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ENHANCEMENT BY A SYNTHETIC ISOPRENOID OF THE TOXICITY OF CONJUGATES OF EPIDERMAL GROWTH FACTOR WITH PSEUDOMONAS EXOTOXIN

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Abstract—A newly synthesized isoprenoid, *N*-solanesyl-*N,N'*-bis(3,4-dimethoxybenzyl)ethylenediamine, has a verapamil-like structure but no calcium channel blocking activity. The isoprenoid enhanced the cytotoxic effect of a conjugate of epidermal growth factor coupled with Pseudomonas exotoxin in human KB cells. By using iodinated epidermal growth factor ([^{125}I]EGF), the effect of the isoprenoid on intracellular transport of EGF was examined. The isoprenoid did not affect the binding and uptake of [^{125}I]EGF by KB cells. The release of radioactivity associated with [^{125}I]EGF into medium was slow in the presence of the isoprenoid. Density gradient fractionation studies using cell homogenates suggest that [^{125}I]EGF accumulates in an undegraded form in lysosomes when cells are treated with the isoprenoid. The pH value in lysosomes of KB cells was 5.5, and SDB did not affect significantly the pH value at the concentrations used to potentiate the cytotoxicity of chimeric toxins. Electron microscopy showed an increased number of electron-dense bodies in KB cells grown for 24 hr with 17–51 μg/ml isoprenoid. The potentiating action of chimeric toxins by the isoprenoid is discussed in relation to the altered lysosomal function in treated cells.

EGF† binds to its membrane surface receptor, moves into an acidic compartment, the endosome or receptosome, and finally into lysosomes where it is degraded [1, 2]. Conjugates (EGF-PE) of EGF and Pseudomonas exotoxin (PE) exert their cytotoxic effect to inhibit protein synthesis by entering the cells through receptor-mediated endocytosis [3]. The intracellular pathway for EGF-PE appears to be similar to that for EGF itself [4]. Both verapamil, a Ca^{2+} channel blocking agent, and thioridazine, a calmodulin inhibitor, have been shown to enhance the cytotoxic effect of EGF-PE and block the release of [^{125}I]EGF degradation products from cultured human cancer KB cells [4–6]. Some Ca^{2+} channel blocking agents also inhibit proteolytic degradation of low density lipoprotein, possibly in lysosomes [7]. The effects of verapamil and thioridazine on the cytotoxicity of EGF-PE and lysosomal function appear to be related. On the other hand, verapamil and thioridazine also overcome the multidrug-resistance phenotype in human epithelial carcinoma KB cells by increasing the intracellular accumulation of anticancer drugs [8, 9]. These effects of verapamil and thioridazine seem not to be directly related to an effect on calcium channel blockage and to calmodulin inhibition respectively [6, 10]. Recently we synthesized two synthetic isoprenoids, *N*-(*p*-methylbenzyl)-decaprenylamine and SDB, and observed that these two isoprenoids almost completely overcome the multidrug resistance in cultured multidrug-resistant KB-Ch^R-24 cells [11]. SDB has a verapamil-like structure, but no calcium channel blocking action, and is less toxic than verapamil [12]. The acute toxicity levels (LD_{50}) on ddY mice for SDB and verapamil are 268.0 and 8.0 mg/kg, respectively, when administered intravenously [12]. In this study, we report that SDB altered the degradation of EGF in lysosomes and potentiated the cytotoxicity of EGF-PE.

MATERIALS AND METHODS

Cell culture and cell line. Human KB epidermoid carcinoma cells were cloned two times; a single recloned cell line, KB-3-1 [13], was used in the present study. Cells were grown in monolayer using minimal essential medium (MEM) (Nissui Seiyaku Co., Tokyo) with 1 mg/ml of Bactopeptone (Difco), 0.292 mg/ml of glutamine and penicillin (100 μg/ml), supplemented with 10% newborn calf serum (NCS) (Flow Laboratories, North Ride). KB-3-1 cells grew with a doubling time of 22 hr, and their relative plating efficiency was approximately 60%.

Chemicals. SDB was synthesized, purified, and used as described previously [12]. EGF-PE was the gift of Dr. Ira Pastan (National Cancer Institute, Bethesda, MD). [^{125}I]EGF (180 μCi/μg) was purchased from New England Nuclear.

Determination of toxicity of EGF-PE by colony formation. Three hundred cells were plated in 60 mm dishes in the absence of any drug and incubated for 24 hr. Then the cells were incubated at 37° with various concentrations of EGF-PE in the presence or absence of SDB for 9 days. Colonies were stained with 0.5% methylene blue in 50% ethanol and counted.

