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Synergistic Effect in Culture of Bleomycin-group Antibiotics and N-Solanesyl-N,N′-bis(3,4-dimethoxybenzyl)ethylenediamine, a Synthetic Isoprenoid

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Like bleomycin and peplomycin, libromycin, a newly developed bleomycin-group antibiotic, was potentiated 130-fold against Chinese hamster V79 cells (V79/S) and 47-fold against its multidrug-resistant mutant (V79/ADM) by N-solanesyl-N,N′-bis(3,4-dimethoxybenzyl)ethylenediamine (SDB-ethylenediamine) at 10 and 3 μg/ml, respectively. But neocarzinostatin, known to cause DNA strand scission as bleomycins do, was potentiated only twofold. This suggests that the high potentiation by SDB-ethylenediamine is unique to the bleomycin-group antibiotics. Isobologram analysis revealed that the combined effect of peplomycin and SDB-ethylenediamine was highly synergistic. SDB-ethylenediamine did not increase the intracellular accumulation of [^{3}H]peplomycin in V79/S cells. Analyses by an alkaline elution method demonstrated that single strand scission in DNA of intact V79/S cells caused by 1-h incubation with peplomycin was greatly stimulated by pre- and co-existence of SDB-ethylenediamine, but DNA strand breaks in isolated nuclei were not affected. Apparently some cytoplasmic constituent(s) is involved in the potentiation mechanism. SDB-ethylenediamine did not block the DNA repair which occurred after the removal of peplomycin from the medium. Two fragments of SDB-ethylenediamine, solanesol (polyprenoid moiety) and a diamine component (verapamil-like moiety), were not synergistic with peplomycin, even when they were mixed together. This indicates that the steric conformation of the intact SDB-ethylenediamine molecule is important for the activity.

Key words: Synthetic isoprenoid — SDB-ethylenediamine — Synergism — Bleomycins — Chinese hamster cells

In the preceding paper,[1] we have reported that SDB-ethylenediamine,[2] a synthetic isoprenoid, inhibits the growth of multidrug-resistant mutant cells to a greater extent than the parental cells, and that it potentiates the cytotoxic activity of almost all kinds of clinically useful antitumor agents *in vitro*, although the degree of potentiation varies with each drug. Of the antitumor agents tested, the activities of bleomycin and peplomycin (PEP) were enhanced most by SDB-ethylenediamine.

Here, we describe the synergistic effect of SDB-ethylenediamine and bleomycin-group antibiotics against Chinese hamster V79 cells *in vitro*.

MATERIALS AND METHODS

Chemicals SDB-ethylenediamine (malate salt) and its components (solanesol and N, N′-bis (3,4-dimethoxybenzyl)ethylenediamine) were kindly supplied by Nisshin Flour Milling Co., Ltd., Tokyo (see Ref. 1 for their chemical structures). These compounds were dissolved in ethanol and diluted with phosphate-buffered saline (PBS) or redistilled water. Bleomycin A_2, PEP, libromycin and [^{3}H]PEP (735 μCi/mg) were generously given by Dr. T. Takita, Nippon Kayaku Co., Tokyo. Neocarzinostatin was a clinical sample from Yamanouchi Pharmaceutical Co., Tokyo, and MTT was from Sigma, St. Louis, MO. [^{3}H]Thymidine (28 Ci/mmol) was purchased from ICN Radio-chemicals, Irvine, CA.

Cells Chinese hamster V79 cells were grown in Eagle's minimum essential medium supplemented with 10% calf serum, in a humidified atmosphere of 5% CO_2 at 37°C. A multidrug-resistant mutant cell line of V79 cells (V79/ADM) was established in our laboratory by stepwise selection during subculturing in increasing concentrations of doxorubicin. The cell line contained amplified DNA and mRNA of *mdr*-1 gene. The colony formation assay and MTT assay were described in the preceding paper.[1] The viable cell number was determined by a trypan blue dye exclusion method. In these determinations, mean values of triplicate assays were calculated. The variation was less than 10%. Nuclei of V79 cells were isolated by a method similar to that described by Pommier *et al.*,[2] except that dithiothreitol was omitted from the nuclei buffer, which was adjusted to pH 7.0. Uptake of [^{3}H]PEP by V79/S cells was determined by a similar method to that described previously.[3]

Alkaline elution method for detection of single-strand breaks in DNA Filter elution procedures were essentially as described by Kohn *et al.*,[4] with slight modifications.

