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CYSTEINE PROTEINASE INHIBITORS AND BLEOMYCIN-SENSITIVE AND -RESISTANT CELLS

GEOFFREY MORRIS, JEHANGIR S. MISTRY, JITESH P. JANI, SAID M. SEBTI and JOHN S. LAZO*

Department of Pharmacology, University of Pittsburgh, School of Medicine, and the Experimental Therapeutics Program, Pittsburgh Cancer Institute, Pittsburgh, PA 15261, U.S.A.

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Abstract—We have isolated a new human head and neck carcinoma cell line (C-10E) that is highly resistant to BLM (40-fold) when compared to the parental (A-253) cell line. Consonant with BLM resistance in the C-10E cell line, we found that this cell line accumulated 2- to 3-fold less BLM A_2 than A-253 cells. Kinetic analyses of BLM A_2 association revealed a decreased V_{max} for C-10E cells with little change in K_a. Furthermore, the BLM-resistant cell line (C-10E) metabolized BLM A_2 to a greater extent than its sensitive counterpart (A-253). Thus, compared to A-253 cells, the C-10E cells exhibited both decreased cellular association and increased metabolism of BLM. Synergistic cytotoxicity was seen when BLM was combined with either E-64 or leupeptin, cysteine proteinase inhibitors known to block BLM metabolism *in vitro*. E-64 inhibited the metabolism of BLM A_2 in both C-10E and A-253 cells, and cellular accumulation of radiolabeled BLM A_2 was increased by leupeptin or E-64 in only A-253 cells. These results suggest that both inhibition of drug metabolism and increased drug accumulation contribute to this synergism.

The bleomycins (BLM†) are a family of related glycopeptide antibiotics derived from *Streptomyces verticillus* [1] and used therapeutically against many solid tumors, including squamous cell carcinoma and malignant lymphoma [2]. The bleomycins are unusual among the cytotoxic anticancer drugs in that they exhibit very little myelosuppressive or immunosuppressive activity; this has led to their popularity, particularly for use in combination with other antineoplastic agents [3]. Unfortunately, one of the major obstacles to chemotherapy has been drug resistance [4]. In an effort to elucidate mechanisms underlying cellular resistance, several cultured cell lines with low levels of BLM resistance have been isolated and these have exhibited either decreased drug association, decreased DNA breakage, or increased drug inactivation [5–7]. With regard to metabolic inactivation, it has been suggested that cells may become resistant to BLM through changes in BLM hydrolase (BH) activity, an enzyme that converts BLM to the inactive metabolite, deamidobleomycin (dBLM; [7–10]).

Recently, BH was identified as a member of the cysteine proteinase family [10]. Cysteine proteinases have been implicated in several important cellular processes including proliferation, cellular migration, polarization and transformation [11–13]. With the advent of highly specific cysteine proteinase inhibitors, it is now feasible to examine directly the effects of enzyme inhibition on BLM metabolism.

E-64 contains an epoxide moiety and inactivates cysteine proteinase irreversibly [14], while leupeptin is a reversible inhibitor of cysteine proteinase [15]. Because of the potential role of BH in BLM resistance, interactions between BLM and inhibitors of this enzyme activity are of interest. We have demonstrated recently that E-64 potentiates the antitumor activity of BLM in human Burkitt's lyphoma xenografts in nude mice by inhibiting BLM metabolism *in vivo* [16]. Nishimura *et al.* [17] reported that E-64 increases the cytotoxic action of peplomycin, a BLM analog, in cultured Chinese hamster lung cells, concomitant with increasing the fraction of unmetabolized drug. It is assumed that inhibition of drug metabolism elevated cellular BLM content. The same laboratory found that either leupeptin or E-64 was effective in prolonging the life span of mice bearing Ehrlich ascites carcinoma cells treated with peplomycin [18]. Because BLM-resistant human tumor cells may have altered BLM metabolism, we have examined the nature of the interaction between cysteine proteinase inhibitors and BLM using a newly characterized, highly BLM-resistant human head and neck squamous cell carcinoma.

MATERIALS AND METHODS

Cells and reagents. A-253 human head and neck epidermoid carcinoma cells were obtained from the

* To whom requests for reprints should be addressed at: E-1346 Biomedical Science Tower, Department of Pharmacology, University of Pittsburgh, School of Medicine, Pittsburgh, PA 15261.

† Abbreviations: BH, bleomycin hydrolase; BLM, bleomycin; dBLM A_2, deamidobleomycin A_2; DMSO, dimethyl sulfoxide; E-64, l-transepoxysuccinylleucylamido(4-guanidino)butane; FBS, fetal bovine serum; IC_{50}, concentration of drug which inhibits cell growth to 50% of control; K_a, association constant; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBS, phosphate-buffered saline; Tris, tris-hydroxymethylaminomethane; and V_{max}, maximal velocity.

