfully separate chemosensitizing activities from calcium antagonistic activities. That is, at an equimolar concentration, BS-300, -304, and -309 enhanced the antitumor activity of ADR on MDR cells more strongly than verapamil, whereas their relaxation effects on arteries previously contracted by KCl were weaker than that of verapamil. The potentiating ability of BS-303 is no greater than that of verapamil, although its calcium antagonistic action is markedly lower.

The structure-activity relationships of dihydropyridines as chemosensitizers are not clear. However, the present data surely suggest the possibilities of investigating a harmless chemosensitizer together with compounds whose structures mimic those of calcium antagonists. For instance, as the structures of BS compounds resemble that of niludipine, we were interested in the difference between effects produced by BS compounds vs niludipine. Niludipine has very strong arterial relaxation activity that is $\geqslant 200$ times as effective as BS compounds, whereas its chemosensitizing activity is lower than that of BS compounds. The mechanism of enhancement of the cytotoxicity of anthracyclines by BS compounds is likely involve the inhibition of a drug-efflux system in MDR cells, as in the case of verapamil.

Recently, binding of antitumor drugs to isolated membrane vesicles in MDR cells has been demonstrated. Cornwell et al. [4] have reported that [^{3}H]-VBL binding to membrane vesicles from multidrug-resistant KB cells was increased to 6 times as high as that of parental KB-cell vesicles. Such binding is inhibited by the addition of anthracyclines, vinca alkaloids, and some chemosensitizers [5]. We obtained a similar result using resistant P388 cells (manuscript to be submitted): BS compounds clearly inhibited [^{3}H]-VBL binding to P388/ADR vesicles. We do not yet know whether the [^{3}H]-VBL binding site of membrane vesicles from P388/ADR cells is the protein(s) that are associated with MDR or how BS compounds inhibit its binding. The present results suggest that BS compounds directly or indirectly affect specific moieties in MDR P388 cells; e.g., P-170 protein proved its existence in MDR cell lines, reversing the resistance.

In conclusion, BS compounds are more effective than verapamil in enhancing the antitumor activities of anthracyclines and vinca alkaloids in vitro. In terms of possible side effects in vivo, the hypotensive effect of BS compounds would be weaker than that of verapamil, because the calcium antagonistic activity of the former is markedly lower than that of te latter. We are now investigating the synergistic antitumor effects of BS compounds in mice bearing MDR tumors.

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