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[2] Abbreviations used in this paper: SDB-ethylenediamine, N-solanesyl-N, N′-bis (3,4-dimethoxybenzyl) ethylenediamine; PEP, peplomycin.

Briefly, V79 cells were labeled with 0.1 μCi/ml of [^{3}H]-thymidine for 24 h and chased for 4 h. After being treated with PEP and/or SDB-ethylenediamine under various conditions, the cells were harvested by pipetting and 1.5~2.0$\times 10^6$ cells were layered on 2-μm pore polycarbonate membrane filters (Nucleopore, Pleasanton, CA), then lysed with a solution of 2% sodium dodecyl sulfate (SDS), 25 m*M* disodium EDTA (pH 9.7) and 0.5 mg/ml of proteinase K (Merck, Darmstadt). The DNAs on the filter were eluted with a solution of 0.1% SDS, 20 m*M* EDTA (acid form), and tetrapropylammonium hydroxide (Sigma), pH 12.1, at a speed of 1.0 ml/20 min/fraction. Radioactivity in each fraction was determined in Scintisol EX-H (Dojin Chemicals, Kumamoto), and the radioactivity remaining on a filter was counted in a Filter-count (Packard Instrument Co., Downers, IL).

RESULTS

Comparison of potentiation of bleomycin-group antibiotics and neocarzinostatin by SDB-ethylenediamine Since SDB-ethylenediamine greatly potentiated bleomycin and PEP, as described earlier,[1] we examined whether its great potentiating activity is restricted to the bleomycin-group antibiotics only, or occurs with all antitumor agents that cause single-strand breaks in DNA. We chose to test libromycin and neocarzinostatin. Libromycin is a newly developed bleomycin-group antibiotic which has been modified to be more lipophilic so it permeates cells more readily than bleomycin or PEP. It is now under clinical trial. Neocarzinostatin, like bleomycin,[5] causes single-strand scission in DNA.[6] As shown in Table I, libromycin, unlike bleomycin and PEP, was similarly cytotoxic to both V79/S and V79/ADM cells. However, its activity was as greatly enhanced by SDB-ethylenediamine as those of bleomycin and PEP were. But neocarzinostatin was less effective on V79/ADM than on V79/S cells, and its activity was enhanced only about twofold by SDB-ethylenediamine. This indicates that the higher potentiation by SDB-ethylenediamine, over 100-fold against V79/S cells, is specific to bleomycin-group antibiotics.

Table I. Potentiation by SDB-ethylenediamine of the Cytotoxic Activity of Bleomycin-group Antibiotics and Neocarzinostatin against V79/S and V79/ADM Cells

(A) V79/S	IC_{50}[a] (μg/ml)		Potentiation ratio[b]
	+SDB-ethylenediamine		
	0	10 (μg/ml)	
Bleomycin A_2	2.1	0.010	210
Peplomycin	2.0	0.017	120
Libromycin	2.0	0.016	130
Neocarzinostatin	0.056	0.030	1.9

(B) V79/ADM	IC_{50} (μg/ml)		Potentiation ratio
	+SDB-ethylenediamine		
	0	3 (μg/ml)	
Bleomycin A_2	0.56 (0.27)[c]	0.039	15
Peplomycin	0.48 (0.25)	0.016	31
Libromycin	1.9 (0.93)	0.040	47
Neocarzinostatin	0.43 (7.7)	0.25	1.7

V79 cells were cultured for 8 days in the presence of various concentrations of antibiotics and the indicated concentrations of SDB-ethylenediamine.
a) IC_{50}, drug concentration required for 50% inhibition of colony-forming capacity.
b) Potentiation ratio was calculated from the IC_{50} values with and without SDB-ethylenediamine.
c) Index of resistance, calculated from the IC_{50} values of drugs against V79/S and V79/ADM cells.