American Type Culture Collection (Rockville, MD) and were grown in McCoy's Medium (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (HyClone, Logan, UT) as previously described [19]. Cells were maintained at 37° in a humidifed incubator with a 95% air, 5% CO_2 atmosphere. The C-10E (BLM-resistant) cell line was derived from C-10 cells [19] by stepwise increase in BLM concentration added to the cultures. This process required 24 months, at which time cell proliferation was unaffected by continuous culturing of cells in 70 ng/mL (50 nM) of BLM. E-64, leupeptin, MTT and DMSO were obtained from the Sigma Chemical Co. (St. Louis, MO). Vincristine sulfate was a gift from Eli Lilly (Indianapolis, IN). Bleomycin A_2 was purified from BLM (Blenoxane; Bristol-Myers Squibb Co., Wallingford, CT) as described previously [20]. [*S*-methyl-^{3}H]BLM A_2 (sp. act. 78.4 Ci/mmol) was purchased from DuPont NEN Research Products (Boston, MA).

Growth inhibition studies. The effects of agents on cellular proliferation were determined using the previously described MTT microculture assay [19]. Briefly, exponentially growing cells were rinsed with PBS and harvested by treatment with trypsin (0.125%, w/v) and centrifugation at 200 *g* for 2 min. The cell pellet was resuspended in McCoy's modified medium with 10% fetal bovine serum (FBS). Cells were counted with a hemacytometer and seeded at a density of 2000 per well in 100 μL volume into 96-well microtiter plates (Costar, Cambridge, MA) using a multi-channel pipet. After 3 hr, drugs (100 μL) were added in serial dilutions to the wells. Drug ratios were chosen to approximate equitoxic concentrations of antineoplastic agent and proteinase inhibitor. After 4 days of growth in the presence or absence of drug(s), the medium was replaced with 100 μL of MTT (1 mg/mL) in McCoy's modified medium with 10% FBS. The tetrazolium/formazan reaction was allowed to proceed for 3 hr at 37°, after which time the solution containing the unreacted MTT was removed and replaced with 100 μL DMSO. After 5 min of shaking to solubilize all dye, the absorbance at wavelength 540 nm was determined spectrophotometrically (Titertek Multiskan; Flow Laboratories, McClean, VA). To evaluate possible drug interactions, isobolograms were generated for each drug combination using the dose-effect software package obtained from Elsevier-BIOSOFT (Cambridge, U.K.; [21]). Each isobole point was obtained by interpolation from a separate experiment designed to determine the concentration–response for each drug separately and for the drug combination.

Cellular drug association studies. The effect of proteinase inhibitor pretreatment on cellular association of [^{3}H]BLM A_2 was determined by previously described methods [19]. Exponentially growing A-253 cells were preincubated at 37° with E-64 (2 mM) or leupeptin (2 mM) for 72 hr prior to exposure to 1 μM [^{3}H]BLM A_2 for various time intervals to ensure complete enzyme inhibition. Drug incubation was terminated by pipetting 100 μL of cell suspension into 10 mL of ice-cold PBS, followed by centrifugation at 12,000 *g* for 30 sec. The resulting cell pellet was resuspended and centrifuged through an oil–aqueous layer as previously described [19]. For kinetic analyses, cells were incubated with radiolabeled BLM A_2 for 30 sec; the concentration of BLM A_2 was varied from 3.33 to 100 μM, and the amount of radioactivity associated with the cellular fraction was calculated for each drug concentration. Binding studies were performed at 4° in the same manner as the drug association studies.

Metabolism studies. Exponentially growing A-253 and C-10E cells were preincubated with or without E-64 (2 mM) for 72 hr. After preincubation, control and E-64-pretreated cells were treated with 1 μM [^{3}H]BLM A_2 (0.50 μCi/mL; sp. act. 78.4 Ci/mmol) and further incubated for 1 or 4 hr. The radioactive medium was removed and the cells were washed twice with 10 mL of ice-cold PBS. Cells were then harvested by treatment with PBS containing trypsin (0.05%, w/v) and EDTA (2 mM) and centrifuged at 200 *g*. Cell pellets were washed once with 5 mL of ice-cold PBS and homogenized in 1 mL of 20 mM ice-cold Tris–HCl (pH 6.5). Proteins were precipitated with 6 M ice-cold trichloroacetic acid. After addition of an equal volume of 0.5 M tri-*n*-octylamine in 1,1,2-trichlorotrifluoroethane to the supernatant fraction, the aqueous phase was filtered through a 0.2 μm ACRO filter, concentrated, and subjected to reverse phase ion-pair HPLC analysis as described earlier [20].

