Jpn. J. Cancer Res. 81, 298–303, March 1990

Cytocidal Activity of a Synthetic Isoprenoid, N-Solanesyl-N,N′-bis(3,4-dimethoxybenzyl)ethylenediamine, and Its Potentiation of Antitumor Drugs against Multidrug-resistant and Sensitive Cells *in vitro*

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A synthetic isoprenoid, N-solanesyl-N,N′-bis(3,4-dimethoxybenzyl)ethylenediamine (SDB-ethylenediamine), inhibited the colony formation of multidrug-resistant mutant cell lines derived from Chinese hamster V79 (V79/ADM) and human hepatoma PLC/PRF/5 (PLC/COL) cells to a greater extent than that of the parental cells. When combined with other clinically useful antitumor agents, it potentiated the cytotoxic activity of almost all kinds of drugs tested including adriamycin (ADM), actinomycin D, vincristine, cytosine arabinoside, and 5-fluorouracil (5-FU), and the potentiation ratios were higher against V79/ADM cells than against V79/S cells. Among the antitumor agents tested, the activities of bleomycin-group antibiotics were more strongly enhanced by SDB-ethylenediamine and the potentiation was higher in the parental cells than in V79/ADM cells. SDB-ethylenediamine enhanced the uptake of ADM and daunorubicin into V79/ADM and its parental cells, but it did not increase the uptake of 5-FU or peplomycin, indicating that different mechanisms operate for potentiation in the cases of the latter drugs, i.e., not simply an increase of intracellular drug uptake. Two fragments of SDB-ethylenediamine, solanesol (polyprenoid moiety) and the diamine component (verapamil-like moiety), showed neither cytotoxic activity nor potentiator activity, even if they were mixed together, indicating that the steric conformation of intact SDB-ethylenediamine molecule is important for these two activities.

Key words: Synthetic isoprenoid — SDB-ethylenediamine — Cytotoxicity — Potentiation of antitumor drug — Multidrug resistance

Studies on overcoming antitumor drug resistance by potentiating antitumor drugs with non-antitumor agents have been done in many laboratories and several chemicals such as verapamil,[1] cepharanthine,[2] and dipyridamole[3] were reported to reverse multidrug resistance. Several kinds of synthetic isoprenoids were tested for potentiation of antitumor drugs by Kuwano's group,[4,5] and a few compounds, including SDB-ethylenediamine, were found to be effective with DNR, VLB, and ACT-D against multidrug-resistant KB cells *in vitro*,[6] and were therapeutically effective in combination with VCR against P388/VCR *in vivo*.[7] We found that SDB-ethylenediamine possessed greater cytocidal activity against multidrug-resistant cells than against sensitive cells, and also that it potentiated almost all kinds of clinically useful antitumor drugs *in vitro*.

Abbreviations used in this paper: SDB-ethylenediamine, N-solanesyl-N,N′-bis(3,4-dimethoxybenzyl)ethylenediamine; ADM, adriamycin; DNR, daunorubicin; ACR, aclarubicin; ACT-D, actinomycin D; 5-FU, 5-fluorouracil; Ara-C, 1-β-D-arabinofuranosylcytosine; CDDP, cisplatin; MTX, methotrexate; MMC, mitomycin C; ACNU, nimustine; VCR, vincristine; VLB, vinblastine; BLM, bleomycin A_2; PEP, peplomycin.

