In Vivo Circumvention of Human Colon Carcinoma Resistance to Bleomycin

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ABSTRACT

Metabolic inactivation of bleomycin (BLM) by cysteine proteinase-like enzymes is thought to be a major mechanism of BLM tumor resistance. We now report that the human colon carcinoma COLO-205 is highly resistant to BLM and that E-64, a cysteine proteinase inhibitor, sensitizes COLO-205 to BLM. Treatment of COLO-205-bearing nude mice with either E-64 (40 mg/kg) or BLM (10 mg/kg) alone did not inhibit the COLO-205 growth. However, pretreatment with E-64 prior to BLM prevented these xenografts from growing. Analysis by high performance liquid chromatography of in vivo BLM metabolism following [3H]BLM A2 treatment of COLO-205-bearing nude mice showed a different metabolic profile among the various organs and the tumor. Whereas [3H]BLM A2 was the only major radioactive peak detected in sera and tumors, several metabolites, including deamido-BLM, were found in kidney, liver, and lung as early as 15 min. Pretreatment of mice with E-64 inhibited tumor, kidney, and lung BLM metabolism. Furthermore, pretreatment with E-64 increased BLM A2 accumulation in tumors (6.1-fold), kidney (4.0-fold), lung (2.8-fold), liver (1.7-fold), and serum (1.7-fold). E-64 pretreatment did not enhance the major toxicity of BLM, pulmonary fibrosis, as determined by both lung hydroxyproline levels and histopathology. Thus, the cysteine proteinase inhibitor E-64 affects the metabolic fate and the levels of accumulation of BLM in tumors (6.1-fold), kidney (4.0-fold), lung (2.8-fold), liver (1.8-fold), and serum (1.7-fold). E-64 pretreatment did not enhance the major side effect of BLM, suggesting a possible clinical use of this combination therapy.

INTRODUCTION

Intrinsic and acquired resistance to antineoplastic drugs is a major obstacle in the successful treatment of most human cancers (1, 5). The antitumor antibiotic BLM3 is used to treat human malignancies (4), but its clinical use is limited due to drug resistance (2, 3, 6, 7) and the dose-dependent production of pulmonary fibrosis (2, 8). Several mechanisms have been proposed to define the biochemical basis of BLM resistance, i.e., decreased drug accumulation, increased metabolic inactivation, and increased DNA repair (2, 3, 6, 9–11). Umezawa et al. (5, 12–14) postulated that hydrolysis of BLM to deamido-BLM, an inactivating metabolite, by the enzyme BLM hydrolase may be involved in tumor resistance. However, due to difficulties in the purification and characterization of this labile enzyme, no further attempts to link BLM hydrolase and human tumor resistance to BLM were pursued. Recently, we (15–18) and others (19) purified to homogeneity and characterized bleomycin hydrolase. Our molecular cloning and biochemical studies revealed that BLM hydrolase is a cysteine proteinase with cathepsin H-like substrate specificity and is inhibited by cysteine proteinase-specific inhibitors, such as E-64 (17).

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3 The abbreviations used are: BLM, bleomycin; E-64, L-trans-epoxy succinyl-Leu-Val-Cys(OMe)-4-guanidino)butane; HPLC, high performance liquid chromatography.
combination of E-64 and BLM. Five weeks after the last injection, mice were sacrificed and right lung collagen content was determined by colorimetric assay of hydroxyproline, as previously described (19). Left lungs were excised, immersion-fixed in formaldehyde, and embedded in methacrylate. Complete sections through individual lobes were cut at a thickness of 1–2 μm, mounted on glass slides, and stained in methylene blue. They were viewed in a Nikon Microphot photomicroscope. For pathological grading, slides were mixed randomly (one slide/mouse), assigned arbitrary code numbers, and examined by an independent observer. Each slide was graded on a scale of 0 to 3+, for the following features: (a) perivascular cellular infiltrate; (b) microvascular and interstitial cellular infiltrate; (c) alveolar macrophages; and (d) organized lesions and fibrosis.