RESULTS

Resistant cell line. To address the problem of drug resistance at the cellular level, we developed a human cell line with a higher degree of resistance than previously reported [6, 7, 19, 22]. The C-10E cell line was generated from the previously described 4-fold BLM-resistant C-10 cell line (Materials and Methods), which was derived from the parental (A-253) cell line by mutagenesis and subsequent stepwise increase in BLM exposure [19]. The C-10E line displayed a 40-fold resistance when compared to A-253 cells over a wide range of BLM A_2 concentrations (Fig. 1). Table 1 summarizes the relative sensitivities

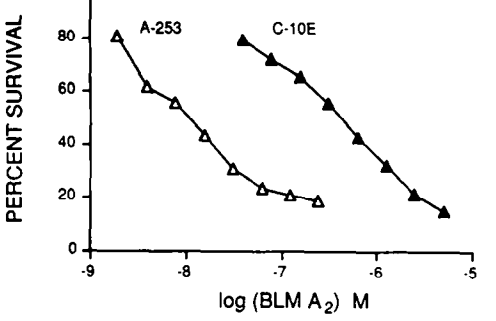

Fig. 1. Concentration–response of A-253 and C-10E cells to BLM. Cells were seeded in 96-well plates at a density of 2000 cells/well in 200 μL of McCoy's medium supplemented with 10% FBS. BLM was added after 2 hr and after 4 days the cell number was determined spectrophotometrically (540 nm) with MTT as described in Materials and Methods. Each point is the average of at least 24 determinations.

Table 1. Drug sensitivities of A-253 and C-10E cells

Drugs	IC_{50} (nM) A-253	IC_{50} (nM) C-10E	Resistance index
Bleomycin	11 ± 0.5	440 ± 49	40*
Doxorubicin	214 ± 4	194 ± 4	0.91
Cisplatin	1190 ± 79	460 ± 9	0.39†
Vincristine	13 ± 0.5	15 ± 0.5	1.15

Cells were exposed to drugs for 4 days and growth inhibition was determined spectrophotometrically as described in Materials and Methods. The resistance index was calculated as the ratio of IC_{50} for C-10E cells compared to the IC_{50} for A-253 cells for each drug. Values are means ± SEM, N = 16 or more individual determinations.

*† Significant differences between A-253 and C-10E cells as determined by Student's *t*-test: * $P < 0.001$, and † $P < 0.05$.

of these two cell lines to a variety of anticancer drugs. Although C-10E cells were 40-fold resistant to BLM A_2, there was no cross-resistance to the structurally dissimilar antitumor drugs tested. C-10E cells also displayed some collateral sensitivity (2.6-fold) to cisplatin. Furthermore, the BLM-resistant phenotype was stable for 2 months in the absence of chronic BLM exposure after which time the cell line began to regain sensitivity to BLM (data not shown). The volume of both cells as measured using an electronic counter was similar for both cell lines (12–16 μm^3).

Cellular association of [^{3}H]*BLM* A_2. To evaluate one possible mechanism of resistance to BLM, we first investigated the importance of cellular drug association to the resistant phenotype. C-10E cells exhibited markedly less cellular association of BLM A_2 when compared to the sensitive A-253 cell line (Fig. 2A). After 10 min, the level of BLM A_2 associated with A-253 cells was nearly double that associated with C-10E cells; this difference increased so that at 30 min, when association plateaued for both cell lines, A-253 cells had nearly 3-fold more radioactivity than C-10E cells. A kinetic analysis of the initial cellular association (Fig. 2B and inset) revealed a higher V_{max} for A-253 cells (183 pmol/10^7 cells/min) than for C-10E cells (105 pmol/10^7 cells/min) with no change in K_a (75.0 vs 74.6 μM). At a concentration of 20 μM BLM A_2 there was a 50% blockade of association with 100-fold excess cold BLM A_2 (data not shown).

Binding of [^{3}H]BLM A_2 at 4° (Fig. 2C) also revealed a pattern similar to cellular association (Fig. 2A). Binding was rapid, with maximal cellular levels occurring within 20 min. The absolute values for binding were lower (approximately 0.5 to 0.25) than for cellular association of drug, and A-253 cells bound over 3-fold more [^{3}H]BLM A_2 than C-10E cells.