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† Abbreviations: EGF, epidermal growth factor; EGF-PE, conjugates of EGF and Pseudomonas exotoxin (PE); and SDB, *N*-solanesyl-*N,N'*-bis(3,4-dimethoxybenzyl)ethylenediamine.

Cellular uptake of [^{125}I]*EGF.* KB-3-1 cells (2×10^6) were plated in 60 mm dishes. After incubation overnight at 37°, 34 μg/ml SDB was added to half of the dishes and incubated for 18 hr at 37°. At this time, medium was replaced with serum-free medium with or without SDB and incubated for 30 min at 37°. Then the medium was replaced again with serum-free medium containing 2 mg/ml bovine serum albumin (BSA) with or without SDB, and [^{125}I]EGF (8.0×10^4 cpm) was added to each dish. In some dishes, 20 μl of 100 μg/ml cold EGF was added before adding [^{125}I]EGF to determine nonspecific binding. Cells were incubated for the indicated times at 37° and washed three times with cold phosphate-buffered saline (PBS) at 4°. Cells were harvested in 1 ml of 1 N NaOH, and cell-associated radioactivity was determined in a gamma counter.

Binding of [^{125}I]*EGF by KB-3-1 cells and kinetics of release of* [^{125}I]*EGF from the cells.* KB-3-1 cells (2×10^6) were plated in 60 mm dishes and incubated for 24 hr. Medium was changed to fresh medium with or without SDB (34 μg/ml) and further incubated for 24 hr. At this time medium was replaced with serum-free medium, and the incubation was continued for 30 min with or without SDB. Then the medium was replaced with 2 ml of serum-free medium with 2 mg/ml BSA containing 0.025 μCi/ml of [^{125}I]EGF and incubated at 4° for 2 hr in the presence or absence of SDB. After incubation, the cells were washed four times with ice-cold PBS, then medium containing 10% newborn calf serum was added, and the incubation was continued for various times at 37°. Cells were harvested with 1.5 ml of 1 N NaOH at the indicated time. Media were also harvested separately and their radioactivities were determined.

Analysis by colloidal silica gradients. KB-3-1 cells (5×10^6) were plated in 100 mm dishes and incubated for 24 hr. Then SDB (34 μg/ml) was added to half of the dishes and incubated for 24 hr. At this time medium was replaced with serum-free medium with or without SDB and incubated for 30 min at 37°. Then the medium was replaced with serum-free medium with 2 mg/ml BSA containing [^{125}I]EGF (0.06 μCi/ml, 0.3 μCi/dish) and incubated for 1 hr at 4° in the presence or absence of SDB. After the incubation, cells were washed four times with ice-cold PBS, fresh medium containing 10% newborn calf serum was added, and the incubation was continued for various times at 37° in the presence or absence of SDB. To end the incubation, media were removed, and cells were cooled on ice and washed with ice-cold PBS. Cells were removed from plates with a rubber scraper and suspended in 2 ml of TES (10 mM triethanolamine, pH 7.5, 1 mM EDTA, 0.25 M sucrose) buffer as described previously [4, 7].

Cell suspensions were placed in a nitrogen cavitation bomb [14] and pressurized to 35 psi for 10 min. After partial disruption by slow release, the cells were homogenized in a Dounce homogenizer (Kontes) with 10 strokes. After centrifugation of the homogenates at 3000 *g* for 10 min to pellet nuclei and unbroken cells, 0.5 ml of the supernatant fraction was layered over 9 ml of 25% isoosmotic Percoll (Pharmacia) TES buffer. The bottom of the tube contained a 0.5 ml cushion of 2.5 M sucrose. After centrifugation (Hitachi RP65 rotor) at 25,000 *g* for 1 hr, the *in situ* generated density gradient was collected from the top. Fractions were assayed for density using density marker beads (Pharmacia), for radioactivity using a gamma counter, and for β-hexosaminidase activity as a marker for lysosomes as described previously [10, 14].

Measurement of lysosomal pH. A standard curve relating the ratio of fluorescence intensities at 520 nm with excitation at 495 and 450 nm was constructed according to the method of Ohkuma and Poole [15] using FD (Wako Chemical, Osaka, Japan) (10 μg/ml) in 0.2 M sodium citrate (pH 4.5 to 6.0), sodium phosphate (pH 6.5 to 7.5) or Tris–HCl (pH 8.0) buffer.

To load FD into lysosomes, cells on coverslips were incubated with FD (5 mg/ml in MEM) at 37° overnight and chased for 1 hr in MEM. The cells were then washed extensively with PBS, and the coverslip was mounted in the holder designed by Ohkuma and Poole [15]. Fluorescence intensities were then measured with a Shimadzu Spectrofluorophotometer RF540 as described previously [16, 17].