**In Vitro Isolation and Biological Characterization of the Major BLM Metabolic Component in Colon Carcinoma.** Cultured colon carcinoma (COLO-205) cells were harvested and homogenized in 20 mm Tris-HCl (pH 7.5), and the 105,000 x g supernatant fraction was isolated. One mg of protein from the supernatant fraction was incubated with 500 μg of BLM A2 and 2 μCi of Cu²⁺-free [³H]BLM A2 (specific activity, 78.4 Ci/mmol) at 37°C for 21 h. The details of isolation of BLM metabolites have been described previously (20). The biological activity of the major BLM component, which eluted with the column dead volume, was determined by its ability to degrade plasmid DNA, as described earlier (20). Briefly, various dilutions of samples and standard BLM A2 solutions were incubated with pGEM-3Z DNA (0.6 pmol; molecular size, 2.74 kilobases) at 4°C for 10 min. The reaction was terminated by addition of 5 μl of Na₂EDTA (50 mm), and samples were loaded on 1.2% agarose gels and electrophoresed. The ability of the material eluting with the column dead volume to inhibit the growth of A-253 cells was also determined. Exponentially growing cells were harvested by treatment with trypsin (0.125%, w/v) and were seeded in 96-well microtiter plates (100 μl, 2000 cells/well). After 3 h, different dilutions of sample or standard BLM A2 solution were added and the plates were incubated for 4 days. Growth inhibition of A-253 cells was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, as described previously (24).

**RESULTS**

**Sensitization of COLO-205 Xenografts to BLM with the Cysteine Proteinase Inhibitor E-64**

COLO-205 xenograft growth rates in nude mice treated with only E-64 or only BLM were similar to that seen with saline (Fig. 1). This indicates that COLO-205 is resistant to *in vivo* treatments of BLM and E-64 when given separately. In contrast, xenograft growth in animals pretreated with E-64 followed by BLM was significantly inhibited (Fig. 1). For example, the average size of the xenografts of mice receiving both E-64 and BLM on day 12 was only 122 mm³, compared with that of the three other groups, which reached an average tumor size of 700 mm³ (Fig. 1). The difference in xenograft size was evident as early as the fourth day of the treatment (*P < 0.05*), at which time xenograft growth in the E-64/BLM group stopped (Fig. 1). All the treatments were discontinued on day 10, and tumor progression was followed for an additional 15 days. The average tumor size in animals 15 days after the discontinuation of treatment was 1557 ± 380 (saline), 1118 ± 588 (E-64), 1141 ± 300 (BLM), and 297 ± 81 mm³ (E-64 and BLM). The average tumor sizes of the saline-, BLM-, and E-64-treated groups of animals were not significantly different. In contrast, the average tumor size of animals treated with the combination of E-64 and BLM was significantly smaller than that of tumors from the other three groups (*P < 0.001*).

**Effects of E-64 on *in vivo* BLM Metabolism and Accumulation by COLO-205 Xenografts**

We previously found that inhibition of BLM metabolism after E-64 treatment was the primary mechanism responsible for the sensitization of Burkitt's lymphoma to the antitumor action of BLM (20). We, therefore, investigated the effect of E-64 on BLM metabolism and accumulation in COLO-205 xenografts. Nude mice bearing human colon carcinoma xenografts were injected with [³H]BLM A2, as described in Materials and Methods. At 15, 30, and 60 min after BLM A2 administration, xenografts were isolated and analyzed for [³H]BLM A2 and its metabolites. A typical HPLC profile of BLM A2 and its metabolites, obtained from xenografts after 60 min of BLM A2 administration, is shown in Fig. 2A. Besides deamido-BLM A2, the only previously characterized BLM A2 metabolite, small amounts of four other unknown peaks, which eluted from the HPLC column at fractions 2–7, 39–47, 62–68, and 72–82, were also detected. Similar HPLC profiles were found after 15 and 30 min of BLM treatment (data not shown). The major metabolic component of this mixture at all time points was the peak that eluted with the column void volume, fractions 2–7 (Fig. 2A). The ability of this component to degrade DNA *in vitro* and to inhibit the growth of human head and neck A-253 carcinoma in culture was determined as described in Materials and Methods. This component did not degrade plasmid DNA *in vitro* and was unable to inhibit the growth of cultured A-253 tumor cells (data not shown). Bio-Gel P-2 (Bio-Rad) gel exclusion chromatography indicated that the void volume material has a molecular weight of 1000–1500.

