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Novel Mechanism of N-Solanesyl-N,N′-bis(3,4-dimethoxybenzyl)ethylenediamine in Potentiation of Antitumor Drug Action on Multidrug-resistant and Sensitive Chinese Hamster Cells

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The mechanism of the synthetic isoprenoid N-solanesyl-N,N′-bis(3,4-dimethoxybenzyl)ethylenediamine (SDB-ethylenediamine) in potentiating antitumor drug action against multidrug-resistant cells was comparatively studied with other potentiators such as verapamil and cepharanthine. SDB-ethylenediamine increased the accumulation of [^{3}H]daunorubicin (DNR) in Chinese hamster V79 (V79/S) and its multidrug-resistant mutant (V79/ADM) cells. Even after SDB-ethylenediamine was removed from the medium, its effect continued. But when verapamil was removed from the medium, its effect disappeared immediately. Unlike verapamil and cepharanthine, SDB-ethylenediamine did not greatly inhibit the efflux of [^{3}H]DNR from V79/ADM, the binding of [^{3}H]vinblastine to membrane vesicles of V79/ADM, or the binding of [^{3}H]azidopine to P-glycoprotein in the cytoplasmic membrane of V79/ADM. It did stimulate the influx of [^{3}H]DNR into the ATP-depleted cells of V79/S and V79/ADM. Thus, SDB-ethylenediamine uniquely potentiates antitumor drugs. The increased intracellular accumulation of antitumor drugs in the presence of SDB-ethylenediamine is due not only to the inhibition of active efflux but also to the stimulation of the influx of antitumor drugs.

Key words: Synthetic isoprenoid — SDB-ethylenediamine — Mechanism of potentiation — Multidrug resistance — Chinese hamster cells

In a previous paper,[1)] we reported that the synthetic isoprenoid, SDB-ethylenediamine,[2] is cytocidal preferentially to multidrug-resistant cells. It also potentiates the cytotoxic activity of almost all kinds of clinically useful antitumor drugs against multidrug-resistant and sensitive cells *in vitro*. Among the antitumor drugs tested, the bleomycin-group antibiotics were more strongly activated by SDB-ethylenediamine than other antitumor agents and the synergistic effect of the bleomycin-group antibiotics and SDB-ethylenediamine was described.[2)] Drugs which are associated with pleiotropic drug resistance were potentiated more strongly against multidrug-resistant V79 cells than against sensitive cells. The mechanism of potentiation by SDB-ethylenediamine can not be simple, because it increases the uptake of adriamycin[1)] but not peplomycin.[2)] We studied the mechanism of potentiation of the drugs involved in multidrug resistance by SDB-ethylenediamine, comparing it with other potentiators such as verapamil and cepharanthine, and found that the activity of SDB-ethylenediamine in overcoming multidrug resistance is unique.

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[2] Abbreviations used in this paper: SDB-ethylenediamine, N-solanesyl - N,N′-bis(3,4 - dimethoxybenzyl)ethylenediamine; DNR, daunorubicin; VLB, vinblastine; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; MDR, multidrug resistance.

MATERIALS AND METHODS

Chemicals SDB-ethylenediamine (malate salt) was kindly supplied by Nisshin Flour Milling Co., Ltd., Tokyo. It was dissolved in ethanol and diluted with phosphate-buffered saline (PBS) or H_2O, and the same amount of ethanol was added to the control culture or the reaction mixture. VLB and rotenone were products of Sigma Chemical Co., St. Louis. Mo. Verapamil was obtained from Wako Pure Chemical Industries, Osaka and cepharanthine was from Kaken Shoyaku Co. Ltd., Tokyo. [^{3}H]VLB (23 Ci/mmol), [^{3}H]azidopine (45.2 Ci/mmol) and [^{32}P]dCTP (approximately 3,000 Ci/mmol) were purchased from Amersham Japan, and [^{3}H]DNR (3.1 Ci/mmol) was from New England Nuclear, Boston, Mass.

Cells Chinese hamster V79 (V79/S) and its multidrug-resistant mutant cells (V79/ADM), established in our laboratory, were grown in Eagle's minimum essential medium (EMEM) supplemented with 10% calf seum in a humidified atmosphere of 5% CO_2 at 37°C. Both cell lines were tumorigenic in nude mice when 3×10^7 cells were implanted subcutaneously.