Electron microscopy. KB-3-1 cells were preincubated with SDB for the indicated times, then trypsinized and washed two times with PBS. The cell pellets for transmission electron microscopy were fixed in cacodylate-buffered (pH 7.4) 2% osmium tetroxide and 0.5% potassium ferrocyanide at 4° for 2 hr. The specimens were dehydrated, immersed in Qr-1, and embedded in Epon epoxy resin. Thin sections were mounted on copper grids and doubly stained with uranyl acetate and lead citrate. The sections were examined in a JEM-100 CX or LEM-2000 electron microscope, and pictures were taken and enlarged photographically as described previously [18].

Short-term assay of cell survival determination. Exponentially growing cells (5×10^4/dish) were plated and incubated overnight at 37°. Medium was then replaced with serum-free MEM, and the cells were incubated for 5 hr with various doses of EGF-PE in the absence or presence of SDB. Then the medium was refreshed with agent-free MEM containing 10% NCS, and incubation was continued for 2 days. The number of viable cells was determined by a Coulter counter as described previously [6].

RESULTS

Enhancement of cytocidal action of EGF-PE. Verapamil and thioridazine, which overcome multiple drug resistance [8, 9], potentiate the cytotoxic effect of EGF-PE [4, 6]. Verapamil also enhances the cytocidal effect of an immunotoxin, a conjugate of anti-transferrin receptor antibody with Pseudomonas exotoxin [5]. SDB restores the sensitivity to anticancer agents of multiple drug resistant KB-Ch^R-24 cells [11]. The chemical structure of SDB was found to be partly similar to that of verapamil (Fig. 1). We thus examined whether SDB could potentiate EGF-PE. The growth of KB-3-1 cells was inhibited by EGF-PE; 50% inhibition of colony formation was observed at 12 ng/ml. SDB was not toxic to KB-3-1 cells at concentration up to 51 μg/ml when it was incubated with cells for 9 days. Therefore, we used 17 and 34 μg/ml SDB with various concentrations of

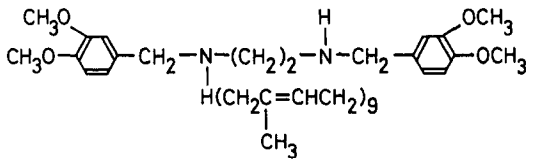

Fig. 1. Chemical structure of *N*-solanesyl-*N*,*N*'-bis(3,4-dimethoxybenzyl)ethylenediamine.

EGF-PE. The cytotoxic activity of EGF-PE was enhanced when combined with SDB (Fig. 2). EGF-PE alone at 12 ng/ml inhibited colony formation by 50%. When combined with SDB, colony formation was inhibited by 50% at concentrations of 2.5 ng/ml EGF-PE (17 μg/ml SDB) and 0.6 ng/ml EGF-PE (34 μg/ml). These results show that SDB enhanced the toxic effect of EGF-PE 5- to 20-fold.

Figure 2 shows the cytotoxic effect of SDB and EGF-PE on colony formation in KB-3-1 cells incubated for 9 days with SDB and EGF-PE. We also tested whether the cytotoxicity of EGF-PE was enhanced by SDB when treated for a short time with EGF-PE in the absence or presence of SDB. KB-3-1 cells were incubated for 5 hr with various doses of EGF-PE in the absence or presence of 34 μg/ml SDB, followed by incubation for 2 days in the absence of any agent (Table 1). The percentage of viable cells was decreased only slightly to 93 ± 3 and 89 ± 2% in the presence of 100 and 500 ng/ml EGF-PE alone. By contrast, the percentage of viable cells was decreased to 69 ± 5 and 46 ± 3% in the presence 34 μg/ml SDB and 100 or 500 ng/ml EGF-PE respectively. SDB alone at 34 μg/ml did not affect cell growth (Table 1). Although enhancement of EGF-PE-induced cytotoxicity by SDB appeared to be less in comparison with that in Fig. 2, apparent enhancement by SDB was observed after treatment for 5 hr.

Effect of SDB on cellular uptake of EGF. EGF-PE binds specifically to EGF-receptor, and the intracellular pathway for EGF-PE appears to be similar to that for EGF itself [4]. The effect of SDB on the metabolism of EGF was examined. SDB at 34 μg/ml did not affect significantly the initial uptake rate of EGF, but the maximum uptake of EGF was about 30% higher in SDB-treated cells than in the absence of the drug (Fig. 3). Figure 3 shows a peak of cell-associated radioactivity at 80 min in the absence of the drug, which then decreased rapidly. When cells were pretreated with the isoprenoid, the cell-associated radioactivity did not fall as rapidly as the control.