MATERIALS AND METHODS

Chemicals SDB-ethylenediamine (malate salt, Fig. 1A) and its components (solanesol and N,N′-bis(3,4-dimethoxybenzyl)ethylenediamine, Figs. 1B and 1C) were kindly supplied by Nisshin Flour Milling Co., Ltd. When the compounds were added to a culture medium, they were dissolved in ethanol and diluted with phosphate-buffered saline (PBS), and the same amount of ethanol was added to the control culture (the final concentration of ethanol was less than 0.5%). Antitumor drugs used in this study were obtained from the following sources: ADM and MMC from Kyowa Hakko Kogyo Co., Ltd., Tokyo; ACR from Institute of Microbial Chemistry, Tokyo; ACT-D from Banyu Pharmaceutical Co., Tokyo; 5-FU, Ara-C, DNR, and VCR from Sigma Chemical Co., St. Louis, Mo.; CDDP, etoposide, BLM, and PEP from Nippon Kayaku Co., Tokyo; ACNU from Sankyo Co., Tokyo; MTX and mitoxantrone from Lederle (Japan) Ltd., Tokyo. [^{3}H]DNR (118.4 GBq/mmol) was purchased from New England Nuclear, Boston, Mass.

Cells Chinese hamster V79 and human hepatoma PLC/PRF/5 cells were grown in Eagle's minimum essential medium supplemented with 10% calf serum in a

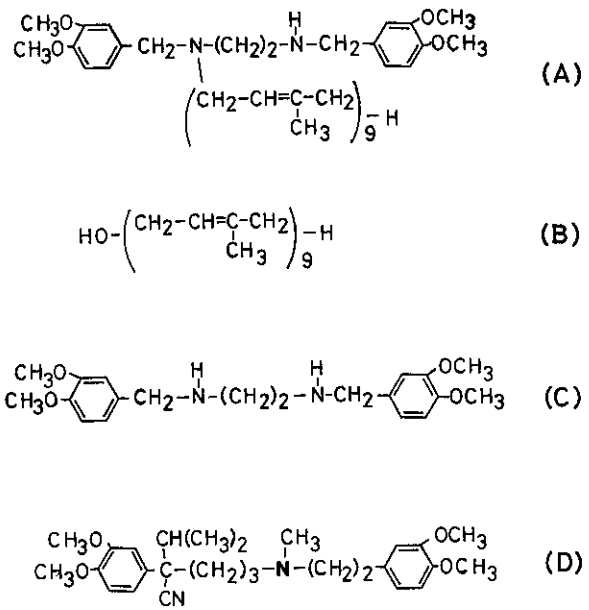

Fig. 1. Chemical structures of SDB-ethylenediamine (A), the solanesol component (B), the diamine component (C), and verapamil (D).

humidified atmosphere of 5% CO_2 at 37°C. Multidrug-resistant mutant cells from both cell lines (V79/ADM and PLC/COL) were established in our laboratory by stepwise selection during subculturing in increasing concentrations of ADM or colchicine, respectively. The cells contained amplified DNA and mRNA of MDR-1 gene (unpublished data).

Colony formation of cells SDB-ethylenediamine and other antitumor drugs were added to the culture medium of V79 and PLC/PRF/5 cells which had been plated on 60 mm plastic dishes (200–300 cells/plate) and incubated for 20 h. After 7–8 days of incubation for V79 cells and 2–3 weeks for PLC/PRF/5 cells, cells were fixed with 10 % formaldehyde solution and stained with crystal violet to enumerate colonies.

Uptake of ADM The uptake of ADM in V79 cells was determined as described by Kunimoto *et al.*[8] V79 cells, which had been plated (about 8×10^5/35 mm dish) and incubated for 18 h at 37°C, were treated with 10 μg/ml of ADM in the presence or absence of SDB-ethylenediamine (10 μg/ml) at 37°C for indicated periods. After removal of the medium, cells were washed twice with cold PBS, and harvested by treatment with trypsin-EDTA solution (Gibco Laboratories). Then, ADM was extracted from the cell suspension (0.6 ml) by adding 0.3 ml of 40% trichloroacetic acid and 0.1 ml of 10% bovine serum albumin. After centrifugation, the fluorescence intensity (590 nm emission excited at 500 nm) of the supernatant was measured and the amount of ADM was calculated by comparison with standard solutions.

Uptake of [^{3}H]DNR Cellular uptake of [^{3}H]DNR was determined by the same procedure as used for ADM, except that cells were solubilized with Protozol (New England Nuclear) and the radioactivity was measured in Scintisol EX-H (Dojin, Kumamoto).