Northern hybridization The total RNA of the V79 cells was isolated by the guanidine thiocyanate/CsCl method and the poly A fraction was obtained from total RNA by oligo dT-cellulose column chromatography. The probe was prepared by labeling a fragment of the cDNA (926

kb)[3] of the human MDR1 gene with [^{32}P]dCTP by a random hexamer method[4] using a multiprime DNA labeling kit 1601Y (Amersham). Northern hybridization was carried out as described by Roninson *et al.*[3] except that it was done at 65°C in 6×SSC/5×Denhardt's solution/1% SDS containing denatured salmon sperm DNA at 100 μg/ml.

Accumulation of [^{3}H]DNR Accumulation of [^{3}H]DNR in V79 cells, which had been diluted 10-fold with cold DNR, was determined as described previously.[1]

Efflux of [^{3}H]DNR To obtain significant amounts of [^{3}H]DNR in V79/ADM cells for determination of [^{3}H]DNR efflux, the cells (5×10^5 cells/well) were preincubated with SDB-ethylenediamine (10 μg/ml) or verapamil (3 μg/ml) for 2 h, then [^{3}H]DNR (0.5 μCi/ml) was added to the culture. After 1 h of incubation with [^{3}H]DNR, the cells were washed twice with cold PBS and incubated in prewarmed serum-free EMEM in the presence or absence of SDB-ethylenediamine or verapamil at the same concentrations as used in the preincubation. After the indicated incubation period, the cells were solubilized with Protosol (New England Nuclear) and the remaining radioactivity in the cells was measured in Scintisol EX-H (Dojin, Kumamoto).

Influx of [^{3}H]DNR into ATP-depleted cells Cells were preincubated for 2 h with SDB-ethylenediamine (10 μg/ml) or verapamil (3 μg/ml) and ATP-depleted cells were prepared by the method of Hammond *et al.*,[5] by incubation with rotenone and 2-deoxyglucose. The ATP-depleted cells (1×10^6/0.5 ml in PBS) were incubated with [^{3}H]DNR (0.5 μCi/ml) at 37°C for 10 min in the presence or absence of SDB-ethylenediamine or verapamil at the same concentrations used in the preincubation. The radioactivity in the cells was determined as described above.

Preparation of membrane vesicles Membrane vesicles were prepared from V79/S and V79/ADM cells by the method of Cornwell *et al.*[6] using nitrogen cavitation. Na^+,K^+-dependent ATPase activity determined by the method of Schoot *et al.*[19] was 4.4- and 4.6-fold higher in the membrane vesicles than in the cell lysates of V79/S and V79/ADM, respectively. Protein concentrations of the vesicles were determined by the method of Bradford[7] on 0.1% Triton X100-solubilized samples. Bovine serum albumin was used as a standard.

Binding of [^{3}H]VLB to membrane vesicles The binding of [^{3}H]VLB to membrane vesicles was assayed by the method of Cornwell *et al.*[6] Membrane vesicles (40 μg protein) were incubated with 0.25 μCi of [^{3}H]VLB in the presence or absence of SDB-ethylenediamine or verapamil at 25°C for the indicated periods, in a final volume of 100 μl of binding buffer (0.01 *M* Tris-HCl, pH 7.5, 0.125 *M* sucrose, and 5 m*M* $MgCl_2$). The reaction was stopped by adding 4 ml of the ice-cold binding buffer and the vesicles were collected on glass-fiber filters (Whatman GF/C) which had been wetted in 3% bovine serum albumin. The filters were washed once with 4 ml of the ice-cold binding buffer and the radioactivities on the filters were measured in a scintillation counter. Nonspecific binding was determined by the addition of 100 μ*M* unlabeled VLB.

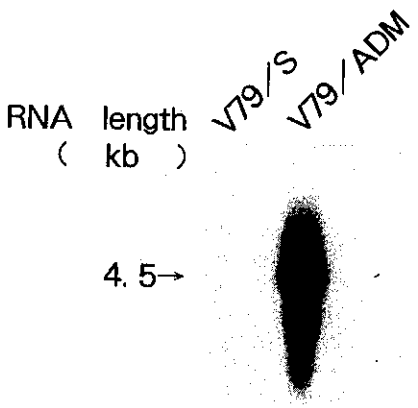

Fig. 1. Northern blot hybridization of V79/S and V79/ADM with an MDR1 probe. Twenty μg/ml of poly A-RNA was hybridized with a ^{32}P-labeled probe.