Effect of SDB on binding and release of EGF in KB-3-1 cells. To examine in more detail the fate of the growth factor, the kinetics for EGF was followed in both medium and cell-associated fractions (Fig. 4). The binding and release of [^{125}I]EGF by KB-3-1 cells were examined after exposing the cells to [^{125}I]EGF at 4° for 2 hr and then incubating the cells for various times at 37°. Preincubation of KB-3-1 cells with 34 μg/ml SDB for 18 hr did not affect the amount of [^{125}I]EGF bound to KB-3-1 cells. The cell-associated radioactivity in control cells decreased rapidly, and radioactivity in the medium increased concomitantly during incubation at 37°. When KB-3-1 cells were preincubated with 34 μg/ml SDB, cell-associated radioactivity decreased slowly after 40 min of incubation, and the radioactivity in the medium increased slowly. At 140 min, most of the EGF was released into the medium from control cells, but only 60% as much radioactivity was released from the SDB-treated cells, and three times more radioactivity was retained in the treated cells (Fig. 4).

Effect of SDB on the intracellular location of EGF. To investigate a change in intracellular distribution of EGF caused by SDB, experiments comparing the subcellular fractions containing [^{125}I]EGF in control and SDB-treated cells were carried out by using density gradient centrifugation of cell fractions on colloidal silica (Fig. 5). After 5 min of incubation at 37° of both control and drug-treated cells, there appeared a broad peak of high radioactivity at 1.034 g/ml corresponding to the surface membrane fraction. At 20 min, the peak moved to the fraction

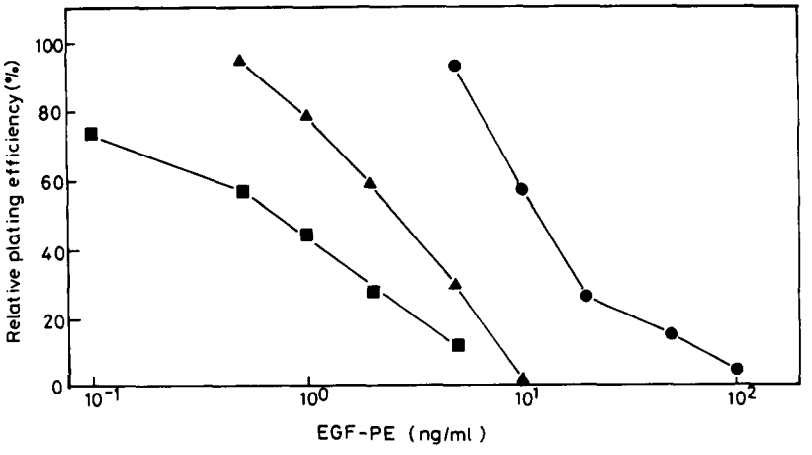

Fig. 2. Dose response of KB-3-1 cells to EGF-PE in the absence or presence of SDB, assayed by colony-forming ability. Cells (300/60 mm dish) were plated and exposed to various concentrations of EGF-PE alone (●) or in combination with 17 μg/ml (▲) or 34 μg/ml (■) SDB for 9 days. Values represent the mean of duplicate determinations.

Table 1. Effect of EGF-PE and SDB on cell growth after short-term treatment

SDB (μg/ml)	Cell survival (%) EGF-PE (ng/ml) 0	50	100	500
0	100	97 ± 1	93 ± 3	89 ± 2
34	100	96 ± 4	69 ± 5	46 ± 3

KB-3-1 cells (5×10^4 per dish) were treated for 5 hr with various doses of EGF-PE in the absence or presence of SDB and incubated for 2 days in the absence of any agents, and then the number of viable cells was determined. Cell number in the absence of EGF-PE corresponding to 100% was 4×10^5 cells per dish in the absence of SDB and 4×10^5 cells per dish in the presence of SDB alone respectively. Relative cell survival (%) is presented when normalized by cell number in the absence of EGF-PE, and each value is the average of duplicate dishes.