Cellular metabolism of [^{3}H]*BLM* A_2. We next determined the intracellular metabolism of BLM in sensitive and resistant phenotypes. Figure 3 shows the HPLC profiles of [^{3}H]BLM A_2 and its metabolites in intact A-253 and C-10E cells after various times of exposure to 1 μM [^{3}H]BLM A_2. After a 1-hr

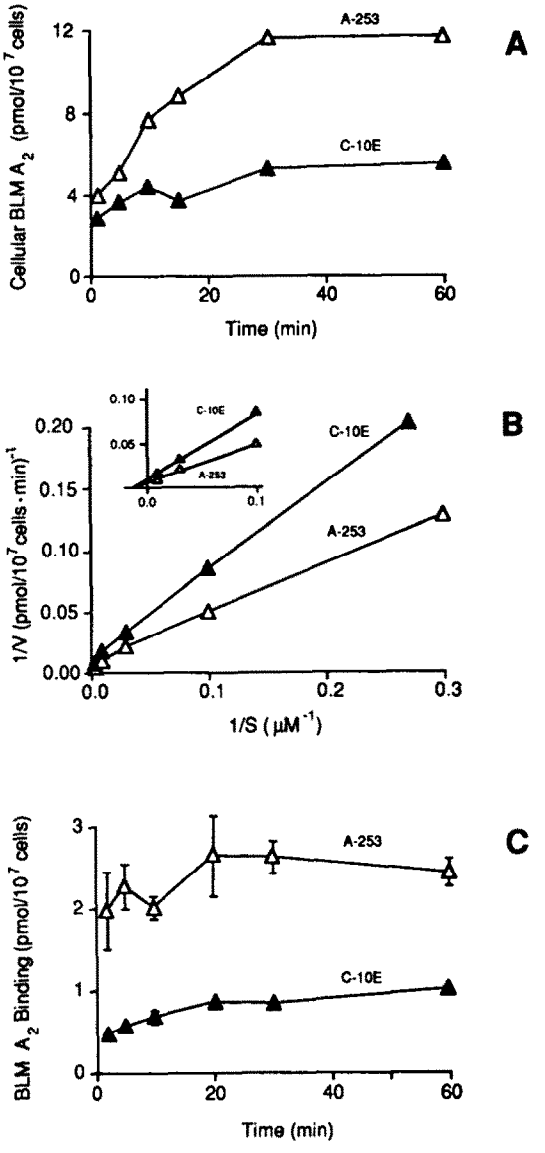

Fig. 2. (A) Cellular association of [^{3}H]BLM A_2 in A-253 and C-10E cells. Cells were exposed to 1 μM [^{3}H]BLM A_2 for various time intervals as described in Materials and Methods. Each point is the average of triplicate determinations. (B) Double-reciprocal plot of [^{3}H]BLM A_2 association in A-253 vs C-10E cells. For kinetic analyses, a single time of incubation with radiolabel of 30 sec was selected. The concentration of BLM A_2 ranged from 3.33 to 333.3 μM, and the assay for cellular association was performed as described in Materials and Methods. The equation for the best-fit line through the double-reciprocal graph (inset) was used to determine the V_{max} and K_a values for cellular drug association. The data are representative of two experiments. (C) Binding of [^{3}H]BLM A_2 in A-253 and C-10E cells. After cells were harvested, McCoy's medium chilled to 4° containing radioactive 1 μM [^{3}H]BLM A_2 was added. At various time intervals aliquots were removed, washed with ice-cold PBS, suspended in medium chilled to 4° and centrifuged at 9000 *g* in a microcentrifuge tube containing perchloric acid/silicone oil/mineral oil mixture. Each point is the average of six determinations; the SEM is included within the symbols unless noted by bars.