Photoaffinity labeling of membrane vesicles with [^{3}H]-azidopine Membrane vesicles (50 μg protein) were incubated with 0.1 μCi [^{3}H]azidopine for 15 min at room temperature with or without various amounts of SDB-ethylenediamine or cepharanthine in a final volume of 50 μl as described by Safa *et al.*[8] After irradiation of the incubation mixtures at 366 nm for 20 min at room temperature, they were solubilized in SDS-containing buffer and analyzed by 4–20% SDS-PAGE containing 4.5 *M* urea. The gel was fluorographed.

RESULTS

Northern hybridization of RNA from V79 cells with MDR1 cDNA Expression of the MDR gene in V79 cells which codes P-glycoprotein was examined by Northern hybridization of poly(A)-containing RNA from cells with an MDR1 probe labeled with ^{32}P. V79/ADM cells expressed a large amount of the 4.5 kb mRNA, which was barely detectable in the parent V79 cells (Fig. 1). The degree of MDR1 mRNA expression was roughly estimated by comparing the density of serially diluted samples of V79/ADM with that of V79/S. The expression of V79/ADM was approximately 100-fold higher

than that of V79/S. This indicates that V79/ADM is a typical multidrug-resistant cell line.

Effect of SDB-ethylenediamine on accumulation of [^{3}H]DNR in V79/S and V79/ADM cells (1) Preincubation and washing: SDB-ethylenediamine at a concentration of 10 μg/ml increased the amounts of [^{3}H]DNR incorporated during 1-h incubation into V79/S and V79/ADM 1.4- and 3.3-fold, respectively. When cells were preincubated with SDB-ethylenediamine for 4 h, the [^{3}H]DNR in both cell lines was increased much more (1.7- and 4.3-fold), and the effect of SDB-ethylenediamine did not diminish after its removal from the medium before incubation with [^{3}H]DNR (Fig. 2).

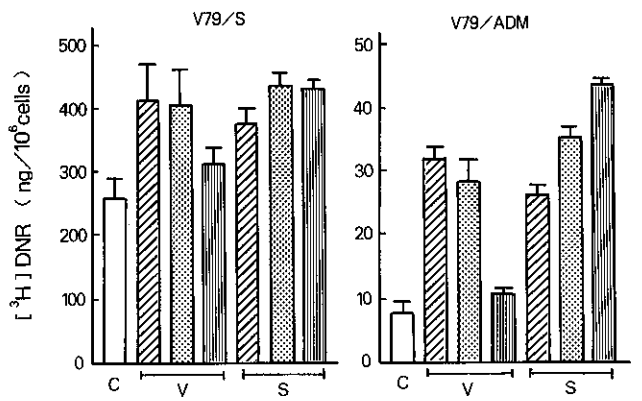

Fig. 2. Effects of SDB-ethylenediamine and verapamil on the accumulation of [^{3}H]DNR by V79/S and V79/ADM. Cells treated with the drugs under various conditions were incubated with [^{3}H]DNR (0.5 μCi/ml) for 1 h. C, control cells; V, verapamil at 3 μg/ml; S, SDB-ethylenediamine at 10 μg/ml; ▨, V or S was added simultaneously with [^{3}H]DNR (no preincubation); ▩, 4 h preincubation with V or S; ▥, 4 h preincubation with V or S followed by washing. Bar, standard deviation (SD).

Verapamil, a well known potentiator,[9] stimulated the uptake of [^{3}H]DNR by V79/S and V79/ADM, 1.6- and 4.0-fold, respectively, at 3 μg/ml, but had no preincubation effect and its stimulation disappeared when it was removed from the medium. Thus, the potentiating mechanisms of antitumor drugs by SDB-ethylenediamine and verapamil are not the same.