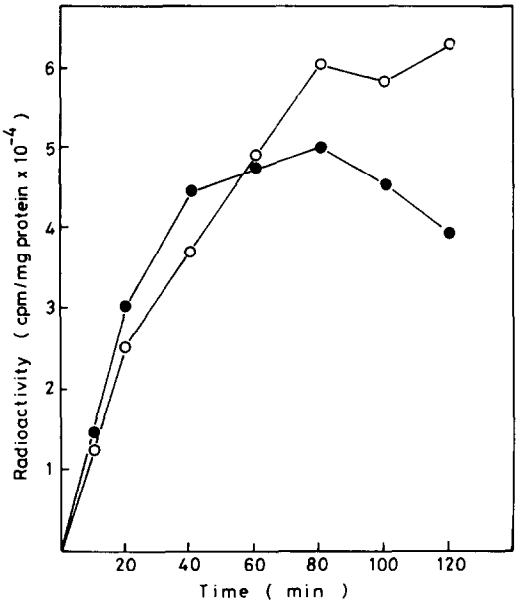

Fig. 3. Effect of pretreatment of KB-3-1 cells with SDB on the cellular uptake of [^{125}I]EGF. KB-3-1 cells were pretreated with 0 μg/ml (●) or 34 μg/ml (○) SDB, and then incubated with [^{125}I]EGF in the absence (●) or presence of 34 μg/ml (○) SDB for the indicated times. Radioactivity was counted after washing with PBS as described in Materials and Methods. Values represent the mean of duplicate determinations.

at 1.043 to 1.046, which was thought to be the endosome/Golgi region. A main peak of radioactivity appeared at 1.054 g/ml, which corresponds to the lysosomal fraction in untreated and treated cells after 60 min of incubation. Radioactivity in the lysosomal regions of untreated cells decreased, and only slight, if any, activity was observed at 120 min. By contrast, in SDB-treated cells significant amounts of radioactivity were observed in the lysosomal region after 120 min of incubation.

The amount of radioactivity of [^{125}I]EGF cosedimenting with lysosomes in Percoll gradients was quantified and expressed as a percentage of the total amount of ligand recovered from the gradient (Fig. 6). At 5 and 20 min, only 8–10% of the total radioactivity on the gradient was detected in the lysosomal fraction of untreated and drug-treated cells. At 60 min, 25 and 31% of total radioactivity accumulated in lysosomes of untreated and treated cells respectively. With time, EGF in lysosomes of untreated cells was degraded and released, and the radioactivity in lysosomes decreased to 10% at 120 min. The percentages from treated cells were 31 and 32% at 90 and 120 min, respectively, maintaining levels similar to those at 60 min, suggesting inhibition of the degradation of EGF in lysosomes by SDB.

The size of [^{125}I]EGF in lysosomes was measured with a PD-10 column (Pharmacia, Sephadex G-10). The peak of radioactivity appeared in the void volume of the column as does intact [^{125}I]EGF, and no other peaks of radioactivity were detected. The result demonstrated that the [^{125}I]EGF that accumulated in lysosomes was not degraded (data not shown).

Effect of SDB on lysosomal pH. The intensity of fluorescence with two different excitation wavelengths and the relative rate of I_{495}/I_{450} were measured. The pH values of the lysosomes were estimated from the standard curve of the relative rate against pH as described previously [17]. The pH value of lysosomes was 5.5 in untreated KB cells. SDB at a concentration of 17–51 μg/ml did not elevate the pH value significantly (Table 2). Addition of a lysosomotropic amine, NH_4Cl, rapidly increased the pH to 6.9.

Ultrastructural change of KB cells exposed to SDB. The lysosomes of SDB-treated cells were examined by electron microscopy. SDB induced characteristic ultrastructural changes in KB-3-1 cells (Fig. 7). Electron-dense bodies that were irregular in size and shape accumulated in KB-3-1 cells when they were treated with more than 17 μg/ml SDB for 24 hr. As shown in Fig. 7, the electron-dense bodies exhibited lamellated figures. An increased number of the dense bodies developed in the cells when they were treated with 51 μg/ml SDB for 24 hr (Fig. 7D). It was also found that more than 34 μg/ml SDB caused the appearance of dilated endoplasmic reticulum. Other organelles like mitochondria and nuclei appeared to maintain their normal structure in SDB-treated cells.

DISCUSSION

In our previous study, we found that verapamil potentiates the toxicity of EGF-PE and immunotoxin [4]. A disturbance of lysosomal function is a possible mechanism of action of verapamil in potentiating immunotoxin activity [5]. However, clinical application of calcium channel blockers such as verapamil may be difficult because of their induction of cardiac arrhythmias. Accordingly, we newly synthesized an isoprenoid, SDB, which carries 9 isoprene chains and has a verapamil-like structure but is not an effective calcium channel blocker in guinea pig ventricular myocytes [12]. Since KB-3-1 did not show any voltage-sensitive calcium channel activity (unpublished data), we did not test whether SDB affected the calcium channel in KB-3-1 cells. In the present study, we found that SDB potentiated the toxicity of the chimeric toxin, EGF-PE, as does