The absorption of BLM A2 was rapid, with the highest level of BLM A2 in the xenografts being attained within 15 min, after which time it declined by 79% in the next 15 min (Table 1). Tumor-associated BLM A2 was 2.94 pmol/g of protein within 15 min of BLM injection, declining to 0.27 pmol/g of protein over the next 45 min. This represents a 91% decrease in tumor-associated drug. When mice bearing COLO-205 xenografts were given injections of E-64 (40 mg/kg, i.p.) 30 min...
A typical HPLC profile after 60-min exposure to BLM A2 is shown in Fig. 2A. The absorption of BLM A2 was rapid, with the highest serum level of $[^{3}H]BLM$ A2 (3.04 pmol/ml) being found within 15 min of BLM administration (Table 1). The serum BLM A2 level at 30 min declined by 32% and at 60 min by 68%. Pretreatment of nude mice with E-64 (40 mg/kg, i.p.) 30 min prior to $[^{3}H]BLM$ A2 treatment increased the level of serum BLM A2 at 60 min by 1.7-fold, from 0.96 pmol/ml to 1.66 pmol/ml (Fig. 3B; Table 1).

Kidney. Although BLM A2 was the major component in the kidney at all times, at least three BLM A2 metabolites were detected. One of these radioactive metabolites coeluted with deamido-BLM A2, the only previously characterized BLM A2 metabolite; the other two metabolites, eluting at fractions 8–11 and 36–46, are of unknown nature (Fig. 2C). A peak of radioactive material that eluted with the void volume of the HPLC column is also of unknown nature, was present in COLO-205 xenografts (see above), and has been found previously in other human tumor xenografts (20).

The levels of $[^{3}H]BLM$ A2 in the kidney at various times after BLM A2 administration are shown in Table 1. The highest level of $[^{3}H]BLM$ A2 (26.64 pmol/g of protein) was found within 15 min of BLM administration, and this level decreased with time. Forty five min later, the amount of $[^{3}H]BLM$ A2 in kidney decreased to 9.99 pmol/g of protein. Pretreatment of animals with E-64 (40 mg/kg, i.p.), 30 min prior to BLM A2 administration, resulted in complete inhibition of $[^{3}H]BLM$ A2 metabolism to deamido-BLM A2 and a marked reduction in all other metabolites (Fig. 3C). E-64 pretreatment increased the proportion of BLM A2 from 38% to 71% and the amount of $[^{3}H]BLM$ A2 in kidney by 4-fold (41 pmol/g of protein), compared with saline control (9.99 pmol/g of protein) (Table 1).

Lung. In lungs the major component of the total radioactivity was also $[^{3}H]BLM$ A2 at all times. The profile of $[^{3}H]BLM$ A2 metabolites found in the lung samples was similar to that found in kidney samples. The three unknown metabolite peaks of BLM A2 that eluted with the column void volume and in fractions 8–11 and 36–46 of kidney samples (Fig. 2C) were also found in lung (Fig. 2D). In contrast to kidney, however, deamido-BLM A2 was barely detected in lung. An additional unknown metabolite that was not detected in kidney eluted in fractions 49–57 from the HPLC column for lung samples. The highest level of $[^{3}H]BLM$ A2 (4.43 pmol/g of protein) in lung was reached within 15 min of BLM A2 administration and declined with time (Table 1). Sixty min after BLM A2 administration, only 1.46 pmol of BLM A2/g of protein was retained by lung. Pretreatment with E-64 decreased the overall metabolism of BLM A2 and increased the percentage of BLM A2, relative to its metabolites, from 34 to 52% (Table 1). In animals pretreated with E-64, the level of $[^{3}H]BLM$ A2 accumulation at 60 min was 2.8-fold higher than saline-treated control (Table 1).