(2) Time course of [^{3}H]DNR accumulation in V79/ADM cells preincubated with SDB-ethylenediamine: As shown in Fig. 3A, [^{3}H]DNR in the control cells reached a plateau after 60 min of incubation. It increased quickly in the first 30 min and gradually increased until 120 min when the cells were preincubated with SDB-ethylenediamine at 10 μg/ml for 2 h and the potentiator was kept in the medium during incubation with [^{3}H]DNR. Three times more [^{3}H]DNR was accumulated than that in the control cells. In the cells which had been preincubated with SDB-ethylenediamine at 10 μg/ml for 2 h and transferred to SDB-ethylenediamine-free medium before incubation with [^{3}H]DNR, the amount of incorporated [^{3}H]DNR also increased quickly up to 45 min as in the SDB-ethylenediamine-containing medium. But then it gradually decreased (Fig. 3B), indicating that the effect of SDB-ethylenediamine lasts at least 2 h after its removal from the medium, but is reversible.

Effects of SDB-ethylenediamine and verapamil on efflux of [^{3}H]DNR from V79/ADM cells Because the uptake of [^{3}H]DNR by V79/ADM is very low as described above, the cells were first preincubated for 2 h with SDB-ethylenediamine or verapamil before incubation with [^{3}H]DNR for 1 h to get enough radioactivity in the cells, then they were transferred to [^{3}H]DNR-free medium for the efflux experiment. As shown in Fig. 4, in verapamil-preincubated cells, about 90% of the DNR was lost from the cells within 30 min of incubation in the absence of verapamil (line A). But adding verapamil significantly inhibited the efflux of the drug (lane B). On

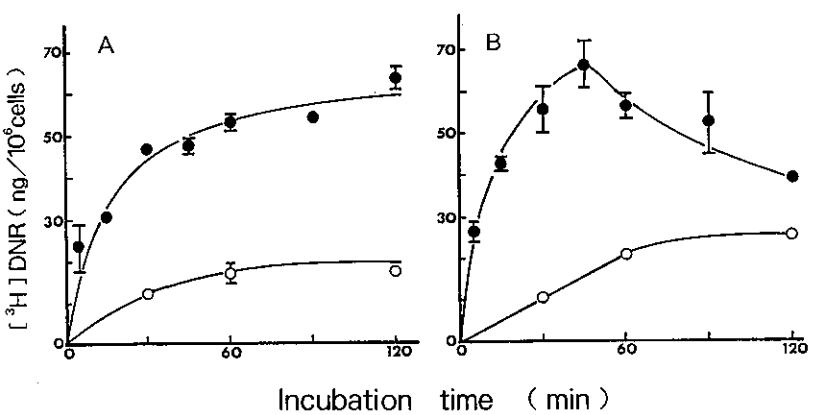

Fig. 3. Time dependency of accumulation of [^{3}H]DNR by V79/ADM after treatment with SDB-ethylenediamine. V79/ADM cells were preincubated with (●) or without (○) 10 μg/ml of SDB-ethylenediamine for 2 h and then incubated with [^{3}H]DNR (0.5 μCi/ml) immediately (A) or after changing the medium to an SDB-ethylenediamine-free one (B). Bar, SD. The SDs of symbols without SD bars are smaller than the height of the symbol.

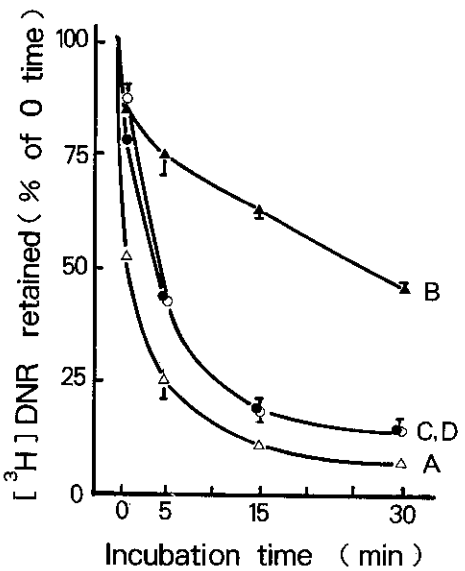

Fig. 4. Effects of SDB-ethylenediamine and verapamil on efflux of [^{3}H]DNR from V79/ADM cells. The experimental details are described in the text. Lines C (○) and D (●), preincubated with SDB-ethylenediamine before labeling with [^{3}H]DNR, then incubated with (●) or without (○) SDB-ethylenediamine. Lines A (△) and B (▲), preincubated with verapamil, then incubated with (▲) or without (△) verapamil. Bar, SD. The SDs of symbols without SD bars are smaller than the height of the symbol.

the contrary, the rate of efflux of DNR from SDB-ethylenediamine-preincubated cells was lower than that of line A, but higher than that of line B, and adding SDB-ethylenediamine to the medium of the efflux experiment did not change it (lines C and D). Apparently, SDB-ethylenediamine's inhibition of the drug efflux is very weak compared to that of verapamil.