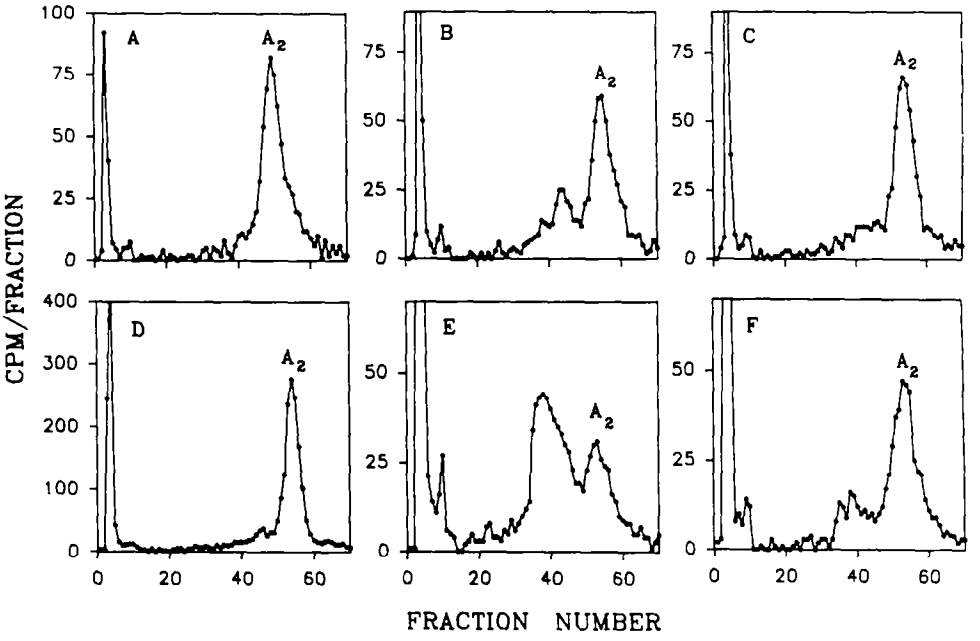

Fig. 3. Metabolism of [^{3}H]BLM A_2 in cultured A-253 and C-10E cells. A-253 cells (A, B and C) and C-10E cells (D, E and F) were treated with 1 μM [^{3}H]BLM A_2 for either 1 hr (A and D) or 4 hr (B, C, E and F). Prior to [^{3}H]BLM A_2 treatment the cells were incubated in the absence (B and E) or presence of 2 mM E-64 (C and F) for 72 hr. After BLM A_2 treatment all cells were harvested and homogenized, and the radioactivity was extracted as described in Materials and Methods. The radioactive material was then separated with a C-8 reverse phase HPLC column as described in Materials and Methods. The greatest cpm for each sample occurred in the solvent front and was: (A) 91.8 cpm, (B) 179 cpm, (C) 163 cpm, (D) 459 cpm, (E) 747 cpm and (F) 434 cpm. BLM A_2 designates the elution position of an authentic standard of BLM A_2.

incubation, there was little metabolism of drug by either cell line (Fig. 3, A and D). The only prominent peak seen other than BLM A_2 was found in the void volume (fractions 3–8) and was present in all samples at approximately the same percentage. After a 4-hr incubation with [^{3}H]BLM A_2, two additional peaks (fractions 9–11 and 36–45) were seen in A-253 cells (Fig. 3B); the majority of the retained radioactivity, however, remained in the unmetabolized BLM A_2 peak. In contrast, exposure of C-10E cells to [^{3}H]BLM A_2 for 4 hr resulted in more extensive metabolism and the majority of the radioactivity was in fractions 36–45, with a smaller peak corresponding to unmetabolized BLM A_2 (Fig. 3E). Based on the elution positions of authentic standards, dBLM A_2 (fractions 23–25), the only BLM A_2 metabolite that has been characterized, was not detected in either A-253 or C-10E cells. The chemical nature of these putative metabolites is not yet known due to small amounts that are available. It is interesting to note, however, that similar metabolites were suggested previously in non-malignant tissues *in vivo* [20], although they are much better resolved with our current HPLC methods. Our preliminary data indicate these metabolites are not able to degrade DNA [16].

Drug interaction studies. Because BLM is thought to be inactivated by a cysteine proteinase, BH, we next examined cell survival in the presence of both BLM and inhibitors of cysteine proteinases. Incubation of A-253 cells with concentrations of E-64 (2 mM) that alone failed to inhibit A-253 cell proliferation markedly increased the cytotoxicity of BLM (IC_{50} = 88 nM in the absence of E-64; IC_{50} = 44 nM in the presence of E-64). Thus, we formally examined whether E-64 or leupeptin could synergistically interact with BLM. Using drug concentration ratios to simulate roughly equitoxic concentrations of BLM and cysteine proteinase inhibitor, we generated isobolograms at the IC_{50} level for each drug combination. With this model, we observed augmentation of cytotoxicity with combinations of BLM and cysteine proteinase inhibitors in both cell lines (Fig. 4). Points falling below the isoeffective (IC_{50}) line indicate enhancement of cytotoxicity created by the drug combination. The combination of E-64 and BLM A_2 resulted in enhanced cytotoxicity at both drug ratios tested in C-10E cells (Fig. 4A). Similarly, the combination of E-64 and BLM A_2 resulted in enhanced cytotoxicity at all ratios tested in A-253 cells (Fig. 4B). The combination of leupeptin and BLM A_2 resulted in synergism of cytotoxicity at the three ratios tested in A-253 cells (Fig. 4C).

Cysteine proteinase and BLM A_2 metabolism and association. Cysteine proteinase inhibitors, such as E-64, can block the metabolism of BLM by isolated BH [10]. We, therefore, examined the effect of E-64 pretreatment on BLM A_2 metabolism in intact cells. Preincubation of A-253 cells with E-64 (2 mM)

for 72 hr prior to [^{3}H]BLM A_2 completely abolished the major metabolite peak (fractions 36–45, Fig. 3C), with all radioactivity detected in either the BLM A_2 peak, fractions 9–11 or the void volume. Preincubation of C-10E cells with 2 mM E-64 for 72 hr (Fig. 3F) greatly reduced the radioactivity in fractions 36–45. When the percent of total radioactivity for each peak was calculated (Fig. 5), a similar pattern emerged. Peaks 1 (solvent front) and 2 (minor metabolite, fractions 9–11) were not changed by pretreatment with E-64 in either cell line. Peak 3 (major metabolite, fractions 36–45), however, was diminished substantially by E-64 pretreatment in both cell lines, with a corresponding increase in unmetabolized BLM A_2. Thus, E-64 was capable of blocking metabolism of BLM A_2 in both sensitive and resistant cell lines.