Isobologram analysis To determine whether the combined effect of PEP and SDB-ethylenediamine was additive or synergistic, we used isobologram analysis according to the method of Steel and Peckham.[7] We analyzed the combined effect of the two drugs at the point of IC_{90} (the drug concentration that inhibits the colony formation of V79/S and V79/ADM cells by 90% of that in drug-free controls). The IC_{90} values of PEP alone were 3.1 μg/ml for V79/S and 0.68 μg/ml for V79/ADM cells. With SDB-ethylenediamine alone, the IC_{90} values were 17 and 4.6 μg/ml for V79/S and V79/ADM, respectively. These concentrations of the two drugs are expressed as 1.0 at the ordinate and abscissa of Fig. 1. The isoeffect curves (I, II_{PEP} and II_{SDB}) were drawn as described by Kano *et al.*[8] When an experimental data point is within the envelope, this combination is considered to be additive and when it falls to the left of the envelope, the two drugs can be considered synergistic. Actual experimental data of three different combinations of PEP and SDB-ethylenediamine are plotted in Fig. 1. They show that the combined effect of PEP and SDB-ethylenediamine is highly synergistic against V79/S and V79/ADM cells.

Combined effect of peplomycin and SDB-ethylenediamine on the growth of V79/S cells in 24 h The inhibitory activities of PEP and SDB-ethylenediamine on the colony formation of V79 cells were obtained after incubation for 7–8 days, so we tested the combined effect after a shorter incubation period of 24 h. Viable cell numbers were determined by a trypan blue dye exclusion method. The doubling time of V79/S cells was 9–11 h and the number of control cells increased to 11.4$\times 10^5$ from 2.0$\times 10^5$ cells per well during 24-h incubation. PEP at 2 μg/ml or SDB-ethylenediamine at 10 μg/ml weakly inhibited growth; the cell numbers after 24-h incubation were 9.1$\times 10^5$ or 8.1$\times 10^5$ per well, respectively. When PEP (2 μg/ml) and SDB-ethylenediamine (10 μg/ml)

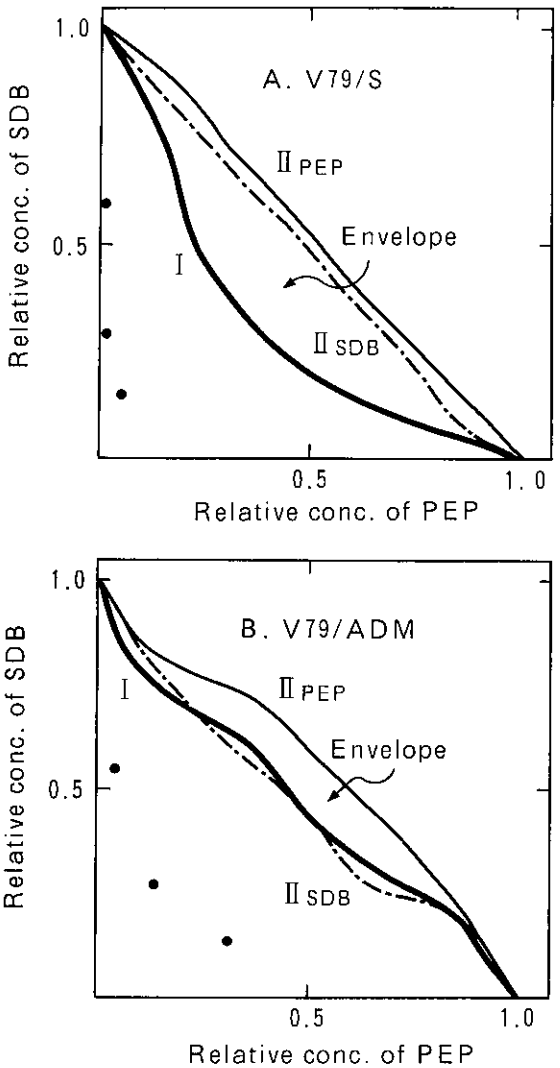

Fig. 1. Isobologram of peplomycin in combination with SDB-ethylenediamine. An envelope of additivity is constructed from the dose-response curves of the effects of the two drugs on the colony formation of V79/S and V79/ADM cells as described by Kano *et al.*[8]