MTT assay An MTT assay was carried out by the method of Carmichael *et al.*,[9] with minor modifications. V79 cells at a density of 2.5×10^3/ml were inoculated (180 μl per well) into 96-well microplates, and 18 h later 20 μl of drug solution in PBS was added to the wells. The plates were incubated for 3 days, then 25 μl of 4 mg/ml MTT in PBS was introduced into each well and the plates were further incubated for 4 h. The medium was completely removed by aspiration and 150 μl/well of dimethylsulfoxide was added. After mixing for 5 min on a plate-mixer, the absorbance at 490 nm was automatically determined with an ELISA analyzer (Toyo Sokki, Tokyo). The relationship of viable cell number and MTT formazan crystal formation was obtained in a separate experiment.

In all experiments, the mean values of three determinations were calculated.

RESULTS

Cytocidal activity of SDB-ethylenediamine The direct cytocidal activity of SDB-ethylenediamine was determined by measuring the inhibition of colony formation of Chinese hamster V79 and human hepatoma PLC/PRF/5 cells. As shown in Fig. 2A, SDB-ethylenediamine prevented the colony formation of parental cells of V79 (V79/S) in a dose-dependent manner with a 50% inhibitory concentration (IC_{50}) of 11.5 μg/ml, and the colony formation of a multidrug-resistant mutant (V79/ADM) was suppressed by the drug to a greater extent than that of the parental cell line; IC_{50} for V79/ADM was 4 μg/ml. Another cell line with multidrug resistance was employed to confirm whether multidrug-resistant cells were more sensitive to the drug than parental cells. As shown in Fig. 2B, the colony formation of multidrug-resistant mutant of human hepatoma PLC/PRF/5 cells (PLC/COL) was markedly suppressed at concentrations over 1.25 μg/ml (IC_{50}, 1.7 μg/ml) of SDB-ethylenediamine, whereas that of the parental cells (PLC/S) was not inhibited at 2.5 μg/ml (IC_{50}, 6.8 μg/ml), indicating again that the drug is more active against multidrug-resistant cells than against sensitive cells.

Potentiation by SDB-ethylenediamine of the cytocidal activity of other antitumor drugs against V79/S and V79/ADM cells Combination effects of SDB-ethylenediamine with various clinically useful antitumor drugs on

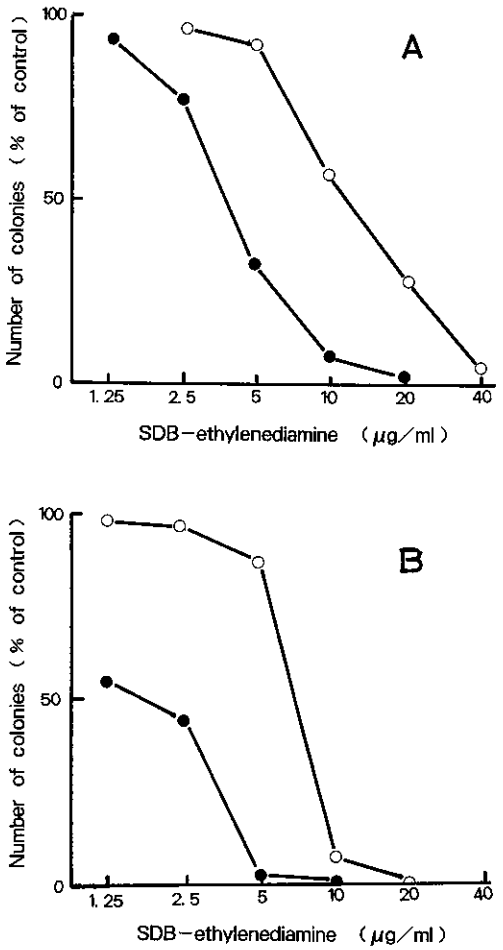

Fig. 2. Cytotoxic activity of SDB-ethylenediamine toward multidrug-resistant and sensitive cells. A: ○, V79/S; ●, V79/ADM. B: ○, PLC/S; ●, PLC/COL.