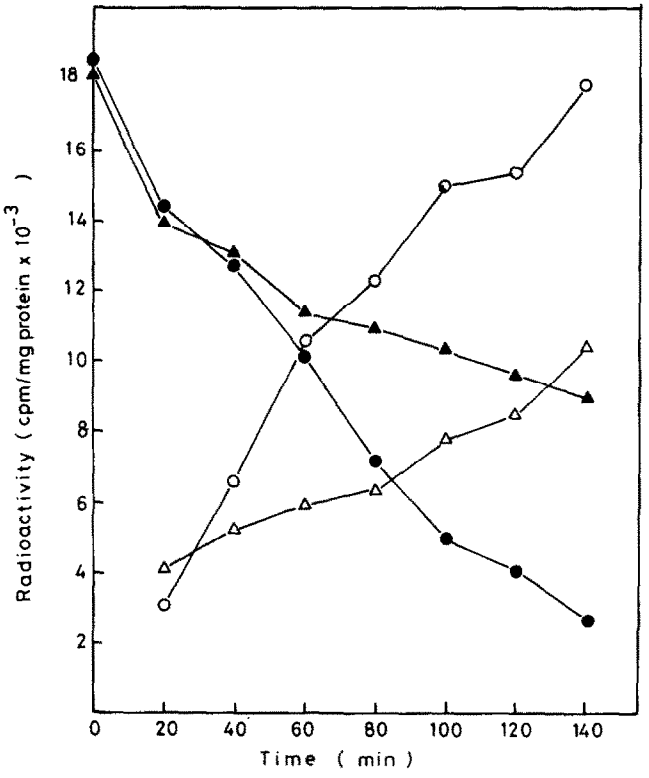

Fig. 4. Effect of SDB on the binding and release of [^{125}I]EGF. KB-3-1 cells were preincubated without (●, ○) or with (▲, △) 34 μg/ml SDB, and then incubated with [^{125}I]EGF at 4°, washed, and warmed to 37° for the indicated periods of time. Total activity in the cell homogenates (●, ▲) and in the medium (○, △) was determined at each time point. Values represent the mean of duplicate determinations.

verapamil. SDB inhibited the degradation of [^{125}I]EGF and the release of degradation products from KB-3-1 cells. In a previous study, thioridazine, a phenothiazine calmodulin inhibitor, was found to enhance the cytotoxicity of EGF-PE [6]. Thioridazine delays both the appearance of [^{125}I]EGF in lysosomes and the disappearance of ^{125}I-EGF from the lysosomes. The potentiation of EGF-PE by thioridazine is discussed in relation to altered lysosomal function in the thioridazine-treated cells. Verapamil, SDB, and thioridazine are all weak bases and seem to belong to lysosomotropic agents.

Several morphological changes were observed after 24-hr treatment with SDB, including dilated vacuoles (endoplasmic reticulum-like structures) and an increased number of lysosome-like structures. To examine cytocidal activity of EGF-PE, colony forming ability in the absence or presence of SDB was determined after incubation for 9 days. By contrast, several experiments including morphological or other assays were done after incubation of the cells for a relatively short period up to 24 hr. We thus determined the effect of the combination of SDB and EGF-PE on cytotoxicity when the cells were treated for 5 hr with EGF-PE in the absence or presence of SDB (Table 1). The short-term assay also showed potentiation of EGF-PE by SDB. These data suggest that the morphological changes of lysosomes or the retardation of EGF-turnover in lysosomes is somehow correlated with SDB-induced potentiation of EGF-PE. The lysosome, which contains more than 40 hydrolytic enzymes, is an important site for intracellular digestion. Intracellular membranes supply lipids or phospholipids to lysosomes through the process of autophagy, and these lipids are thought to be processed and degraded in lysosomes [19]. Interference with lysosomal function, in particular with lipid metabolism, by lysosomotropic agents may cause the appearance of intracellular multimeric vesicles. The intracellular multimeric structures, or lipidosis, have been observed in animals and cultured animal cells that have been treated with lysosomotropic agents such as diethylaminoethoxyhexestrol, chloroquine, thioridazine or inhibitors of thiol-proteases [6, 8, 20–22]. The presence of many autophagosome/lysosome-like vesicles in the cytoplasm in SDB-treated cells may also be caused by delayed digestion of cellular membrane components through inhibition of lysosomal function by SDB. The formation of the aberrant vacuoles has been observed also in thioridazine-treated HeLa cells [6] and intact rats [23]. These data may indicate the ability of these putative lysosomotropic agents to induce lysosomal storage of polar lipids (drug-induced lipidosis). A hypothesis to explain the molecular mechanisms for these phenomena has been presented by Lüllman *et al.* [24].

