Metabolic Inactivation: A Mechanism of Human Tumor Resistance to Bleomycin

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ABSTRACT

The mechanism of human tumor resistance to the antineoplastic drug bleomycin (BLM) is not known. We now provide evidence implicating metabolic inactivation in the resistance of human Burkitt's (Daudi) lymphoma to BLM. Daudi lymphoma and human head and neck squamous cell carcinoma (A-253) cells grown (s.c.) in nude mice were found to be resistant and sensitive to BLM treatment, respectively. Within 1 h of s.c. injection of [3H]BLM A2, Daudi xenografts accumulated less BLM and metabolized this drug to a much greater extent than did A-253 xenografts. The BLM-resistant Daudi xenografts metabolized BLM A2 to at least 6 metabolites and only a small proportion of the drug remained as unmetabolized BLM A2. In the BLM-sensitive A-253 xenografts, however, BLM A2 remained the major component. Incubation of BLM A2 with Daudi cytosolic fractions resulted in a complex mixture of metabolites similar to that formed by Daudi xenografts in nude mice. This BLM metabolite mixture was biologically inactive in plasmid DNA degradation assays. Treatment of mice bearing Daudi xenografts with an inhibitor of BLM hydrolase, L-phenylalanine-leucylamido-(4-guanidino)butane (E-64), prior to [3H]BLM A2 treatment did not affect the amount of BLM accumulated but inhibited BLM A2 metabolism in the xenografts. Furthermore, although E-64 alone did not inhibit the growth of Daudi xenografts, it potentiated the antitumor activity of BLM. These results indicate that Daudi tumors resist BLM by metabolically inactivating it and that inhibition of BLM metabolism in vivo enhances the antitumor activity of BLM and hence overcomes resistance.

INTRODUCTION

Drug resistance is a major barrier to the successful treatment of cancer, and the mechanisms of resistance in vivo to many anticancer drugs are not known (1, 2). The antitumor drug BLM (3) is widely used to treat human cancers such as squamous cell carcinoma, testicular carcinoma, and Hodgkin's disease (4). Other tumors, however, do not respond to BLM, and the mechanism of BLM resistance is not understood (5). Several mechanisms, such as decreased drug accumulation, increased DNA repair, and increased metabolic inactivation, have been proposed to account for tumor resistance to BLM (2, 4, 5). Direct evidence linking any of these mechanisms to BLM resistance, however, is lacking.

The discovery of an enzymatic activity, BLM hydrolase, capable of inactivating BLM led Umezawa et al. (6–9) to suggest that drug metabolism may play a role in tumor resistance to BLM. Further investigations of the importance of BLM hydrolase in tumor resistance were hampered by difficulties both in purifying this labile enzyme and in developing methods to study the in vivo metabolism of BLM. Recently, we (10–13) purified rabbit BLM hydrolase to homogeneity and characterized it at the biochemical level. Our molecular cloning, complementary DNA sequencing, and biochemical studies revealed that BLM hydrolase is a cysteine proteinase with cathepsin H-like activity (12). We have also demonstrated that cysteine proteinase-specific inhibitors such as E-64 inhibit BLM hydrolase (12). In the present study, a high resolution HPLC system and E-64 were used as tools to demonstrate (a) that metabolism of BLM is one mechanism by which human tumors can resist BLM treatment in vivo and (b) that nontoxic agents exist that block metabolism in vivo and increase the antitumor activity of BLM.

MATERIALS AND METHODS

Cells. Human Burkitt's lymphoma (Daudi) cells and human head and neck squamous cell carcinoma (A-253) cells were purchased from the American Type Culture Collection (Rockville, MD). Daudi cells were grown in RPMI 1640 medium (GIBCO, Grand Island, NY) supplemented with 20% heat-inactivated fetal bovine serum (GIBCO), and A-253 cells were grown in McCoy's medium (GIBCO) supplemented with 10% fetal bovine serum. Both cell types were grown in a humidified incubator with 95% air/5% CO2 atmosphere.

Materials. BLM A2 was purified from Bleomax (Bristol-Myers Squibb Co., Wallingford, CT) as previously described (15, 16). [S-methyl-3H]BLM A2 (78.4 Ci/mmol) was purchased from Dupont NEN Research Products (Boston, MA). The cysteine proteinase inhibitor E-64 was purchased from Sigma Chemical Co. (St. Louis, MO). pGEM-3Z plasmid DNA was purchased from Promega (Madison, WI). Female athymic nude mice (NHI nu/nu) were purchased from Frederick Cancer Research Facility, NCI (Frederick, MD).