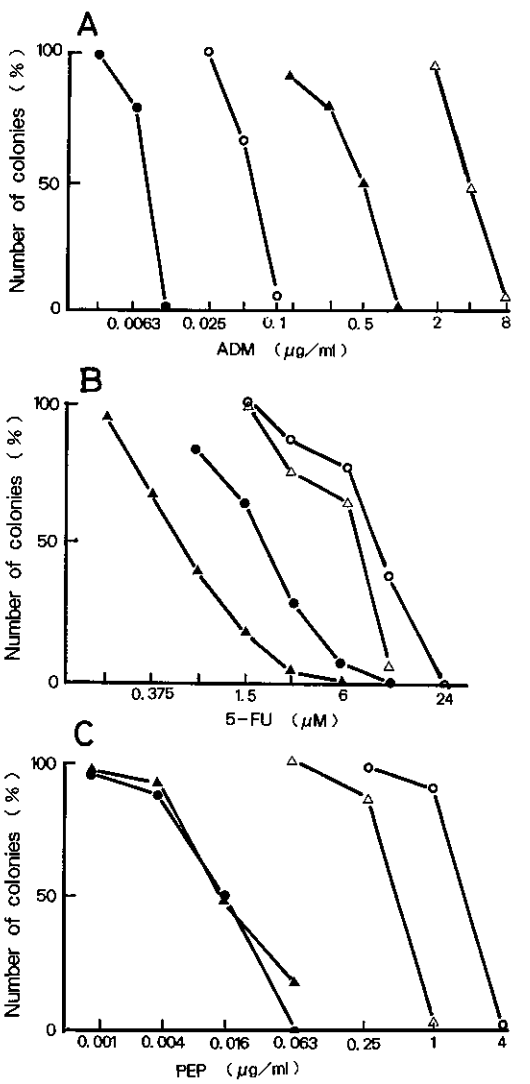

Fig. 3. Typical examples of potentiation of antitumor drugs by SDB-ethylenediamine. V79/S and V79/ADM cells were cultured with various concentrations of ADM (A), 5-FU (B), or PEP (C) with or without SDB-ethylenediamine, and colonies were enumerated after 7–8 days. The concentration of SDB-ethylenediamine was 10 μg/ml for V79/S in all cases and 3 μg/ml (ADM and PEP) or 5 μg/ml (5-FU) for V79/ADM. V79/S: ○, without; ●, with SDB-ethylenediamine. V79/ADM: △, without; ▲, with SDB-ethylenediamine.

the colony formation of V79/S and V79/ADM cells were examined. Since SDB-ethylenediamine itself suppressed the colony formation of V79/ADM more markedly than that of V79/S as described above, SDB-ethylenediamine 10 μg/ml for V79/S cells and 3 or 5 μg/ml for V79/ADM were combined with various concentrations of other antitumor drugs. At these concentrations of SDB-ethylenediamine, the colony formation of both cell lines was inhibited to a similar extent of approximately 40%. As illustrated in Fig. 3A, V79/ADM was highly resistant to ADM; IC_{50} of ADM was 3.9 μg/ml for V79/ADM and 0.057 μg/ml for V79/S, indicating that V79/ADM was 68-fold more resistant to ADM. The addition of SDB-ethylenediamine at the concentration of 3 or 10 μg/ml for V79/ADM or V79/S, respectively, decreased the IC_{50} values to 0.5 and 0.008 μg/ml; the potentiation ratio was 7.6 for V79/ADM and 7.1 for V79/S. Similar potentiation by SDB-ethylenediamine was observed with other antitumor drugs such as ACR, ACT-D, mitoxantrone, etoposide, and VCR, to which V79/ADM was highly resistant, and in all cases, their potentiation ratios were higher in V79/ADM than in V79/S, suggesting that SDB-ethylenediamine is useful to reverse