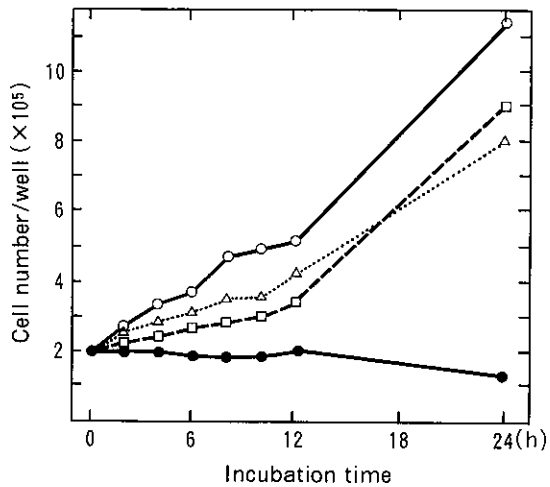

Fig. 2. Combined effect of peplomycin and SDB-ethylenediamine on the growth of V79/S cells in 24 h. Cells were treated without (○) or with peplomycin at 2 μg/ml (△), SDB-ethylenediamine at 10 μg/ml (□) or both drugs at the same concentrations (●), and the viable cell numbers were determined by trypan blue dye exclusion at 2-h intervals up to 12 h and at 24 h. Mean values of triplicate samples were plotted.

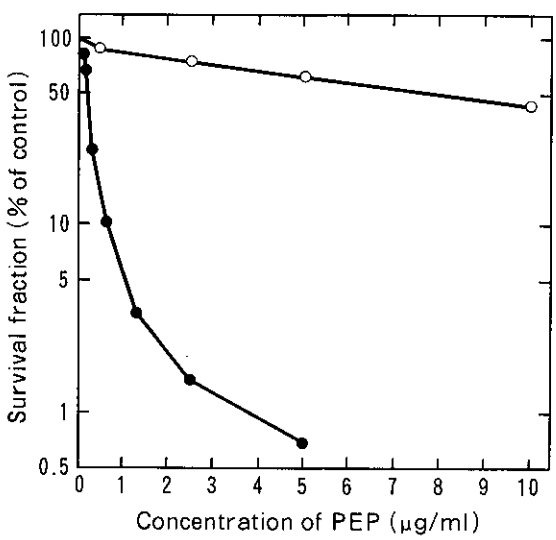

Fig. 3. Effect on colony formation of the short-term exposure of V79/S cells to drugs. The cells were incubated for 1 h with various concentrations of peplomycin with (●) or without (○) 10 μg/ml of SDB-ethylenediamine, and an appropriate number of cells was replated and cultured for 7 days in a drug-free medium.

were added to the medium together, the growth of V79/S cells was completely blocked at as early as 2 h after the start of incubation (Fig. 2). Clearly the combined effect of PEP and SDB-ethylenediamine appears quickly after they are added to a medium, and the strong inhibition is a synergistic effect of the two compounds.

Brief exposure to the drugs V79/S cells were incubated for 60 min with various concentrations of PEP in the presence or absence of SDB-ethylenediamine, then an appropriate number of cells was replated in a drug-free medium, and cultured for 7 days. As shown in Fig. 3, 1-h exposure to PEP alone weakly inhibited the colony formation of V79/S cells (IC_{50}=8.4 μg/ml). An addition of 10 μg/ml SDB-ethylenediamine significantly increased

the activity of PEP (IC_{50}=0.20 μg/ml). The potentiation ratio is 42-fold, which is similar to that of continuous exposure. Apparently the potentiating activity of SDB-ethylenediamine appears irreversibly in the very early period of incubation.

Accumulation of [^{3}H]PEP in V79/S cells Because the intracellular drug concentration is one of the most important factors affecting cytotoxicity, we studied the effect of SDB-ethylenediamine on the uptake of [^{3}H]PEP by V79/S cells. But SDB-ethylenediamine at 10 μg/ml did not increase the intracellular accumulation of [^{3}H]-PEP after preincubation with the cells for 4 h (Table II). Under similar conditions, the uptakes of [^{3}H]daunorubicin and [^{3}H]vinblastine by V79/S cells increased 1.7- and 2.3-fold (data not shown), respectively.

Single-strand breaks in DNA As treatment of PEP with SDB-ethylenediamine for only 1-h had a synergistic effect on the clonogenic activity of V79/S cells, we determined the damage to the DNA of the cells after drug treatment for 1-h using an alkaline elution method. As shown in Fig. 4A, DNA strand breaks were clearly caused by PEP at 10 μg/ml, but not by SDB-ethylenediamine at 40 μg/ml. The single-strand breaks caused by PEP were greatly increased by SDB-ethylenediamine at 10 and 20 μg/ml in a dose-dependent manner and reached a plateau at

Table II. Effect of SDB-ethylenediamine on the Intracellular Accumulation of [^{3}H]PEP in V79/S Cells

Incubation (h)	Intracellular PEP (ng/10^7 cells)	
	control	+SDB-ethylenediamine[a)]
1	2.9	2.6
2	3.4	2.7

a) V79/S cells growing in plastic dishes were incubated with SDB-ethylenediamine at 10 μg/ml for 4 h.