Xenograft Transplantation and Antitumor Studies. A-253 and Daudi xenografts were cultured as described above. The cells were harvested and injected (106 cells/mouse) into the right flank of athymic nude mice. Each control or drug-treated group included 4–5 females (10–12 wk old). Various treatment protocols (see Figs. 1 and 6) were initiated when tumor volumes were 70 to 200 mm3. The width (W) and length (L) of the tumors were measured with Vernier calipers, and the volume was calculated as

\[ V = \frac{W^2 \times L}{2} \]

The data were then plotted as tumor volume in mm3 versus days after initial treatment.

In Vivo Metabolism Studies of Bleomycin. Nude mice bearing A-253 and Daudi xenografts were given i.p. injections of saline or 40 mg/kg E-64. Thirty min later the mice were given s.c. injections of 10 mg/kg [S-methyl-3H]BLM A2 (10 µCi/mouse). One h later the s.c. tumors were removed and homogenized in 20 mM Tris, pH 7.5. Proteins were precipitated with 10% ice cold 6 M trichloroacetic acid and centrifuged, and the supernatant fluid was neutralized with an equal volume of 0.5 M tri-N-octylamine in 1,1,2-trichlorotrifluoroethane. The aqueous phase was filtered through a 0.2-µm ACRO filter, concentrated, and mixed with un labeled BLM A2 and deamido BLM A2 standards (1 µg each) before injection into a C8 reverse-phase HPLC column (25 cm x 4.6 mm, 5-µm particle size; Rainin Dynamax). The BLM A2 metabolites were eluted at 1 ml/min with 13% methanol, 7.5% acetonitrile, acetic acid, heptane sulfonic acid, and triethylamine, pH 5.5. This column and mobile phase provide a separation of BLM A2 from deamido BLM A2 that is markedly better than our previously published methods (10).
One-ml fractions were collected for the first 15 fractions and then 0.2-ml fractions were collected for the remaining fractions. The radioactivity of each fraction was determined by liquid scintillation counting and divided by the total amount of tumor protein as measured by absorbance at 280 nm.

**In Vitro Metabolism of [3H]BLM A₂ and DNA Cleavage Activity.** Cultured Daudi cells were harvested and homogenized in 20 mM Tris-HCl, pH 7.5, and the 105,000 × g supernatants were isolated as described previously (15, 16). One mg protein from the supernatant fractions was incubated with 100 µg BLM A₂ and 2 µCi Cu²⁺-free [3H]-BLM A₂ (specific activity, 78.4 Ci/mmol) in 400 µl of 20 mM Tris buffer (pH 7.5) at 37° for various time intervals. After 1, 3, 5, and 24 h, 100-µl aliquots were precipitated with 100 µl methanol followed by brief centrifugation. Aliquots (50 µl) of each time point were subjected to HPLC analysis as described above. The biological activity of the reaction mixture was determined using a slight modification of a previously described DNA cleavage assay (17). Various dilutions of the above reaction mixtures as well as various concentrations of BLM A₂ were incubated with pGEM-3Z DNA (0.6 pmol; molecular size, 2.74 kilobases) at 4° for 10 min in a buffer containing 80 mM Tris-acetate (pH 8.0), 20 mM MgCl₂, and 25 mM dithiothreitol in a final volume of 20 µl. The reaction was quenched by adding 5 µl of 50 mM Na₂EDTA. Samples were then loaded on 1.2% agarose gel and electrophoresed for 15 h at 30 V in a buffer containing 40 mM Tris-acetate and 1 mM EDTA. The gel was then stained with 1 µg/ml ethidium bromide, and a negative film of the gel was recorded and scanned using an LKB 2400 GelScan XL densitometer.