multidrug-resistance, as reported by Nakagawa *et al.*[6] In addition to the ability to overcome multidrug resistance, SDB-ethylenediamine also potentiated 5-FU (Fig. 3B), AraC, MMC, CDDP, MTX, and ACNU against V79/ADM, which was not resistant to these agents. SDB-ethylenediamine was most efficacious with bleomycin group antibiotics. Although V79/ADM is a typical multidrug-resistant cell line, it showed collateral sensitivity to bleomycin group antibiotics, as reported previously.[10] SDB-ethylenediamine enhanced the cytotoxic activity of PEP more than 100-fold against V79/S cells and more than 30-fold against V79/ADM cells (Fig. 3C). Similar striking enhancement by SDB-ethylenediamine was also observed for BLM. The potentiation ratios for various antitumor drugs are summarized in Table I, along with the IC_{50} values with or without SDB-ethylenediamine.

Effect of SDB-ethylenediamine on uptake of ADM by V79/S and V79/ADM cells The uptake of ADM into V79/S and V79/ADM cells was examined in the presence or absence of SDB-ethylenediamine. As shown in Fig. 4A, SDB-ethylenediamine at 10 μg/ml increased the intracellular level of ADM in both cell lines during a 2-h incubation period. The intracellular accumulation of ADM was greatly enhanced, especially in V79/ADM cells, when the cells were preincubated with SDB-ethylenediamine for 4 h (Fig. 4B).

Comparison of cytocidal activity and enhancement of drug uptake between SDB-ethylenediamine and its structural components SDB-ethylenediamine consists of two moieties of solanesol and a diamine; the latter resembles verapamil (Fig. 1), which reverses multidrug-resistance by interfering with the active efflux of antitumor drugs from resistant cells,[1] and the former partially resembles polyene antibiotics such as amphotericin B, which is well known to kill eukaryotic cells by binding to sterol in the cytoplasmic membrane. Therefore, it was of interest to examine whether the two different activities of SDB-ethylenediamine, cell killing and increasing drug uptake, were apparent with each component. The cell killing activity of SDB-ethylenediamine and its components was determined by MTT assay against V79/S cells. Neither solanesol nor the diamine component showed any significant inhibition of cell growth at 18 μM, and only slight inhibition was observed when both components were added together to the medium. In contrast, SDB-ethylenediamine blocked the growth markedly even at a concentration as low as 2.25 μM (Fig. 5). The enhancement of drug accumulation into V79/S cells was determined by measuring the uptake of [^{3}H]DNR. Again, the uptake of [^{3}H]DNR was not affected by the addition of the components at 9 μM, whereas it was stimulated 3-fold by 9 μM SDB-ethylenediamine (Fig. 6). These results indicate that the two different kinds of activities of SDB-

Table I. Effect of SDB-ethylenediamine on the Cytotoxicity of Various Antitumor Drugs against V79/S and V79/ADM Cells

Drug	V79/S		V79/ADM	
	IC_{50}[a)]	PR[b)]	RR[c)]	PR[b)]
ADM	0.057	7.1	68	7.6
ACR	0.037	1.4	10	4.2
ACT-D	0.017	5.3	38	6.4
Etoposide	0.27	2.8	>74	>2.7
Mitoxantrone	0.19	2.3	19	4.9
VCR	0.018	3.0	27	13
ACNU	7.6	1.4	1.3	3.2[d)]
5-FU (μM)	9.7	4.6	1.0	12[d)]
Ara-C (μM)	0.076	1.4	2.8	6.4[d)]
CDDP	0.37	1.4	1.1	2.5[d)]
MMC	0.020	2.0	2.9	5.2[d)]
MTX	0.017	1.2	0.56	4.4
BLM	2.1	200	0.27	15
PEP	2.0	120	0.25	31

a) IC_{50} is given in μg/ml except for 5-FU and Ara-C (μM).
b) PR: Potentiation ratio calculated from IC_{50} values in the presence (10 or 3 μg/ml for V79/S or V79/ADM, respectively) and absence of SDB-ethylenediamine.
c) RR: Resistance ratio calculated from IC_{50} values of V79/S and V79/ADM.
d) Five μg/ml of SDB-ethylenediamine was used.