Liver. Liver HPLC profiles were similar to those of kidney and lung samples, except that the proportion of $[^{3}H]BLM$ A2 relative to the other peaks was much less, and the relative proportion of the peak eluting in the column void volume was much higher (Fig. 2E). Similar to serum, kidney, and lung samples, the highest level of $[^{3}H]BLM$ A2 was reached within 15 min of BLM A2 administration (Table 1). This level of $[^{3}H]BLM$ A2 declined from 0.36 to 0.16 pmol/g of protein within 45 min. E-64 pretreatment enhanced liver accumulation of $[^{3}H]BLM$ A2 by 1.8-fold and increased the percentage of unmetabolized BLM A2 from 23% to 33% (Table 1).

Brain. The accumulation of $[^{3}H]BLM$ A2 into brain was low at all time points. The major component of the total radioac-

Effects of E-64 on BLM Metabolism and Accumulation in Organs of COLO-205-bearing Nude Mice

Sera, kidneys, lungs, livers, and brains of mice given injections of $[^{3}H]BLM$ A2 (1 mCi/kg, 10 mg/kg, s.c.) and sacrificed 15, 30, or 60 min later were isolated and processed for HPLC analysis, as described in “Materials and Methods.” Typical HPLC profiles of $[^{3}H]BLM$ A2 and other radioactive metabolites in serum, kidney, lung, liver, and brain samples obtained 60 min after BLM A2 administration are shown in Fig. 2, B, C, D, E, and F, respectively.

Serum. Serum samples (100–200 µl) were deproteinized with trichloroacetic acid, and the protein-free filtrates were extracted and analyzed by HPLC for $[^{3}H]BLM$ A2 and its metabolites, as described in “Materials and Methods.” At all time points, $[^{3}H]BLM$ A2 was the major component detected in these samples. A typical HPLC profile after 60-min exposure to BLM A2 is shown in Fig. 2B. The absorption of BLM A2 was rapid, with the relative proportion of BLM A2 increased by only 6% (Fig. 3A; Table 1). However, pretreatment with E-64 enhanced the tumor-associated BLM A2 at 60 min by 6.1-fold (Table 1). In mice pretreated with saline or E-64, the average tumor-associated BLM A2 was 0.27 and 1.65 pmol/g of protein, respectively.

prior to treatment with $[^{3}H]BLM$ A2, the relative proportion of BLM A2 increased by only 6% (Fig. 3A; Table 1). However, pretreatment with E-64 enhanced the tumor-associated BLM A2 at 60 min by 6.1-fold (Table 1). In mice pretreated with saline or E-64, the average tumor-associated BLM A2 was 0.27 and 1.65 pmol/g of protein, respectively.
Pulmonary Toxicity of BLM

DENNIS M. MICHALISZEN, J. WILLIAM BAUGH, JR., ROBERT E. LARSON, AND JOHN C. O'BRIEN

CIRCUMVENTION OF BLEOMYCIN RESISTANCE

Table 1 Amount and percentage of BLM A2 in normal and malignant tissues

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Kidney</th>
<th>Lung</th>
<th>Liver</th>
<th>Brain</th>
<th>Serum</th>
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* Mice were pretreated with E-64 for 30 min prior to [3H]BLM A2 treatment.

Table 2

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<th>Time (min)</th>
<th>Kidney</th>
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<th>Liver</th>
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DISCUSSION

Tumor resistance has limited the clinical usefulness of BLM to a few selected human malignancies, such as head and neck carcinoma, testicular carcinoma, and Hodgkin's disease (4). Overcoming this resistance has been difficult, due to the limited knowledge concerning the mechanism of tumor resistance to BLM.