We next examined the effect of E-64 on cellular association of [^{3}H]BLM A_2 in the A-253 and C-10E cell lines. We did find a significant increase in [^{3}H]BLM A_2 association at all time points ($P < 0.005$) when A-253 cells were preincubated with 2 mM E-64 (Fig. 6A). The cellular association of radioactivity in the presence of E-64 was elevated approximately 2-fold and persisted for the full time-course of this study. Pretreatment with 2 mM leupeptin also resulted in a significant increase ($P < 0.05$) in cellular [^{3}H]BLM A_2 in A-253 cells at all time points (Fig. 6B). Association of [^{3}H]BLM A_2 was increased by nearly 2-fold during the first 30 min of incubation. Cellular association was rapid, peaked at 5 min, and declined slightly over the 60-min time-course. In contrast, E-64 pretreatment had no effect on cellular [^{3}H]BLM A_2 association in C-10E cells (Fig. 6C).

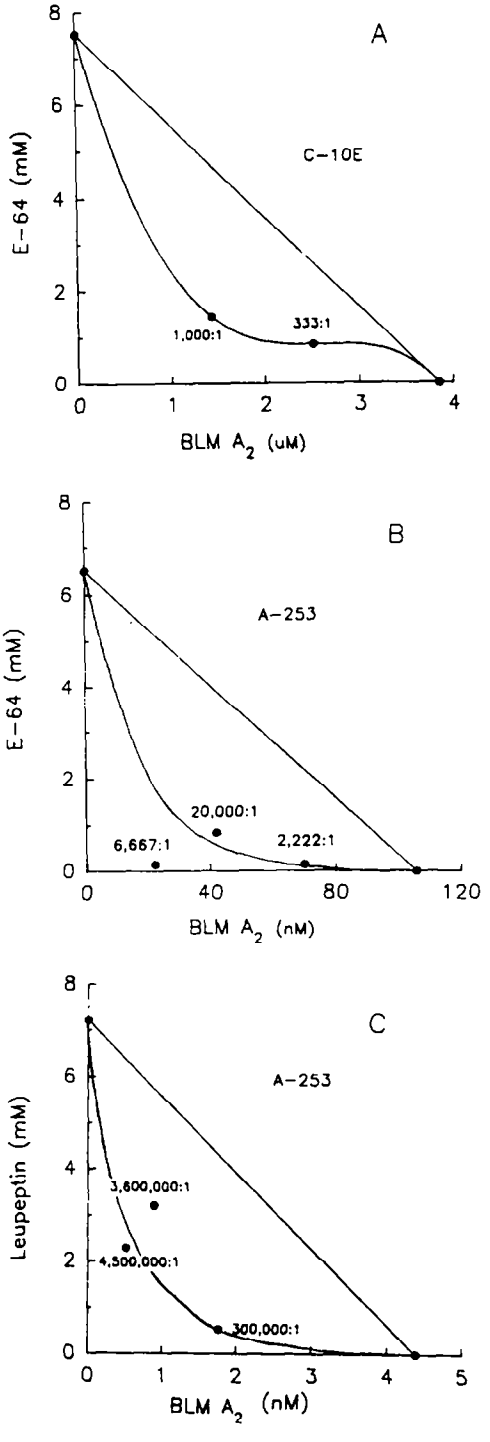

Fig. 4. Synergism with various ratios of cysteine proteinase inhibitors and BLM A_2 in A-253 and C-10E cells. Cells were seeded in microtiter plates and allowed to grow for 4 days in the presence of drug combinations. Cell number was determined spectrophotometrically (540 nm) after staining with MTT. Growth inhibition data were then transformed to IC_{50} isobolograms using the median-effect software package obtained from Elsevier-BIOSOFT (U.K.). Each point is the mean value of three or more determinations, and drug ratios were chosen to bracket equitoxic doses for the two compounds. (A) Synergism with the drug combination of E-64 and BLM A_2 in C-10E cells. The molar drug ratios used were E-64:BLM A_2 of 1,000:1 and 333:1 as indicated. The IC_{50} for E-64 alone in this experiment was 7.5 mM. (B) Synergism to the drug combination of E-64 and BLM A_2 in A-253 cells. Molar drug ratios used were E-64:BLM A_2 of 20,000:1, 6.667:1, or 2,222:1. The IC_{50} for E-64 alone was 6.5 mM. (C) Synergism between leupeptin and BLM A_2 in the growth inhibition assay. A-253 cells were grown in the presence of molar drug ratios of 300,000:1, 3,600,000:1 or 4,500,000:1 for 4 days. The IC_{50} for leupeptin in this experiment was 7.2 mM.