Binding of [^{3}H]VLB to membrane vesicles and its inhibition by verapamil and SDB-ethylenediamine To determine the specific binding of [^{3}H]VLB to membrane vesicles, the nonspecific binding obtained during incubation in the presence of 100 μM nonlabeled VLB was subtracted from the binding without the VLB. Significantly more [^{3}H]VLB than V79/S was bound to the membrane vesicles of V79/ADM, as expected from Fig. 1 (data not shown). The effects of verapamil and SDB-ethylenediamine during 10 min incubation were studied using the membrane vesicles of V79/ADM. Specific binding of [^{3}H]VLB to membrane vesicles of V79/ADM was inhibited dose-dependently by verapamil (IC_{50} was 4.5 μM), in accordance with the report by Cornwell *et al.*[6] But the inhibition by SDB-ethylenediamine was too weak to determine the IC_{50}; the inhibition with 80 μM (81 μg/ml) SDB-ethylenediamine was approximately 30% (Fig. 5).

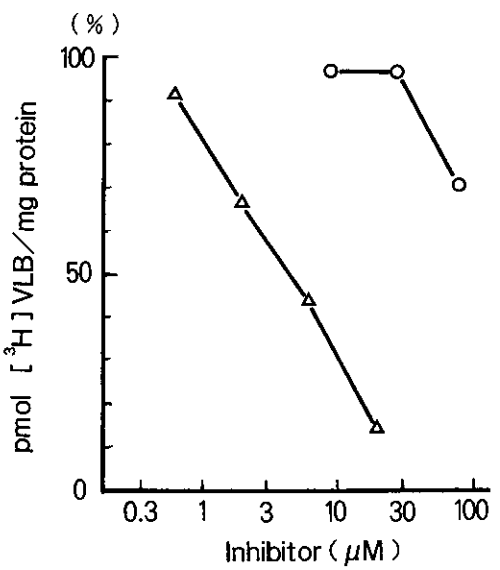

Fig. 5. Effects of verapamil and SDB-ethylenediamine on binding of [^{3}H]VLB to membrane vesicles from V79/ADM cells. Incubation was done for 10 min. ○, SDB-ethylenediamine; △, verapamil. The variation of the duplicate assay was less than 10% and the mean values were plotted.

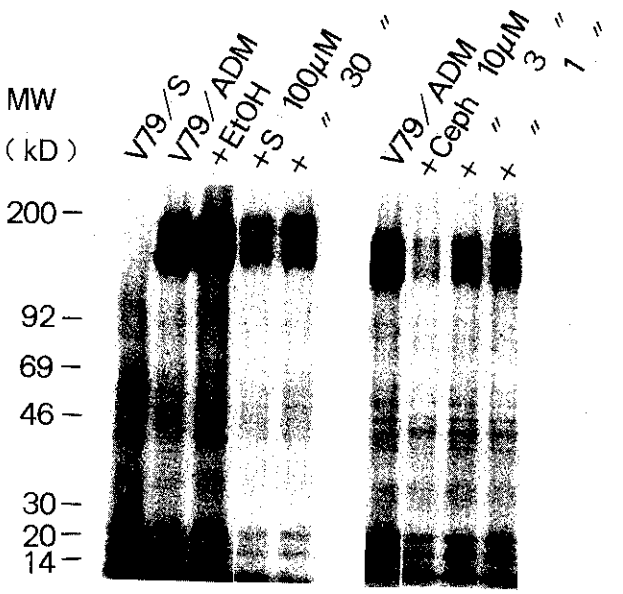

Fig. 6. Effects of SDB-ethylenediamine and cepharanthine on photoaffinity labeling of P-glycoprotein of V79 cells with [^{3}H]azidopine. Because SDB-ethylenediamine and cepharanthine were dissolved in ethanol (EtOH), the same amount (final 8%) of EtOH was added to a reaction mixture as a control (+EtOH). +S, SDB-ethyelenediamine was added, +Cepha, cepharanthine was added.