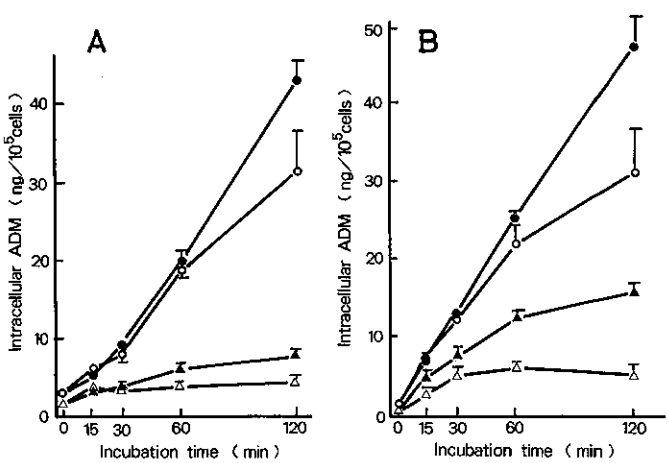

Fig. 4. Intracellular uptake of ADM into V79/S and V79/ADM cells. A. ADM and SDB-ethylenediamine were simultaneously added. B. SDB-ethylenediamine was added 4 h before addition of ADM. The concentrations of both drugs were 10 μg/ml. V79/S: ○, without; ●, with SDB-ethylenediamine. V79/ADM: △, without; ▲, with SDB-ethylenediamine.

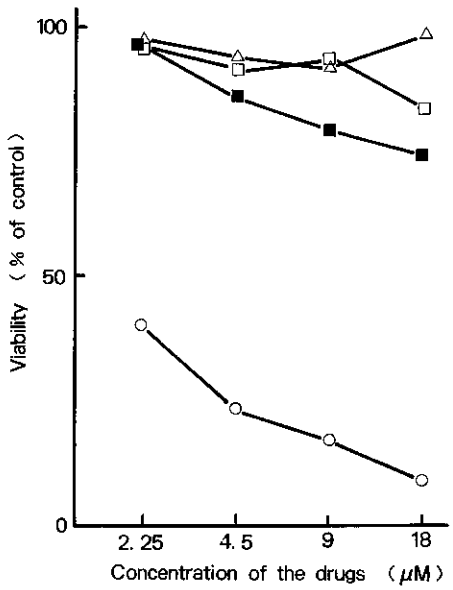

Fig. 5. Comparison of cytotoxic activities of SDB-ethylenediamine and its structural components on V79/S cells measured by the MTT method. ○, SDB-ethylenediamine; △, the solanesol component (S); □, the diamine component (D); ■, S+D.

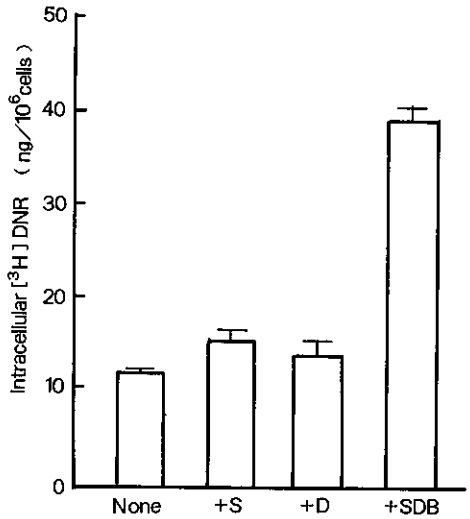

Fig. 6. Uptake of [^{3}H]DNR into V79/S cells in the presence of SDB-ethylenediamine or its components. V79/S cells were preincubated with the drugs at 9 μM each for 2 h and then incubated with [^{3}H]DNR (18.5 kBq/ml, 1.55 μg/ml) for 1 h. S, the solanesol component; D, the diamine component; SDB, SDB-ethylenediamine.

ethylenediamine are not manifested separately by its two components, and the steric conformation of SDB-ethylenediamine is important for both activities.