Lysosomal enzymes have acid pH optima and are active at about pH 5.0 in lysosomes. Lysosomotropic

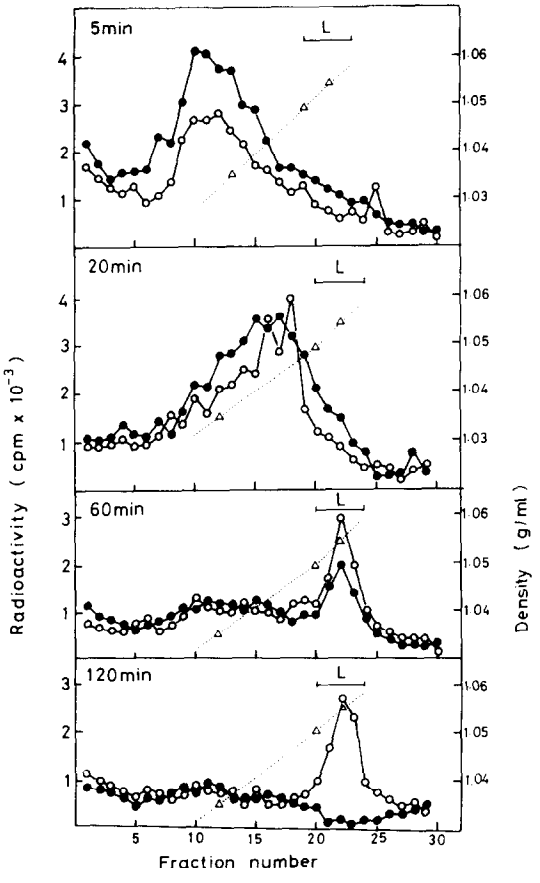

Fig. 5. Kinetics of transfer of [^{125}I]EGF between intracellular compartments as determined by equilibrium density centrifugation and the effect of SDB. Cells were preincubated without (●) or with (○) SDB, and incubated with [^{125}I]EGF at 4°. Cells were washed and warmed to 37° for various times. After homogenization and fractionation on Percoll gradients, ^{125}I-radioactivity was determined. Density marker beads were used to determine the gradient density.

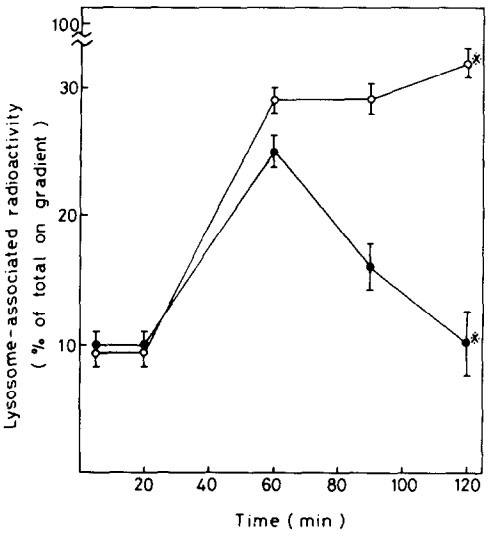

Fig. 6. Kinetics of the appearance of EGF in lysosomes. Using [^{125}I]EGF, the amount of radioactivity cosedimenting with lysosomes from control (●) and SDB-treated cells (○) in Percoll gradients was quantified and expressed as a percent of the total amount of ligand recovered from the gradient. Data points with error bars represent the mean and SE of three separate experiments. An asterisk denotes $P < 0.005$.

Table 2. Effect of SDB on pH in lysosomes of KB cells

SDB (μg/ml)	pH
0	5.5
17	5.3
34	5.2
51	5.5

Measurement of pH in lysosomes was done as described in Materials and Methods. The cells were incubated continuously for 24 hr with SDB at the indicated concentration before the pH assay. Each value represents the mean of duplicate determinations.