### RESULTS

Establishment of a BLM-sensitive/BLM-resistant Human Tumor Model. An in vivo model was developed to investigate the biochemical mechanism of resistance of human tumors to BLM. Two human tumor cell lines, Burkitt's lymphoma (Daudi) and A-253 (A-253), were implanted s.c. in nude mice and their responses to BLM were determined as described in Fig. 1. In the absence of BLM treatment, A-253 and Daudi xenografts grew with average doubling times of 3.5 and 2 days, respectively (Fig. 1). When mice carrying the xenografts were treated with 10 mg/kg BLM, A-253 tumor growth was completely inhibited (Fig. 1A) whereas the growth of Daudi tumors was minimally affected (Fig. 1B). Thus, A-253 xenografts are sensitive and Daudi xenografts are resistant to BLM treatment.

Do BLM-sensitive and BLM-resistant Human Tumors Have Different BLM Metabolic Profiles in Vitro? To investigate whether BLM metabolism is responsible for the resistance of Daudi tumors, we first compared the ability of A-253 and Daudi xenografts to metabolize BLM in vivo using the above xenograft nude mouse model. For these studies we used BLM A₂, the major component of the clinical BLM mixture (3, 15, 16). One h after injection of [3H]BLM A₂, the levels of radioactivity accumulated in A-253 and Daudi tumors were 45, 810, and 19,670 cpm/g, respectively, and the BLM A₂ metabolite profiles of the two tumors were quite different. Only two major metabolite peaks were retained on the HPLC column of the radioactivity associated with A-253 tumors (Fig. 2A). The largest of the two peaks corresponds to the parent compound BLM A₂; and the identity of the other major metabolite (peak 6) is unknown. Several other minor metabolites (peaks 3, 4, and 5) were eluted, but their identity is also not yet known. Deamido BLM A₂, the inactive product generated by BLM hydrolase in vitro, was barely detectable (Fig. 2A). The early-eluting material (peak 1) was present in the void volume and may contain more than one metabolite. In contrast, the metabolism of [3H]BLM A₂ by Daudi xenografts was much more extensive and resulted in a complex mixture of metabolites (Fig. 2B). Only a small proportion of BLM A₂ remained unmetabolized in these tumors after 1 h of BLM treatment. Metabolite peaks 5 and 6 were the major metabolites formed in Daudi xenografts (Fig. 2B). A similar mixture of metabolites was also observed when Daudi cell homogenates were exposed to [3H]BLM A₂ (see below). Although metabolite peaks 2, 3, and 4 were minor, their relative proportion was much higher in Daudi xenografts as compared to A-253 xenografts (Fig. 2). Furthermore, deamido BLM A₂, which was barely detectable in A-253 cells, was present in a much greater amount in Daudi xenografts. Thus, 1 h after [3H]BLM A₂ treatment in vivo, A-253 xenografts had accumulated 2 times more BLM than Daudi xenografts. Furthermore, Daudi xenografts were able to convert a large proportion of BLM A₂ to other metabolites, whereas in A-253 xenografts BLM A₂ remains the major component in the tumor.

Are the BLM A₂ Metabolites Formed by Daudi Tumor Cells Capable of Degrading DNA? We next examined in vitro metabolism of BLM A₂ using the 105,000 × g supernatant fluid of Daudi cells, and we tested the ability of the BLM A₂ metabolites generated by these Daudi cytosolic fractions to cleave supercoiled, covalently closed, linear plasmid DNA (form I) and convert it to single-nicked (form II) and double-nicked linear (form III) DNA. Daudi cytosolic fractions were incubated with

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**Fig. 1.** Treatment of human head and neck squamous cell carcinoma A-253 cells and Burkitt's lymphoma Daudi xenografts with BLM. A-253 (A) and Daudi (B) cells were injected s.c. (10⁶ cells/mouse) into the right flank of athymic nude mice as described in "Materials and Methods." Animals bearing tumors of 100-200 mm³ each were treated with either saline (C) or 10 mg/kg BLM in saline (s.c. in the left flank) (A) every 4 days. Day 0 was the first day of treatment. The tumors were measured with calipers, and the tumor volumes were calculated as described in "Materials and Methods." The data are representative of two or more independent experiments.
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Fig. 2. In vivo metabolism of [3H]BLM A2 by A-253 and Daudi xenografts. Nude mice bearing A-253 (A) and Daudi (B and C) xenografts received injections of 10 mg/kg [S-methyl-3H]BLM A2 (10 μCi/mouse). Thirty min prior to [3H]BLM A2 treatment, mice were given i.p. injections of saline (A and B) or 40 mg/kg of the cysteine proteinase inhibitor E-64 (C). One h after [3H]BLM A2 treatment, the s.c. tumors were removed and processed for HPLC analysis of the radiolabeled BLM A2 metabolites as described in “Materials and Methods.” The total amounts of tumor protein were 0.174 g (A), 0.392 g (B), and 0.152 g (C). The highest radioactivity levels were fraction 57 (510 cpm) (A), fraction 9 (450 cpm) (B), and fraction 60 (310 cpm) (C). A2 and dA2 designate the elution position of the internal standards BLM A2 and deamido BLM A2, respectively. The data shown are representative of two or more independent experiments.