The cell suspensions of control and the SDB-ethylenediamine-treated cells were prepared at a cell density of 3×10^6/ml in the culture medium and incubated with [^{3}H]PEP (0.25 μCi/ml) for 1 or 2 h at 37°C on the oil mixture[3)] in the presence or absence of SDB-ethylenediamine (10 μg/ml). Mean values of triplicate samples are presented.

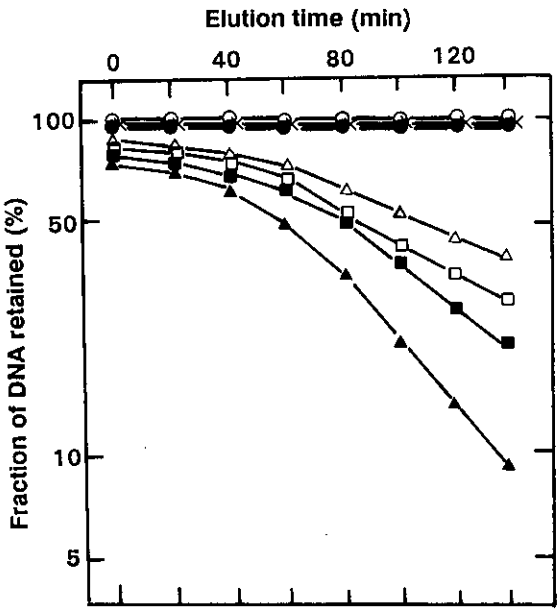

Fig. 5. Effect of pre- or post-treatment of SDB-ethylenediamine on DNA strand scission by peplomycin, detected by alkaline elution. V79/S cells were treated with SDB-ethylenediamine for 5 min (□) or 60 min (■) before a 1-h incubation with peplomycin, or cells were treated with peplomycin for 1 h (△) and incubated with (●) or without (×) SDB-ethylenediamine for another 1 h. Peplomycin and SDB-ethylenediamine were simultaneously added and incubated for 1 h (▲). Control (○). The concentrations of peplomycin and SDB-ethylenediamine were 10 and 20 μg/ml, respectively.

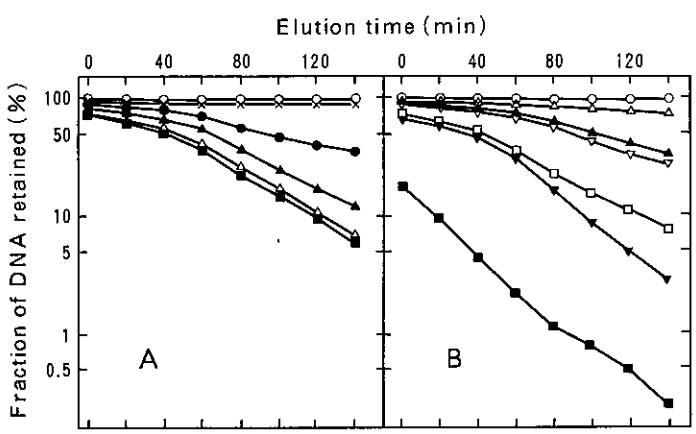

Fig. 4. Alkaline elution for detection of DNA strand breaks. [^{3}H]Thymidine-labeled V79/S cells were treated with drugs for 1 h and alkaline elution was carried out as described in "Materials and Methods." A. Concentration of peplomycin was fixed (10 μg/ml) and 0 (●), 10 (▲), 20 (△), and 40 (■) μg/ml of SDB-ethylenediamine was added. Control (○) and SDB-ethylenediamine at 40 μg/ml alone (×). B. Concentrations of peplomycin were 2.5 (△, ▲), 10 (▽, ▼), and 40 (□, ■) μg/ml, and 20 μg/ml of SDB-ethylenediamine was included (▲, ▼, ■). Control (○).