amines rapidly increase lysosomal pH as well as endosomal pH [15, 25], resulting in inhibition of lysosomal enzyme activity. When the pH in the acid compartment is raised by chloroquine or NH_4Cl, degradation of diphtheria toxin [26] and EGF [1, 27] is inhibited. Chloroquine enhances the inhibition of protein synthesis when mouse 3T3 cells are exposed to EGF conjugated to ricin A chain and chloroquine [28]. Chloroquine also potentiates the killing action on human acute lymphoblastic leukemia cell lines by anti-T cell antibody conjugated to pokeweed antiviral protein [29]. One could thus expect alteration of lysosomal pH in SDB-treated cells, but we could not observe any effect of SDB on lysosome pH (Table 2). Thiol-proteases such as cathepsins are representative enzymes of lysosomes. These enzymes are possible candidates for degradation of EGF-PE and EGF, and SDB may block the activity of these degradating enzymes. Intracellular degradation of EGF was blocked almost completely in the cultured cells treated with leupeptin, an inhibitor of thiol-protease (unpublished data). The cytosol activities of cathepsin B and D of 17 and 34 μg/ml SDB-treated cells were assayed *in vitro*. We found levels of these lysosome enzymes in the SDB-treated cells to be similar to those in untreated cells (data not shown). In this assay system, however, SDB that accumulates in lysosomes may leak from the lysosomes during the extraction of the cytosol fraction and, thereby, restore the enzyme activities. EGF accumulated as its undegraded form in lysosomes of SDB-treated cells (Fig. 5). This result suggests that the degradation of EGF-PE was also retarded in lysosomes of the SDB-treated cells. The chimeric toxin probably remains intact longer in the lysosomes of the SDB-treated cells, increasing the probability that the toxin will escape into the cytosol, inhibit protein synthesis, and result in cell death.

SDB, as well as verapamil and thioridazine, is cationic and amphipathic. It was shown that a strong interaction occurs between cationic amphipathic drugs and certain polar lipids resulting in complexes formed by hydrophobic and electrostatic forces [30].

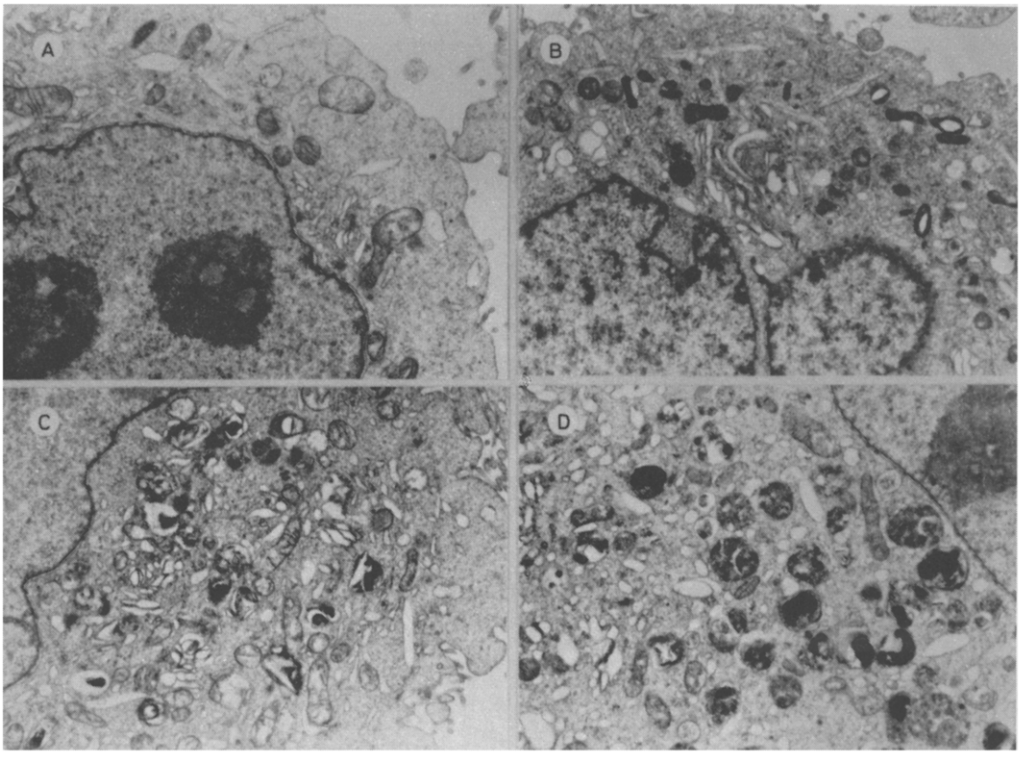

Fig. 7. Electron microscopy of a thin section of KB-3-1 cells cultured with or without SDB. Key: (A) untreated; (B) treated with 17 μg/ml SDB for 24 hr; (C) treated with 34 μg/ml SDB for 24 hr; and (D) treated with 51 μg/ml SDB for 24 hr. Magnification: × 9,775.

We found recently that lysosomotropic amines like chloroquine, propranolol, atropine, amantadine and nicotine can also reverse the multiple drug resistance in human multidrug-resistant KB carcinoma cells in culture [31]. The circumvention of the multidrug resistance by these agents may be correlated somehow with their perturbing effect on membrane, but the precise mechanism remains to be studied.

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