DISCUSSION

We have developed a human squamous carcinoma cell line (C-10E), which is both highly (40-fold) and selectively resistant to BLM. The C-10E cells exhibit two phenotypic properties that distinguish them from the parental A-253 cells: (1) increased metabolism of BLM and (2) decreased cellular association of BLM. The BLM-resistant C-10E cells were not cross-resistant to doxorubicin or vincristine, but were more sensitive to cisplatin (Table 1). Thus, the C-10E cells have acquired a drug resistance profile distinct from cells previously derived from the parental A-253 cell line [19]. We find the collateral sensitivity of C-10E cells to cisplatin intriguing not because there is any apparent mechanistic basis but because BLM is often used with cisplatin and this could have some clinical significance.

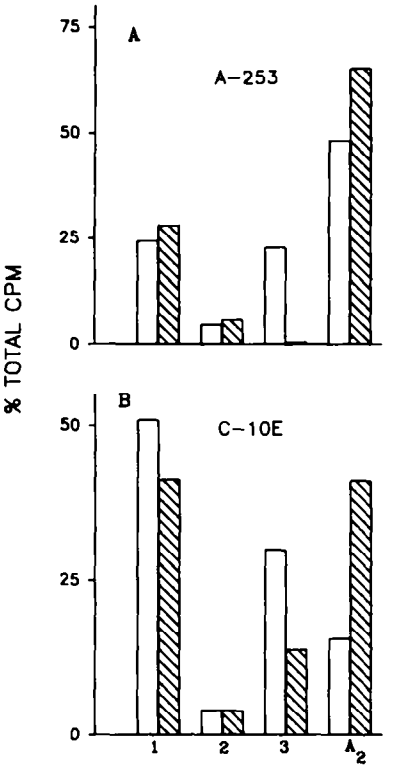

Fig. 5. Effect of E-64 pretreatment on the metabolism of [^{3}H]BLM A_2 in A-253 and C-10E cells. The amount of radioactivity in each peak (from Fig. 3) is expressed as a percentage of the total radioactivity. Peak 1 represents solvent front (fractions 2–5), peak 2 represents minor metabolite (fractions 9–11) and peak 3 represents the major metabolite (fractions 36–45). A_2 represents BLM A_2 (fractions 46–58). (A) Metabolism of [^{3}H]BLM A_2 after a 4-hr incubation with A-253 cells in the absence (open bars) or presence (hatched bars) of 2 mM E-64. The total radioactivity applied was 2063–2760 dpm. (B) Metabolism of [^{3}H]BLM A_2 after a 4-hr incubation with C-10E cells in the absence (open bars) or presence (hatched bars) of 2 mM E-64. Total radioactivity applied in these samples was 3418–4953 dpm.

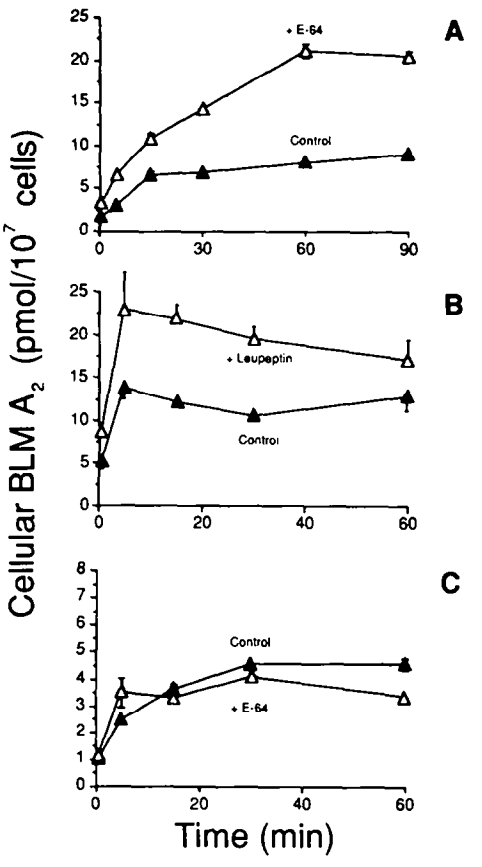

Fig. 6. (A) Enhancement of cellular association of [^{3}H]BLM A_2 in the presence of 2 mM E-64. Control flasks contained subconfluent A-253 cells grown with McCoy's modified medium. Experimental flasks contained the same medium to which 2 mM E-64 was added 3 days prior to assay. Cells were harvested, and the [^{3}H]BLM A_2 association was determined as described in Materials and Methods. (B) Enhancement of radioactive association of [^{3}H]BLM A_2 by leupeptin. A-253 cells were grown in McCoy's medium supplemented with 10% FBS in the presence or absence of 2 mM leupeptin for 3 days prior to harvesting. (C) Effect of E-64 on association of [^{3}H]BLM A_2 in C-10E cells. C-10E cells were grown in the presence or absence of 2 mM E-64 for the 3-day preincubation period. C-10E cell flasks contained no BLM for the preincubation period. Each point in the above figures is the average of six determinations; the SEM is included within the symbols unless noted by bars. Statistical significance was determined between E-64 pretreatment and no pretreatment samples at all time points using an unpaired Student's *t*-test ($P < 0.005$ for panel A and $P < 0.05$ for panel B).

