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INITIAL SINGLE-STRAND DNA DAMAGE AND CELLULAR PHARMACOKINETICS OF BLEOMYCIN A_2*

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Abstract—The cellular association and fate of high specific activity [^{3}H]bleomycin A_2 (BLM A_2) were examined in three previously untreated cultured cell lines. Human head and neck A-253 carcinoma cells were 10-fold more sensitive to a 1-hr exposure to BLM A_2 than either murine leukemic L1210 or human ovarian SK-OV cells. Both murine and human cells displayed rapid drug association with steady-state drug levels being reached within 15–30 min. At steady state, the $T_{1/2}$ of drug dissociation was slow (between 65 and 155 min), unaltered by 100-fold excess of unlabeled BLM A_2, and unrelated to cellular sensitivity to BLM. Approximately 15% of the total cellular drug was found in the nuclei at steady state. In intact cells, BLM hydrolase activity appeared latent; significant BLM hydrolase activity was detected using broken cell homogenates with all cell types, but no extensive drug metabolism was evident in intact cells. Murine L1210 cells differed from both human cell lines in that they had only 50% of the steady-state drug levels, had lower nuclear drug content, and had markedly less initial single-strand DNA damage. Human SK-OV cells had 2.4-fold greater initial single-strand DNA damage despite similar nuclear content and a much lower rate of DNA repair. Thus, cellular or nuclear factors, in addition to BLM A_2 content, affect initial single-strand DNA damage. Collectively, our data support the proposition that lesions other than single-strand DNA breaks contribute to the cytotoxicity of BLM.

Most antineoplastic agents are believed to act by disrupting the structure or functionality of chromosomal DNA. Studies with purified DNA have demonstrated unambiguously that the antineoplastic drug BLM** cleaves DNA [1, 2]. Single-strand DNA damage has been observed with intact eukaryotic cells after brief treatment with BLM [3, 4], although some investigators [5–7] have noted that single-strand DNA damage can only be observed with concentrations of BLM producing extremely high cell death.

Many pharmacological actions reflect the concentrations of the agent at the target site. The process by which BLM damages DNA *in vitro*, however, appears to be highly complex and could require several additional factors in intact cells [1, 2]. Thus, the relationship between DNA damage and cellular or nuclear drug content should be examined.

The central importance of chromosomal DNA damage in the lethality of BLM also has been challenged. Berry *et al.* [8], for example, studied the relationship between growth inhibition and single-strand DNA damage with a variety of congeners and concluded that these two are not directly correlated. Smith [3] found that ferrous ions dramatically elevate BLM-induced single-strand breakage of chromosomal DNA while paradoxically increasing cell survival. BLM can affect mitochondrial DNA *in vitro* [9] and other non-nuclear cellular components [10, 11]; thus, other targets of injury may exist.

Differences in the cellular accumulation of BLM and in its inactivation have been proposed as mechanisms of cellular resistance to BLM [1, 2]. Although the subcellular distribution of BLM has not been examined, it appears that a majority of BLM found associated with cells is not located in the nucleus [12]. The inactivating enzyme, BLM hydrolase, is found in cytosolic preparations [2, 5] but definitive evidence of its activity with intact malignant cells has not been reported. Thus, we have examined the cellular accumulation, nuclear levels, and metabolism of BLM A_2 in three previously untreated cell lines, and also have studied BLM-induced single-strand DNA damage and repair in the same cell lines. Unexpectedly, we found in all cells no significant metabolism of BLM, despite metabolism *in vitro*. In addition, we noted that with human tumor cells neither initial single-strand DNA damage nor cell survival after BLM exposure directly related to cellular or nuclear drug content.

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** Abbreviations: BLM, bleomycin; BLM dA_2, deamido bleomycin A_2; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; and PBS, phosphate-buffered saline.

MATERIALS AND METHODS

Cell culturing techniques. L1210 murine leukemia cells were maintained as previously described [7]. All studies were conducted with mycoplasma-free cells in exponential growth phase (2–5 × 10^5 cells/ml). The A-253 and SK-OV human cells were

obtained from the American Type Culture Collection (Rockville, MD) and have been described previously [13]. A-253 cells were isolated originally from a human squamous cell carcinoma located in the submaxillary gland, a tumor that is usually responsive to BLM, and SK-OV cells originated from a human ovarian adenocarcinoma, a tumor that does not normally respond well to BLM. Both cell lines were maintained as monolayers with McCoy's 5a medium (modified) (Grand Island Biological Co., Grand Island, NY) containing 10% fetal bovine serum. Exponentially growing cells were removed from plastic culture flasks with a 1-min exposure to phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 8 mM Na_2PO_4, pH 7.4) containing 0.25% (w/v) trypsin and 2 mM EDTA. After centrifugation at 200 *g* for 4 min and washing with PBS, the cell concentration was determined with a Coulter Counter. The population doubling time for L1210 cells was 12 hr and for A-253 and SK-OV cells was 24 hr. Apparent cell volume was calculated by the method of Wohlhueter *et al.* [14] using [^{14}C]inulin and [^{3}H]water. The mean volumes were: 0.39 pl/L1210 cell, 2.1 pl/SK-OV cell, and 2.4 pl/A-253 cell.