Efforts have been made during the last decade to understand the biochemical basis of BLM resistance and to circumvent this resistance using a variety of chemical modulators. Results of cell culture experiments have shown that polyene antibiotics (25), anticalmodulin drugs (26-28), local anesthetics (29-31), lysosomotropic agents (32), and calcium channel antagonists (33) can sensitize tumor cells to the cytotoxicity of BLM. None of the aforementioned studies, however, investigated the sensitization of human tumors to BLM in vivo.

The demonstration by our laboratories (17) that a BLM-inactivating enzyme, BLM hydrolase, is a cathepsin-H-like cysteine proteinase provides a rational approach toward over-
coming BLM resistance. Because an increase in BLM metabolism has been suggested as one of the mechanisms responsible for BLM resistance (11, 14), we hypothesized that the cysteine proteinase-specific inhibitor E-64 might sensitize human tumors to BLM by inhibiting BLM inactivation. We have recently demonstrated an inhibition by E-64 of in vivo metabolism of BLM and subsequent sensitization of Burkitt's lymphoma (Daudi) to BLM (20).

The objectives of the present study were to investigate whether E-64 pretreatment also sensitizes human solid tumors to the antitumor action of BLM, to explore the possible mechanism(s) of this sensitization, and to evaluate the effect of E-64 on the pharmacokinetics of BLM and its major side effect, pulmonary fibrosis. Our results clearly demonstrate that (a) human colon carcinoma xenografts are highly resistant to the antitumor action of BLM, (b) E-64 pretreatment sensitizes colon carcinoma to the antitumor action of BLM to such an extent that no growth was observed during treatment and minimal growth was observed even 2 weeks after discontinuation of E-64 and BLM combination treatment, and (c) E-64 enhances COLO-205 tumor accumulation of BLM A2. Our results are consistent with those of Nishimura et al. (34), where E-64 increased the survival time of mice implanted with murine Ehrlich ascites carcinoma and treated with peplomycin, an analogue of BLM. Furthermore, these results are in accordance with our earlier findings (20), which demonstrated that E-64 pretreatment can sensitize a human hematological tumor (Daudi Burkitt's lymphoma) to BLM in vivo. In contrast to our previous studies, the E-64 sensitization of colon carcinoma to BLM was more pronounced than that of Burkitt's lymphoma. Even though Burkitt's lymphoma was sensitized to BLM by E-64, it was still able to grow in nude mice (20), whereas the colon carcinoma growth was completely inhibited by a combination of BLM and E-64 (Fig. 1). Furthermore, the present finding is of potentially greater importance, because colon carcinoma is a BLM-resistant solid tumor and is not responsive to other currently available chemotherapeutic agents.

The absorption of BLM was very rapid, with the highest tumor levels being attained within 15 min or less. Within 1 h of BLM administration, however, >90% of drug in the tumor was either metabolized or eliminated. In addition to the only previously characterized metabolite of BLM A2, deamido-BLM A2, we were able to detect three other minor peaks, of unknown chemical composition. Overall, however, the extent of BLM metabolism was much lower in the colon carcinoma, compared with other human tumor xenografts such as human head and neck carcinoma (A-253) and Burkitt's lymphoma (20). The major metabolic component formed by colon carcinoma in vivo and in vitro corresponded to a radioactive peak that eluted with the void volume of the column. Our in vitro evaluation of the biological activity of this material by plasmid DNA cleavage assay and growth inhibitory effect against BLM-sensitive A-253 cells indicated that this component was biologically inactive.