The role of BLM metabolism in tumor cell resistance is controversial. Some investigators [6, 7, 23] have found that increased drug metabolism correlates with BLM resistance, whereas others [19, 24] have not. Recently, we demonstrated that BLM metabolism plays a major role in Burkitt's lymphoma *in vivo* [16]. The HPLC profile (Fig. 3) and histograms (Fig. 5) for BLM metabolism indicated that the BLM-resistant C-10E cell line did possess an increased capacity to metabolize BLM compared to the sensitive (A-253) cell line. Incubation with [^{3}H]BLM A_2 for 4 hr was required for this difference to be detected and the major metabolite (fractions 36–45) for the C-10E cell line did not appear to be the known metabolite produced by BH, dBLM A_2, which eluted earlier (fractions 23–25). The identity of the component(s) within this peak area is unknown but its formation is clearly cysteine proteinase dependent (see below).

We have also found that the resistant (C-10E) cell line accumulated 2- to 3-fold less BLM than did the drug-sensitive (A-253) cell line (Fig. 2). Although change in cellular drug association has been hypothesized as a means of BLM resistance [23–25], the C-10 [19] and C-10E cell lines are the first in which this has been demonstrated. We measured binding at 4° to differentiate between cell surface bound and internalized drug and found cell surface binding was also lower in resistant cells (Fig. 2C); the diminution in cell binding was approximately the same as the decrease in cellular drug association (2-

to 3-fold). Although the mode by which BLM enters cells is not known, cell surface binding has been suggested by previous microscopic [25] and biochemical [26] data. A previous kinetic study of BLM uptake supported a carrier-mediated transport process with a roughly equal number of specific and non-specific binding sites [26]. We also found that a portion of BLM binding could be blocked with excess unlabeled BLM, consistent with the presence of both non-specific and specific drug-binding sites. In the resistant cell line we found no change in K_a for BLM, but could account for the lower steady-state levels of BLM by a 43% decrease in V_{max} for drug association (Fig. 2B, inset). This observation would be consistent with a lower density for acceptor sites for BLM in the resistant (C-10E) cell line. Thus, the C-10E cells have two phenotypic properties that could produce resistance to BLM; we do not believe, however, that these two differences are related because the C-10E cells were derived from cells (C-10), which exhibited only decreased BLM A_2 association [19]. Continued selection pressure with BLM over 2 years has apparently resulted in the expression of a second mechanism of cellular resistance, increased drug metabolism, in the C-10E cell line.

The cysteine proteinase inhibitor, E-64, has been shown to inhibit BH *in vitro* [10, 27], and to potentiate the cytotoxicity of peplomycin against Chinese hamster lung cells in culture [17] and Ehrlich ascites-bearing mice *in vivo* [18]. Using isobologram analyses to evaluate drug interactions, we report here for the first time true pharmacological synergism between BLM and E-64 in both resistant and sensitive human tumor cell lines (Fig. 4). Nishimura *et al.* [17] suggested that potentiation of peplomycin cytotoxicity by E-64 is due to inhibition of BH; these investigators, however, found no significant change in total cellular content of drug (parent drug plus metabolites). The presence of several metabolites (fractions 9–11, 36–45) and the inhibition of the formation of one of these peaks (fractions 36–45) by treatment with E-64 (Figs. 3 and 5) could signal the existence of another cysteine proteinase capable of metabolizing the parent molecule or could indicate further metabolism of dBLM A_2. We are currently investigating the chemical nature and biological activity of these BLM metabolites.

In addition to effects on drug metabolism, E-64 and leupeptin were capable of increasing BLM A_2 cellular association. A-253 cells responded to either E-64 or leupeptin (Fig. 6, A and B) with a substantial increase in cellular BLM association. Surprisingly, the resistant (C-10E) cell line did not (Fig. 6C). This cannot be explained by a differential sensitivity to E-64 since the IC_{50} values for both cell types were similar (5–7 mM). Thus, two separate cellular mechanisms: (1) inhibition of metabolism and (2) enhancement of cellular drug association may account for the synergism seen between cysteine proteinase inhibitors and BLM.

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