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Inhibition of Intracellular Bleomycin Hydrolase Activity by E-64 Leads to the Potentiation of the Cytotoxicity of Peplomycin against Chinese Hamster Lung Cells

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N-(N-(L-3-*trans*-carboxyoxiran-2-carbonyl)-L-leucyl)-agmatine (E-64), a thiol protease inhibitor, potentiated the cytotoxicity of peplomycin against the Chinese hamster lung (V79) cell. After the treatment of the cells with E-64 (50 μg/ml) for 12 h, bleomycin hydrolase activity of the cells was almost completely inhibited. V79 cells treated with [^{3}H]peplomycin for 24 h in the presence of E-64 (50 μg/ml) accumulated twice as much [^{3}H]peplomycin and five times less [^{3}H]desamidopeplomycin compared with V79 cells treated in the absence of E-64. These results suggest that E-64 increases the sensitivity of V79 cells to peplomycin probably by inhibiting the intracellular bleomycin hydrolase activity.

Key words: Peplomycin — E-64 — Synergism — Bleomycin hydrolase

Bleomycin (BLM)[2] shows remarkable therapeutic efficacy in patients with squamous cell carcinoma and malignant lymphoma. The drug is inactivated by BLM hydrolase (BLMase), which hydrolyzes the carboxamide bond in the pyrimidoblamic acid moiety of the BLM molecule.[1,2] As reported in a preceding paper, we successfully purified BLMase from rabbit liver tissues using a monoclonal antibody.[3] Sebti *et al.* purified BLMase from the rabbit lung tissues.[4] We, moreover, found that BLMase activity is inhibited by E-64,[3] which was isolated from the extracts of *Aspergillus japonicus* grown on a solid medium.[5] There are publications showing that the cytotoxic activity of several antitumor agents is significantly potentiated when the agents are combined with some membrane-active agents. Verapamil and isoprenoids or amphotericin B were demonstrated to enhance the cytotoxicity of some antitumor agents by decreasing their efflux,[6,7] or by increasing their influx,[8] respectively. Another type of antitumor combination might be a drug that enhances the effectiveness of an antitumor drug by interfering with its metabolism, but is not itself an antitumor drug. Expecting such a synergistic combination effect, we have examined possible potentiation by E-64 of the cytotoxicity of peplomycin (PEP), a BLM derivative, against V79 cells, which have high BLMase activity in the cytosol.

MATERIALS AND METHODS

Materials PEP and [^{3}H]PEP (phenyl-*m*-[^{3}H], 250 μCi/mg)[9] were kindly provided by Dr. T. Takita of Nippon Kayaku Co., Tokyo. E-64 was obtained from the Peptide Institute, Osaka. A V79 cell line was generously given by Prof. S. Okada, Faculty of Medicine, University of Tokyo. McCoy 5A medium, calf serum, and fetal calf serum were supplied by GIBCO Laboratories, Chagrin Falls, OH.

Drug sensitivity by colony formation Drug sensitivity was measured by plating 200 V79 cells in each well of 6-well plastic plates containing 2 ml of McCoy 5A medium supplemented with 10% calf serum and 5% fetal calf serum and various concentrations of PEP and E-64 dissolved in PBS(20 μl). After incubation at 37°C for 7 days, the plates were stained with 0.1% crystal violet and colonies were counted.

Assay of BLMase activity The enzyme activity was determined by using high-performance liquid chromatography (HPLC).[10,11] Six microliters of a PEP solution (16 mg/ml) was mixed with 54 μl of the 105,000*g* supernatant (0.5 mg protein/ml) of V79 cell homogenate prepared in 10 m*M* phosphate buffer (pH 7.4). The mixture was incubated at 37°C for 2 h, and then 180 μl of ice-cold methanol and 0.6 mg of cupric carbonate were added to terminate the reaction. After centrifugation for 5 min at 10,000*g*, the supernatant was recovered and injected into an Aquasil SS-352N HPLC column (4.6 ×250 mm), which was equilibrated and eluted with a mobile phase consisting of methanol-acetonitrile-20% ammonium acetate-acetic acid (560:440:100:0.5) at room temperature. Two peaks with the retention times of 7 and 10 min (flow rate 1.5 ml/min) were found in the eluate, and identified as PEP and desamido-PEP, respectively. The quantities of PEP and desamido-PEP were separately determined from the intensity of ultraviolet absorption at 290 nm. The amount of BLMase that hydrolyzes 1 μg of PEP/min at 37°C was defined as 1 unit.