[3H]BLM A2 for various lengths of time, and aliquots of the reaction mixture were analyzed by HPLC as well as for their ability to convert form I to form II and III pGEM-3Z DNA by gel electrophoresis, as described in “Materials and Methods.” Fig. 3 shows the HPLC profile of the [3H]BLM A2 metabolites after a 5- or 24-h incubation with Daudi homogenate. Daudi cytosolic fraction metabolized BLM A2 to a complex mixture of metabolites similar to that formed by Daudi cells in vivo (Fig. 2B). After a 5-h incubation with [3H]BLM A2, all metabolites formed in vivo were present in the in vitro reaction mixture (Fig. 3A), and by 24 h 90% of BLM A2 was metabolized (Fig. 3B). Fig. 4 shows the agarose gel electrophoresis profile of the 3 forms of pGEM-3Z DNA after incubation with reaction mixtures of BLM A2 and Daudi cytosolic fractions for various lengths of time. Lanes 3–6 show that increasing concentrations of BLM A2, in the absence of Daudi cytosolic fractions, cleaved pGEM-3Z DNA and converted, in a concentration-dependent manner, form I (uncut) to form II (single-strand break) and form III (double-strand break). As the time of incubation with Daudi cytosolic fractions increased, the ability of BLM A2 to degrade form I was reduced (lanes 7–14). Fig. 5 shows that the time-dependent loss of BLM A2 due to its metabolism, determined from the radioactivity associated with the BLM A2 peak from HPLC profiles, correlated closely with the percentage of form I remaining after incubation with the Daudi cytosol reaction mixtures (Fig. 4). For example, after 1 h of exposure to Daudi cytosolic fractions, 50% of BLM A2 was metabolized and 40% of form I remained undegraded. By 24 h only 10% of BLM A2 remained unmetabolized and more than 80% of form I remained intact. Incubation of BLM A2 without Daudi cytosolic fractions or with cytosolic fractions boiled (100°, 5 min) prior to incubation with BLM A2 both resulted in no BLM A2 metabolism and in DNA degradative profiles similar to those generated by intact BLM A2 (data not shown). Thus, Daudi cytosolic fractions metabolized BLM A2 to metabolites unable to degrade DNA.

Can Cysteine Proteinase Inhibitors Block BLM Metabolism in Vivo? If metabolism is responsible for the resistance of Daudi tumors to BLM, inhibition of BLM metabolism should lead to sensitization of these tumors to BLM. Recently, we showed through molecular cloning and complementary DNA sequencing that BLM hydrolase, a BLM-inactivating enzyme, is a cysteine proteinase and that the cysteine proteinase inhibitor E-64 (18) is an effective blocker of this enzyme in vitro (12). We used E-64 in the present studies to inhibit in vivo metabolism of [3H]BLM A2 in the Daudi xenograft nude mouse model. Fig. 2C shows the HPLC elution profile of [3H]BLM A2 and its metabolites from Daudi xenografts 1 h after [3H]BLM A2 injection into Daudi tumor-bearing nude mice. The animals were treated
Fig. 4. Plasmid DNA cleavage by BLM A2 and its metabolites. Aliquots of reaction mixture similar to those in Fig. 3 were analyzed for their ability to cleave plasmid DNA. The loss of covalently closed circular pGEM-3Z DNA (form I) and the appearance of single-nicked (form II) and linear (form III) DNA were determined by agarose gel electrophoresis as described in “Materials and Methods.” Lane 1: HindIII-digested λDNA fragments as molecular weight markers; lane 2: pGEM-3Z DNA incubated without any drug or reaction mixture; lanes 3–6: treatment with 0.625, 2.5, 10, and 25 ng BLM A2, respectively; lanes 7–8: treatment with 1:100 dilutions of the reaction mixture at 1 h (6.25 and 25 ng BLM A2 equivalents); lanes 9, 11, and 13: treatment with 1:50 dilutions (12.5 ng BLM A2 equivalents) of the reaction mixture at 3, 5, and 24 h, respectively.