Effect of SDB-ethylenediamine on binding of [^{3}H]-azidopine to P-glycoprotein in V79/ADM cell membrane [^{3}H]Azidopine, a photoactive dihydropyridine derivative, binds specifically to P-glycoprotein in multidrug-resistant cells.[8] Thus, plasma membranes from V79/S and V79/ADM were photoaffinity-labeled with [^{3}H]-azidopine and analyzed by fluorography after SDS-PAGE. Like Safa *et al.*,[8] we detected a radiolabeled doublet (150–180 kDa) in membranes from multidrug-resistant (V79/ADM) cells but not from sensitive (V79/S) cells (Fig. 6), indicating that these bands are P-glycoproteins bound covalently with [^{3}H]azidopine. There were several distinct but weakly labeled bands at similar levels in both cell lines. Cepharanthine, which reverses multidrug resistance by inhibiting active drug efflux from resistant cells,[10] inhibited the binding of [^{3}H]azidopine to P-glycoprotein of V79/ADM clearly over 1 μM and almost completely at 10 μM. On the other hand, inhibition by SDB-ethylenediamine was weak and even at 100 μM (101 μg/ml) it was only partial.

Effect of influx of [^{3}H]DNR into V79/S and V79/ADM cells Despite the enhanced accumulation of [^{3}H]DNR in the cells, SDB-ethylenediamine, unlike verapamil or cepharanthine, did not greatly inhibit the active efflux of [^{3}H]DNR or binding of [^{3}H]azidopine to P-glycoprotein. So the effect of SDB-ethylenediamine on the influx of [^{3}H]DNR into the cells was determined using ATP-depleted cells. As shown in Fig. 7, during 10 min incubation at 37°C, [^{3}H]DNR was incorporated into the control cells of V79/ADM at a level similar to that in the case of V79/S. This was quite different from the level obtained in intact V79/ADM cells (Fig. 2), indicating that the active efflux was almost completely eliminated from cells by rotenone and 2-deoxyglucose treatment.

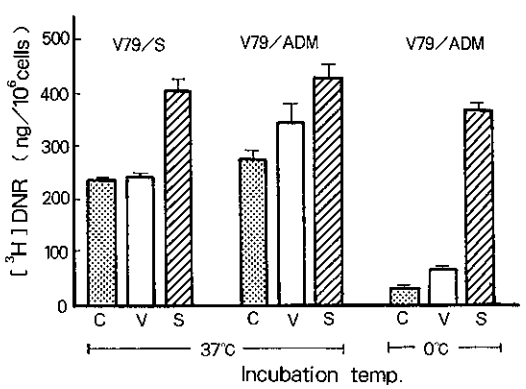

Fig. 7. Effects of SDB-ethylenediamine and verapamil on influx of [^{3}H]DNR into V79/S and V79/ADM. ATP-depleted cells were incubated with [^{3}H]DNR for 10 min in the presence of SDB-ethylenediamine (S, 10 μg/ml) or verapamil (V, 3 μg/ml) at 37°C or 0°C. C, control.

Verapamil at 3 μg/ml did not affect the intracellular level of [^{3}H]DNR in V79/S and slightly increased it in V79/ADM cells. In contrast, SDB-ethylenediamine stimulated the uptake of [^{3}H]DNR into V79/S and V79/ADM cells 1.7- and 1.6-fold, respectively. [^{3}H]DNR uptake was greatly (9.8-fold) stimulated when ATP-depleted V79/ADM cells were incubated with SDB-ethylenediamine at 0°C, although such great stimulation did not occur with verapamil. These results indicate that SDB-ethylenediamine increases the intracellular level of [^{3}H]DNR mainly by stimulating simple influx of the drug and partly by inhibiting active efflux of the drug, while verapamil and cepharanthine increases it only by inhibiting the active efflux.