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[2] Abbreviations: BLM, bleomycin; BLMase; BLM hydrolase, PEP, peplomycin; HPLC, high-performance liquid chromatography.

Labeling of V79 cells with [^{3}H]PEP The V79 cells at a concentration of ca. 5×10^6 cells in each 100-mm-diameter dishes were labeled with [^{3}H]PEP (0.2 μg/ml) in culture medium at 37°C for various intervals. Then the cells were trypsinized, harvested by centrifugation and rapidly washed twice with cold PBS and lysed with 2 ml of methanol. The extract with methanol was centrifuged for 5 min at 10,000g, and the resultant supernatant was concentrated in an evaporator and injected into a HPLC column as described above. The radioactivities of [^{3}H]-PEP and desamido-[^{3}H]PEP were determined separately in a liquid scintillation spectrometer.

RESULTS

Effect of E-64 on the activity of BLMase extracted from V79 cells The BLMase activity of the cellular extracts prepared from the V79 cells was significantly inhibited by E-64 (Fig. 1). Fifty percent inhibition was produced by 0.5 μg/ml of E-64 and complete inhibition by 10 μg/ml of the protease inhibitor. Even after E-64 had been removed from the reaction mixture by dialysis, no BLMase activity was recovered (data not shown), suggesting that BLMase activity was irreversibly inhibited by E-64.

Effect of E-64 on PEP cytotoxicity against V79 cells The cytotoxicity of PEP toward V79 cells was enhanced in the presence of 50 μg/ml of E-64 (Fig. 2). When exposing cultures of V79 cells to PEP in a single use, 1.1 μg/ml of the drug was required to reduce the surviving fraction of the cell population to a level of one-half of that of untreated control cultures. A similar extent of cytotoxic effect was induced by 0.36 μg/ml of PEP when combined with 50 μg/ml of E-64, that itself had no cytotoxicity.

Effect of E-64 on BLMase activity in V79 cells Table I shows the results of experiments in which the BLMase activity of cellular extracts prepared from V79 cells that had been treated with 50 μg/ml of E-64 for 12 h was compared with that from untreated control cells. A substantial BLMase activity of 3.8 unit/mg protein was recovered from the untreated cells, whereas the enzymatic activity was scarcely detected from the E-64-treated cells.

Subsequently, we attempted to examine whether metabolic degradation by V79 cells of PEP taken up from the ambient medium with resultant formation of its inactive metabolite, desamido-PEP, is inhibited when E-64 is added in combination. For this purpose, experiments were conducted as follows: V79 cells were incubated with 0.2 μg/ml of [^{3}H]PEP for various intervals up to 24 h in

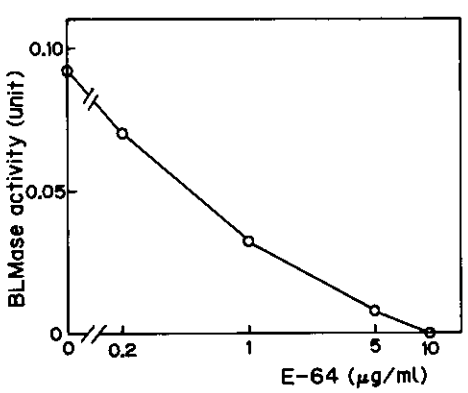

Fig. 1. Effect of E-64 on BLMase activity from V79 cells. V79 cells were homogenized with 10 mM phosphate buffer (pH 7.4) and the homogenate was centrifuged at 105,000g for 1 h. E-64 at various concentrations was added to the resulting supernatants, mixed thoroughly, and incubated at 37°C for 2 h. Then BLMase activity in the mixture was assayed in the presence of E-64 as described under "Materials and Methods."

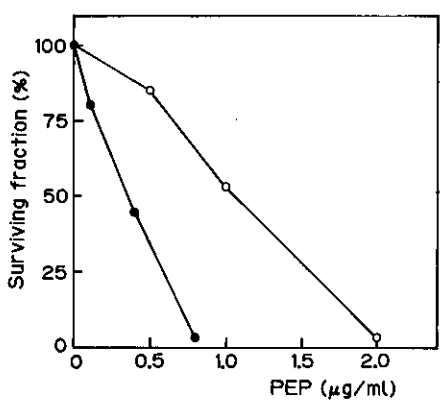

Fig. 2. Survival curves of V79 cells treated with PEP alone or in combination with E-64. Colonies of V79 cells treated with various concentrations of PEP alone (○) and in combination with E-64 (●) were counted.