Fig. 5. Relationship between in vitro [3H]BLM A2 metabolism by Daudi cytosolic fraction and DNA damage. The percentage of BLM A2 remaining in the reaction mixture after 1, 3, 5, and 24 h was calculated from the HPLC profiles based on the radioactivity associated with the BLM A2 peak. The percentage of form I DNA remaining after incubation of pGEM-3Z plasmid DNA with the reaction mixture aliquots at various time intervals was calculated from the peak area of the densitometer scans of the form I DNA band of a negative film obtained after gel electrophoresis.

as in Fig. 2B except that, 30 min prior to [3H]-BLM A2 treatment, mice were pretreated with the cysteine protease inhibitor, E-64). Pretreatment with E-64 (40 mg/kg) did not alter the levels of [3H]BLM A2 accumulated by the tumors (19,670 cpm/g in the absence of E-64 and 21,710 cpm/g in the presence of E-64). As described in Fig. 2B, in the absence of E-64 Daudi xenografts metabolized a large proportion of [3H]BLM A2 to a complex mixture of metabolites. When nude mice were pretreated with E-64, however, the formation of all metabolites was inhibited and the only major peak retained on the HPLC column was the parent unmetabolized drug, BLM A2. The amount of unmetabolized BLM A2 in E-64-treated tumors (9600 cpm/g protein) was 2.5 times higher than that of E-64-untreated tumors (3870 cpm/g protein). Thus, E-64 was able to effectively inhibit the conversion of BLM A2 to its metabolites so that most of the drug remaining in the tumor was the unmetabolized BLM A2.

Can the Resistance of Daudi Xenografts to BLM Be Overcome by Inhibition of BLM Metabolism? We reasoned that E-64, the inhibitor of BLM metabolism, should potentiate the antitumor activity of BLM if drug metabolism is responsible for BLM resistance of Daudi xenografts. We tested this hypothesis in the human tumor xenograft model described in Fig. 1B. In the absence of any treatment, Daudi xenografts grew to an average tumor size of 3,000 mm³ in 10 days (Fig. 6, open circles). Treatment of the tumor-bearing nude mice with the cysteine protease inhibitor, E-64, had very little effect on tumor growth (Fig. 6, closed circles). Therefore, E-64 by itself did not inhibit the growth of Daudi tumors. BLM alone was able to inhibit Daudi xenograft growth only at days 8 and 10 (P < 0.01 for day 10 and P < 0.05 for day 8) (Fig. 6, closed triangles). In the presence of E-64, however, BLM inhibited the growth of these tumors to a much greater extent than in the absence of E-64 (compare open to closed triangles, respectively). Although BLM alone showed some antitumor activity only at days 8 and 10 of treatment, in the presence of E-64, BLM significantly inhibited tumor growth as early as 4 days after treatment (P < 0.001 at day 10, P < 0.01 at days 8 and 6, and P < 0.05 at day 4). By day 10, tumors from mice receiving a combination of E-64 and BLM had an average size of only 1,200 mm³, and those receiving BLM alone had an average size of 2,050 mm³ (P < 0.01). The tumors receiving E-64 alone or just saline had an average size of 3,500 and 3,000 mm³, respectively. A combination of E-64 and BLM inhibited the ability of Daudi xenografts to grow by 60% whereas BLM alone resulted in only 30% growth inhibition. Thus, E-64 potentiated the antitumor activity of BLM against Daudi xenografts by 2-fold.
DISCUSSION

Many human tumors exhibit intrinsic resistance to antineoplastic drugs. Overcoming drug resistance remains a formidable task but an essential goal for broadening the spectrum of tumors that can be treated with the presently available antineoplastic drugs. In the present report we have shown that resistance to the anticancer drug BLM can be overcome in human Burkitt’s lymphoma in vivo. We have demonstrated that metabolism of BLM is involved in the resistance of Daudi xenografts to BLM and that inhibition of BLM metabolism results in sensitizing the tumors and thus overcoming resistance.