DISCUSSION

Several compounds, such as verapamil,[9] cepharanthine,[10,11] dipyridamole,[12] cyclosporin A,[13] quinacrine,[14] AHC-52,[15] and other dihydropyridine analogs,[16,17] have been reported to potentiate antitumor drugs and to reverse multidrug resistance *in vitro* and *in vivo*. Apparently these substances block the active efflux of antitumor drugs from the cells by competitively inhibiting the binding of antitumor drugs to the P-glycoprotein which appears in the cytoplasmic membranes of multidrug-resistant cells. Thus, the antitumor drugs that can be potentiated by these agents are only those involved in typical multidrug resistance, such as anthracyclines, vinca alkaloids, actinomycin D, etoposide, etc.

SDB-ethylenediamine was first reported by Kuwano's group[18] to reverse the resistance of doxorubicin and vincristine in multidrug-resistant cells. However, we found that the agent is unique, having a direct cytotoxic activity preferentially against multidrug-resistant tumor cells. And it potentiates almost all kinds of clinically useful antitumor drugs, not only those involved in multidrug resistance but also others, such as peplomycin, mitomycin C, methotrexate, 5-fluorouracil, and cytosine arabinoside.[1] SDB-ethylenediamine may have multiple potentiation mechanisms, since drug uptake was stimulated in the case of anthracyclines and vinca alkaloids but not peplomycin or 5-fluorouracil.[1] We further studied the precise mechanism of SDB-ethylenediamine in stimulating the uptake of anthracyclines and vinca alkaloids, compared with those of other typical potentiators such as verapamil and cepharanthine. In the experiments on uptake and efflux of [^{3}H]DNR with intact V79/S and V79/ADM cells, we used SDB-ethylenediamine at 10 μg/ml. Although the drug at 10 μg/ml strongly inhibited the colony formation of both cell lines after continuous exposure for 7–8 days,[1] it did not affect the viability of either of the cell lines during a short incubation period of 1–4 h, as measured by the trypan

blue dye exclusion method. We found several differences between SDB-ethylenediamine and other potentiators as follows. 1. SDB-ethylenediamine stimulated DNR accumulation even after its removal from the medium, but the verapamil stimulation disappeared immediately after it was removed (Fig. 2). 2. Preincubation of cells with SDB-ethylenediamine effected greater accumulation of DNR, but preincubation with verapamil did not (Figs. 2 and 3). 3. The active efflux of [^{3}H]DNR (Fig. 4), the binding of [^{3}H]VLB to membrane vesicles from V79/ADM (Fig. 5) in which P-glycoprotein is abundant (Fig. 1), and the binding of [^{3}H]azidopine to P-glycoprotein (Fig. 6) were weakly inhibited by SDB-ethylenediamine compared with verapamil and cepharanthine. 4. The influx of [^{3}H]DNR in ATP-depleted cells was greatly enhanced by SDB-ethylenediamine in both drug-sensitive and -resistant cells, but the effect of verapamil on this system was negligible (Fig. 7). All this indicates that SDB-ethylenediamine is not similar to the typical potentiators, in spite of its similarity in stimulating the accumulation of anthracyclines and vinca alkaloids in tumor cells.

One site of action of SDB-ethylenediamine may be P-glycoprotein. However, its affinity is not as strong as that of verapamil or cepharanthine. The great increase in the influx of [^{3}H]DNR into the ATP-depleted cells indicates that SDB-ethylenediamine damages the cytoplasmic membrane somehow to facilitate the entry of some drugs into the cells, and this membrane injury may be the cause of the direct cytotoxicity of SDB-ethylenediamine. Further studies are needed to determine the target molecules of the agent. We found that the agent greatly potentiates bleomycin-group antibiotics without stimulating the cellular uptake of the drugs, indicating that there must be another mechanism for potentiating bleomycin-group antibiotics. The details have been reported elsewhere.[2)]

The V79/ADM cell line we employed in the present study is an extremely highly MDR mutant and its MDR could not be overcome completely by verapamil or SDB-ethylenediamine *in vitro*. However, it should be noted that more MDR cells would be killed by the combination of antitumor drugs and a higher concentration of SDB-ethylenediamine, because SDB-ethylenediamine itself exerts preferential cytotoxicity against MDR cells in addition to the effect of increased accumulation of the antitumor drugs in MDR cells. Clearly, SDB-ethylenediamine is effective in several experimental animal tumor systems in combination with some other antitumor drugs. It is hoped that it will be of clinical use.

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