We found earlier that complete inhibition of BLM metabolism after E-64 pretreatment sensitizes Daudi xenografts to BLM (20). In the present study, although E-64 minimally inhibited BLM A2 metabolism, increased accumulation of BLM A2 appears to be the major factor in colon carcinoma xenograft sensitization. To our surprise, we found 6-fold higher levels of BLM A2 in xenografts pretreated with E-64 (Table 1), compared with untreated xenografts. This was unexpected, because in Daudi xenografts E-64 had no effect on tumor accumulation of BLM (20). Furthermore, the 6-fold increase in tumor accumulation of BLM cannot be explained solely by the 1.7-fold increase in plasma levels of BLM. A direct effect of E-64 on tumor BLM uptake and/or efflux may be operating. This is consistent with our in vitro studies, where E-64 increased the accumulation of BLM A2 in cultured human head and neck carcinoma cells (A-253) (24).

The results of the organ distribution, accumulation, and metabolism studies of BLM showed the highest levels of BLM A2 in serum, kidney, lung, liver, and brain at the earliest timepoint, 15 min after [3H]BLM A2 administration. The greatest accumulation of BLM A2 was found in kidney, followed by lung, brain, and liver. Kidney, lung, and liver all metabolized BLM A2 as early as 15 min after BLM administration. Deamido-BLM A2 was a major metabolite in kidney but was barely detectable in other organs. At least three minor metabolites of unknown nature, which were found in COLO-205 xenografts, were also found in the other organs. Biological and chemical characterization of individual BLM metabolites was not possible, due to their limited amounts.

Pretreatment of nude mice with E-64 greatly inhibited the metabolism of BLM A2 by kidney and lung and increased the proportion of BLM A2, relative to its metabolites. E-64 also increased the amount of radioactivity accumulated by lung and kidney by 2-fold. The decreased metabolism and increased accumulation resulted in a 3–4-fold increase in the amount of active BLM A2 in these organs. Furthermore, E-64 also increased the amount of BLM A2 in serum by 1.7-fold. This is consistent with our preliminary results, which indicate that E-64 decreases urinary excretion of BLM.4 Although BLM alone increased the levels of lung hydroxyproline and induced fibrotic morphological changes, the combination of E-64 and BLM did not enhance these effects. Thus, E-64 sensitized colon carcinoma tumors to BLM without augmenting its pulmonary toxicity. The lack of increased lung toxicity despite inhibition of BLM metabolism is a novel observation and suggests that other mechanisms, such as efficient DNA repair, may be operating. Furthermore, our preliminary results show that neither BLM alone nor the combination of E-64 and BLM alters the levels of blood urea nitrogen,5 suggesting that, despite the 4-fold increase in BLM A2 levels in kidney, renal function was not affected. Similarly, the combination of E-64 and BLM treatment was not hepatotoxic, as judged by estimation of serum glutamate-oxaloacetate transaminase and glutamate-pyruvate transaminase.5

Thus, E-64 was able to sensitize colon carcinoma to BLM without increasing its toxicity, consequently improving its therapeutic index. These results warrant additional studies to de-

### Table 2 Lung hydroxyproline levels and histopathological findings

<table>
<thead>
<tr>
<th>Hydroxyproline (mg/right lung)</th>
<th>Control</th>
<th>E-64</th>
<th>BLM</th>
<th>BLM/E-64</th>
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<td>Mononuclear infiltrate (peri-vascular)</td>
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<tr>
<td>Mononuclear infiltrate (microvascular/interstitial)</td>
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<td>Macrophages (intra-alveolar)</td>
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<tr>
<td>Organized granulomas/fibrosis</td>
<td>0</td>
<td>0</td>
<td>+</td>
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*P < 0.05 (control/BLM).
Fig. 4. Photomicrographs of the subpleural region of lungs from C57BL6 mice. Mice (six mice/group) were treated with BLM and E-64, as described in "Materials and Methods." A, saline; B, BLM, showing an example of an advanced focal granulomatous lesion with fibrosis; not all regions showed this level of organization, and many resembled the lesion shown in D; C, E-64; and D, E-64 and BLM, showing an earlier stage of organization. Arrowheads, pleura x 1700.
termine the clinical usefulness of cysteine proteinase inhibitors such as E-64 in combination with BLM in the treatment of human malignancies that are resistant to BLM.

REFERENCES

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