DISCUSSION

With the aim of overcoming drug resistance of tumor cells, we have been searching for new microbial products with selective toxicity against resistant tumor cells and discovered COTC,[11] lactoquinomycin[12] and resorthiomycin,[13,14] which are effective against multidrug-resistant tumor cells, and cadeguomycin,[15] which potentiates cytosine arabinoside. Of these compounds, resorthiomycin was unique because it exhibited not only preferential inhibition against multidrug-resistant tumor cells but also augmentation of cytotoxicity of ACT-D and VCR, although it failed to enhance the activities of ADM and 5-FU. Therefore, we tried to find another drug able to potentiate more antitumor drugs, and also possessing direct cytotoxic activity. SDB-ethylenediamine appeared to be such a drug. In addition to the cytotoxic activity preferentially exhibited against multidrug-resistant tumor cells (Fig. 2), SDB-ethylenediamine enhanced the activities of various antitumor drugs, especially against multidrug-resistant V79 cells. V79/ADM cells are highly resistant to ADM, ACR, ACT-D, mitoxantrone, etoposide, and VCR, but are not resistant to ACNU, 5-FU, and CDDP, and show collateral sensitivity to MTX and bleomycin group antibiotics. It is of interest that SDB-ethylenediamine could potentiate almost all clinically useful drugs, although the ratio varied between drugs (Table I).

The reasons why SDB-ethylenediamine potentiates such a wide range of antitumor drugs, and why it exhibits inhibitory activity to a greater extent against multidrug-resistant cells than against sensitive cells are unclear. One of its potentiation mechanisms is the enhancement of accumulation of drugs in cells as shown with ADM and DNR (Figs. 4 and 6), which accords with the results of Nakagawa *et al.*[6] However, it did not increase the uptake of 5-FU or PEP in V79/S cells (data not shown) under conditions where the potentiation of 5-FU and PEP was clearly observed (Fig. 3), indicating that other mechanisms are involved in the potentiation of these drugs. Because SDB-ethylenediamine consists of two moieties, a verapamil-like component and an isoprenoid component, we examined whether the two different properties of SDB-ethylenediamine, direct cytotoxicity and potentiation, resided on separate components. No such properties, however, were observed with the components, even when they were mixed together, and the activities were found only in the intact SDB-ethylenediamine molecule (Figs. 5 and 6). Nakagawa *et al.*[6] reported that SDB-ethylenediamine inhibited active efflux of DNR and

VCR from multidrug-resistant human KB cells, and Yamaguchi *et al.*[7] observed that it had no calcium channel-blocking activity, unlike verapamil. We did not find any evidence that SDB-ethylenediamine blocks the efflux of antitumor drugs from V79/ADM cells at concentrations that enhanced drug accumulation. Because the combination of ADM or PEP with SDB-ethylenediamine at a concentration at which the drug alone did not inhibit the growth of V79/S cells exhibited strong inhibition (data not shown), antitumor agents and SDB-ethylenediamine are thought to work synergistically on tumor cells. However, the precise potentiation mechanisms of SDB-ethylenediamine for various kinds of antitumor drugs, as well as its mechanism of cytocidal action against tumor cells, remain to be elucidated.

ACKNOWLEDGMENTS

This work was supported in part by a Grant-in-Aid for Cancer Research from the Ministry of Education, Science and Culture, Japan, and by a grant from Lederle (Japan) Ltd. We thank Nisshin Flour Milling Co. Ltd. for providing SDB-ethylenediamine and its components, and Professor Hideyo Yamaguchi for his encouragement during the present study.

(Received November 14, 1989/Accepted January 18, 1990)

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