40 μg/ml. PEP at 2.5, 10, and 40 μg/ml caused DNA strand breaks dose-dependently and adding of SDB-ethylenediamine at 20 μg/ml significantly increased the degree of single-strand scission at all concentrations of PEP (Fig. 4B); the degree of DNA strand scission by PEP (10 μg/ml) plus SDB-ethylenediamine (20 μg/ml) was greater than that by 40 μg/ml of PEP alone and the potentiation ratio was more than 4-fold. With PEP at 40 μg/ml plus SDB-ethylenediamine, damage to DNA was so severe that only 18% of the total DNA remained on a filter at the start of alkaline elution. On the other hand, DNA strand breaks caused by neocarzinostatin were not stimulated by SDB-ethylenediamine (data not shown).

Effect on DNA strand scission of sequential treatment with peplomycin and SDB-ethylenediamine The effects of SDB-ethylenediamine added alone to the medium before or after PEP treatment were examined. As shown in Fig. 5, pretreatment with SDB-ethylenediamine, which was removed from the medium before the addition of PEP, increased the DNA strand scission even with only 5-min pretreatment. But cotreatment with PEP and SDB-ethylenediamine caused the highest degree of DNA strand breaks in V79/S cells. DNA strand scission caused by PEP during 1-h incubation disappeared after 1-h incubation in a drug-free medium. This shows that DNA damage was repaired during the incubation, and the addition of SDB-ethylenediamine at this stage did not inhibit the DNA repair.

Effect of SDB-ethylenediamine on isolated nuclei The potentiation by SDB-ethylenediamine of the DNA strand scission by PEP was studied with isolated nuclei of V79/S cells. Figure 6 shows that PEP caused DNA strand scission in isolated nuclei in a dose-dependent manner; but unlike in intact cells, SDB-ethylenediamine did not increase the PEP effect. These results suggest that some cytoplasmic factor(s) are involved in the potentiating activity of SDB-ethylenediamine.

Potentiating effects of the components of SDB-ethylenediamine SDB-ethylenediamine has two components, an isoprenoid moiety (solanesol) and a verapamil-like moiety (diamine component). Since some polyprenoids were reported to potentiate bleomycin[9] and verapamil overcomes multidrug resistance,[10] the potentiation by each SDB-ethylenediamine component of the growth inhibition by PEP was examined. After cultivation with each drug for 3 days, the growth of V79/S cells was

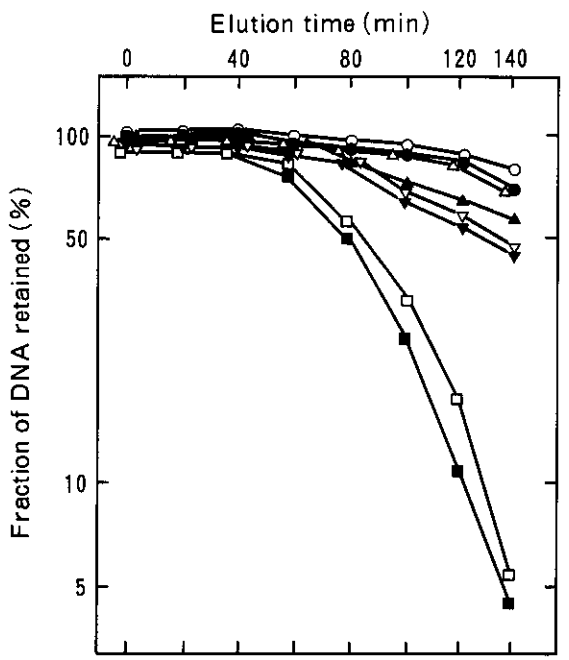

Fig. 6. DNA strand breaks in isolated nuclei. Nuclei were isolated from [^{3}H]thymidine-labeled V79/S cells and incubated with drug(s) for 1 h. Alkaline elution was carried out similarly with intact cells. Concentrations of peplomycin were 0 (○, ●), 2.5 (△, ▲), 10 (▽, ▼), and 40 (□, ■) μg/ml, and SDB-ethylenediamine (20 μg/ml) was included (●, ▲, ▼, ■).