The metabolism of BLM in human tumors grown in vivo has not been previously studied. Human tumor cell metabolism of BLM has been investigated only in homogenates (2, 19, 20). In tumor homogenates from any species, deamido BLM, an inactive metabolite of BLM, is the only metabolite reported (2, 8). Deamido BLM is metabolically generated by BLM hydrolase, a BLM-inactivating enzyme that we recently characterized biochemically and molecularly (10-13). Surprisingly, in the present study we found that BLM metabolism in human Burkitt’s lymphoma grown in vivo is complex. Within 1 h of BLM treatment, tumor xenografts were able to form at least 6 BLM metabolites. This is consistent with previous observations from our laboratory that suggested complex in vivo metabolism in nonmalignant tissue such as murine liver and kidneys (15). Deamido BLM, the only previously reported BLM metabolite in tumors, was only a minor metabolite found in vivo in the high-metabolizing Daudi xenografts and was barely detectable in A-253 xenografts. These studies show for the first time that human tumors grown in vivo are able to metabolize BLM and that the metabolite mixture formed is very complex. The chemical structure of these new metabolites is not yet known.

Studies attempting to link BLM metabolism to tumor resistance have been conducted mainly with homogenates and have been inconclusive (2, 4). While some showed a good correlation between BLM hydrolase levels in various tumor homogenates and their levels of resistance (20-23), others did not (9, 24). Recently, Nishimura et al. (25) provided additional evidence using E-64 to support the notion that this enzyme may play an important role in regulating the toxicity of BLM against Chinese hamster lung cells (25). This work, however, was carried out in normal cultured hamster lung cells, which metabolized peplomycin, a BLM-analogue, to a single metabolite, deamido peplomycin, in contrast to the human tumor xenografts of the present study, which have a complex metabolic profile. In addition, we have used human tumors grown in vivo to investigate the importance of drug metabolism in BLM tumor resistance. We found that BLM-resistant Daudi xenografts in nude mice metabolize BLM A2 in vivo to a much greater extent than do the BLM-sensitive human A-253 xenografts. The metabolites formed by Daudi tumors in vivo were also formed when BLM A2 was incubated with cytosolic fractions from Daudi cells, and these metabolites were unable to degrade DNA, the presumed molecular target of BLM (Figs. 3-5). Our results with an inhibitor of BLM metabolism also strongly suggest the involvement of BLM metabolism in Daudi tumor resistance. Pretreatment of nude mice bearing Daudi xenografts with a cysteine proteinase-specific inhibitor, E-64, prior to BLM treatment resulted not only in the inhibition of the known metabolite generated by BLM hydrolase, deamido BLM, but also in the complete inhibition of all BLM metabolites formed in vivo (Fig. 2C). These results suggest that either BLM hydrolase is capable of generating BLM metabolites other than deamido BLM in vivo or that other cysteine proteinase inhibitor-sensitive enzymes are also involved in BLM metabolism in vivo. It is also conceivable that some of these metabolites are generated from deamido BLM. Regardless of the origin of these BLM metabolites, we were able to abolish BLM metabolism in vivo by E-64. Daudi tumors from mice that were pretreated with E-64 contained 2.5 times more unmetabolized BLM per g tumor protein than did those tumors that were treated with BLM alone. Furthermore, although E-64 alone did not inhibit the growth of Daudi tumors, it enhanced the ability of BLM to inhibit this growth by 2-fold. The amount of active, unmetabolized BLM in Daudi xenografts is therefore directly proportional to its antitumor activity. This is in agreement with our in vitro DNA degradation studies, which showed a direct correlation between BLM A2 metabolism and its biological activity in this system. Thus, the ability of E-64 to inhibit BLM metabolism in vivo coupled with its capacity to potentiate the antitumor activity of BLM indicate that BLM metabolism plays an important role in the resistance of Daudi tumors to BLM.

Thus, our results with human tumor xenografts demonstrate that Burkitt’s lymphoma is able to extensively metabolize BLM and hence escape the toxic effects of this group of antineoplastic drugs. Our results also demonstrate that human tumor resistance to BLM can be overcome by potentiating the antitumor activity of BLM with inhibitors of BLM metabolism. If such a combination is found not to elevate known toxicities of BLM to normal tissues, compounds like E-64 could potentially increase the therapeutic efficacy of BLM-like drugs.

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