Table I. BLMase Activity Recovered from V79 Cells Treated with E-64 and Untreated Control Cells

Treated with	BLMase activity[a] (unit/mg protein)
none	3.8
E-64	0.2

a) V79 cells incubated with or without 50 μg/ml of E-64 for 12 h were homogenized with 10 mM phosphate buffer (pH 7.4). The homogenates were centrifuged at 105,000g for 1 h, and BLMase activity in the supernatant was assayed as described in "Materials and Methods."

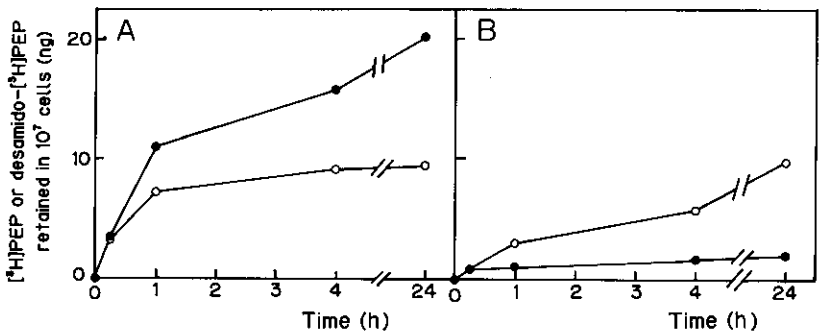

Fig. 3. Intracellular content of [^{3}H]PEP (panel A) and desamido-[^{3}H]PEP (panel B) in E-64-treated and untreated V79 cells. V79 cells were incubated with or without 50 μg/ml of E-64 for 4 h and then labeled with [^{3}H]PEP (0.2 μg/ml). After various times, samples were taken from both E-64-treated cultures (●) and untreated cultures (○) for radioactivity assay of [^{3}H]PEP and desamido-[^{3}H]PEP.

the presence and absence of 50 μg/ml of E-64, and then [^{3}H]PEP and desamido-[^{3}H]PEP was separated chromatographically for radioactivity assay. The treatments of V79 cells with the drugs showed scarcely any cytotoxic effect (data not shown). As illustrated in Fig. 3A, initially, both E-64-treated and untreated cells rapidly took up [^{3}H]PEP for up to 15 min after the onset of incubation, and then incorporation of the drug into the cells continued at decreasing rates for 24 h or longer, irrespective of the presence or absence of E-64. However, in this second phase of incorporation, the intracellular level of [^{3}H]PEP in the E-64-treated cells was always 1.5- to 2-fold higher than that in the untreated cells, when compared at any time point during the incubation period. Consistent with this, the intracellular level of desamido-[^{3}H]PEP in the untreated cells became increasingly higher than that in the E-64-treated cells (Fig. 3B). There was no significant difference in the rate of PEP efflux between the E-64-treated and untreated cells (data not shown).

DISCUSSION

We reported in our preceding papers that E-64 inhibited the enzymatic activity of purified BLMase from rabbit tissues,[3] and that, as compared with Chinese hamster ovary cells and L5178Y cells, V79 cells showed high BLMase activity in the cytosol.[12] Later, we found that E-64 inhibited the enzymatic activity of cellular extracts prepared from the V79 cells. This result leads us to the possibility that the protease inhibitor potentiates the cytotoxicity of PEP against V79 cells. This postulate was supported by the results of the present study showing that a synergistic cytotoxic action is exerted between PEP and E-64 against V79 cells grown *in vitro*.

Since metabolic degradation of PEP into desamido-PEP catalyzed by BLMase occurring in V79 cells was profoundly inhibited by E-64, which was added to the medium together with PEP, the combination effect may be due to the consequent maintenance of higher levels of the active antitumor agent in the cells. This may also be the mechanism by which E-64 potentiates the cytotoxicity of PEP against Ehrlich ascites carcinoma in mice.[13] The combination effect as seen in the present study of the active drug and the inhibitor of the drug-inactivating enzyme is analogous to that with β-lactam antibiotics and an inhibitor of β-lactamase in antibacterial chemotherapy.[14]

It is established that PEP displays remarkable therapeutic activity only toward tumors whose cells contain little BLMase. However, when PEP is combined with some specific BLMase inhibitor, such as E-64, PEP may become therapeutically effective against other species of tumor which are insensitive or resistant to PEP because of high BLMase activity.

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