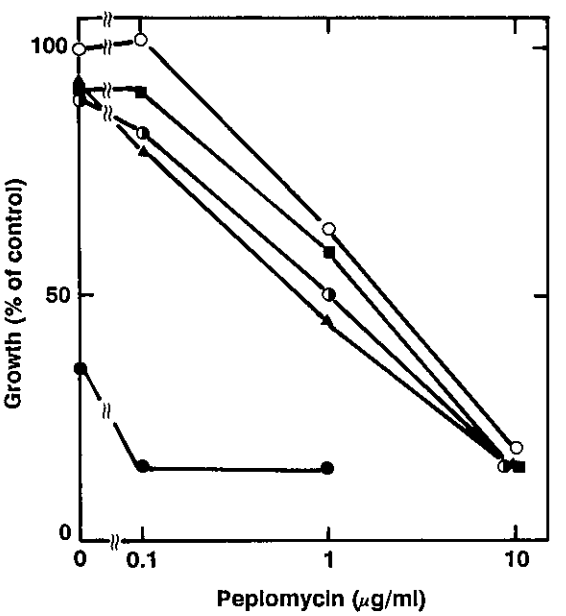

Fig. 7. Comparison of synergistic activity with peplomycin between SDB-ethylenediamine and its structural components against V79/S cells measured by the MTT method. Cells were treated with different concentrations of peplomycin in the presence of SDB-ethylenediamine or its components at a concentration of 4.5 μM each. ○, peplomycin alone; ●, + SDB-ethylenediamine; ▲, +solanesol; ■, +diamine component; ◑, +solanesol and diamine component.

determined by the MTT method. As shown in Fig. 7, SDB-ethylenediamine at 4.5 μM (5 μg/ml) alone inhibited the growth of cells and significantly potentiated the activity of PEP, whereas each component at 4.5 μM failed to exhibit these activities, even when they were mixed together, indicating that the conformation of the SDB-ethylenediamine molecule is important for its activity.

DISCUSSION

SDB-ethylenediamine has unique properties, such as the direct cytotoxic activity preferentially exhibited against multidrug-resistant tumor cells and the potentiation of almost all kinds of clinically useful antitumor agents.[1] Among antitumor drugs tested, potentiation ratios for bleomycin and PEP were far higher than those for other agents. Thus, we focused on bleomycin-group antibiotics to elucidate the mechanism of potentiation by SDB-ethylenediamine.

The high potentiation was confirmed to be peculiar to bleomycin-group antibiotics, since it was observed in libromycin but not in neocarzinostatin (Table I). Several chemical agents enhance the activity of bleomycin-group antibiotics. Ikezaki *et al.*[9] reported that some synthetic polyprenoids potentiated the activity of bleomycin severalfold against V79 cells; Suzuki *et al.*[11] reported that glycerol enhanced the activity of bleomycin against V79 cells; and we found that E-64, an inhibitor of a bleomycin-inactivating enzyme, also increased the activity of PEP.[12] Compared with these agents, SDB-ethylenediamine is unique because it is directly cytotoxic. Also it potentiates bleomycin-group antibiotics far more effectively than others. Isobologram analysis showed potent synergism between PEP and SDB-ethylenediamine against V79/S and V79/ADM cells (Fig. 1), and the synergism was exerted very soon after exposure of the cells to the two drugs (Fig. 2). Although SDB-ethylenediamine did not cause DNA strand breaks, it greatly increased the breakage by PEP (Fig. 4). SDB-ethylenediamine increased intracellular accumulation of doxorubicin in V79 cells.[1] But it did not affect that of [^{3}H]PEP (Table II). A bleomycin-inactivating enzyme affected the cytotoxicity of PEP against V79/S cells which contained a large amount of the enzyme.[9] We compared the enzyme activity between SDB-ethylenediamine-treated (10 μg/ml for 16 h) and untreated V79/S cells and found that SDB-ethylenediamine did not inhibit the enzyme activity in cells (data not shown). It did not block the repair of DNA strand breaks caused by PEP (Fig. 5). Thus, the reason why SDB-ethylenediamine potentiates the bleomycins is not yet known, and neither is the reason why it is directly cytotoxic to tumor cells. However, we obtained evidence that some cytoplasmic constituent(s) must be involved in the potentiation by SDB-ethylenediamine, since the effect was not exerted on isolated nuclei (Fig. 6). Analysis of the factors in cytoplasmic fractions concerning the potentiation by SDB-ethylenediamine is now under way.

Combination therapy using PEP with SDB-ethylenediamine prolonged the life span of tumor-bearing mice. The results of *in vivo* experiments will be published elsewhere.

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