Drugs and chemicals. Copper-free BLM A_2 was isolated by our previously described methods [15] from Blenoxane (Bristol Myers Co., Wallingford, CT) and converted to the Cu(II) form by incubation with an equal molar concentration of $CuSO_4$. In all studies, unless otherwise noted, the Cu(II) complex of BLM A_2 was used, since this is believed to be the form that is present in blood and that enters cells [1]. Moreover, previous results indicate that the cytotoxicities of both the metal-free and Cu(II) complex of BLM are similar [6] as are the binding properties to purified DNA [12]. High specific activity Cu(II)[5-*methyl*-^{3}H]BLM A_2 (38 Ci/mmol) was obtained from NEN Research Products (Boston, MA) and stored in the copper-form at 4° in 30% ethanol:H_2O. This radiolabeled material co-migrated with unlabeled Cu(II)BLM A_2 on HPLC and TLC [15] and produced a concentration-dependent growth inhibition that was equivalent to that of unlabeled drug (data not shown).

Cellular sensitivity to BLM A_2. A-253 and SK-OV cells were detached from flasks as described above and resuspended in fresh medium containing serum. L1210 cells also were resuspended in fresh medium containing serum. All cells were incubated with various concentrations of Cu(II)BLM A_2 for 1 hr, washed, and plated in soft agar as previously described [16]. The number of colonies formed were counted after 2 weeks. In some studies, cells were exposed to BLM A_2 for 3 days and the inhibition of cell proliferation was assessed as described previously [17].

Cellular radioactivity and nuclear isolation. L1210 cells were resuspended (1×10^7 cells/ml) in Fischer's medium with 10% horse serum and 10 mM Hepes buffer (pH 7.4). Detached A-253 and SK-OV cells were resuspended (1×10^7 cells/ml) in McCoy's medium with fetal bovine serum and 10 mM Hepes (pH 7.4). The cell association of [^{3}H]BLM A_2 was determined as previously described [5].

Nuclei were isolated by a modification of the method of Kennedy *et al.* [18] and Roy and Horwitz [12]. L1210, A-253 and SK-OV cells were suspended at a concentration of 2–8 $\times 10^7$ cells/ml in medium containing 10% serum, 10 mM Hepes (pH 7.4), and 1 μM Cu(II)[^{3}H]BLM A_2 (30 μCi/ml). After a 30-min incubation at 37°, the cells were centrifuged at 1000 *g* for 2 min, washed with 12 ml of drug-free medium, and centrifuged as above. The resulting cell pellet was resuspended in 5 ml of ice-cold distilled water and 0.5% (v/v) Nonidet P40 was added. The nuclei were isolated by centrifugation through a 2.2 M sucrose cushion at 40,000 *g* for 60 min (4°), resuspended in distilled water (200 μl), and dissolved in Protosol (3 ml; NEN Research Products) by heating overnight at 60°; the radioactivity in the sample was determined by liquid scintillation counting. Addition of 100 μM nonradiolabeled BLM A_2 did not affect the nuclear recovery data. Recovery of nuclei from cells was greater than 85% as judged by recovery of radioactivity in DNA labeled with [^{3}H] thymidine. The preparations of nuclei were found to be free of contamination by cytoplasmic organelles based upon electron microscopy.

DNA damage and repair. Previously described alkaline elution techniques [5, 7] were used to measure single-strand DNA breakage and DNA repair in the three cell lines.

HPLC of cell-associated and medium radioactivity. L1210, A-253 and SK-OV (1×10^7 cells/ml) cells were incubated at 37° with 1 μM Cu(II)[^{3}H]BLM A_2 (10 μCi/ml; 10 Ci/mmol) for 60 min. The incubation was terminated by washing the cells twice with 15 ml of ice-cold PBS. For dissociation studies, cells incubated with 1 μM Cu(II)[^{3}H]BLM A_2 for 1 hr were washed once with 15 ml of ice-cold medium, resuspended in medium, and reincubated for various times at 37°. Cells were then washed once with 15 ml of ice-cold PBS. For all procedures the cell suspensions were centrifuged at 1000 *g* for 2 min, and the medium was removed completely. Thereafter, 200–300 μl of distilled water was added, and the cells were lysed by sonication (2×5 sec). The sonicate was centrifuged at 15,600 *g* for 5 min, and the supernatant fraction was stored at −20°. For HPLC analysis, the cell sonicate or culture medium was passed through a 0.45 μm ACRO LC13 membrane filter (Gelman Sciences Inc., Ann Arbor, MI), injected into a Microsorb C_8 column (6.4 × 150 mm; Rainin Instrument Co., Inc., Woburn, MA) and eluted with $CH_3OH:CH_3CN:H_2O:CH_3COOH$ (130:72:790:8) containing 2 mM heptanesulfonic acid and 25 mM triethylamine (pH 5.5) as described previously [15]. With this method the separation between Cu(II)BLM A_2 and Cu(II)BLM dA_2 is sufficient to allow the detection of $<5\%$ BLM dA_2 in a mixture [15].

BLM hydrolase assay. BLM A_2 metabolism by BLM hydrolase was studied *in vitro* using a slight modification of our previously described methods [15]. Metal-free BLM A_2 was used because metal-bound drug, such as Cu(II)BLM A_2, is not a substrate for BLM hydrolase [1, 19].

RESULTS

Cellular sensitivity to BLM A_2. With a 1-hr exposure, the concentration of BLM A_2 required to

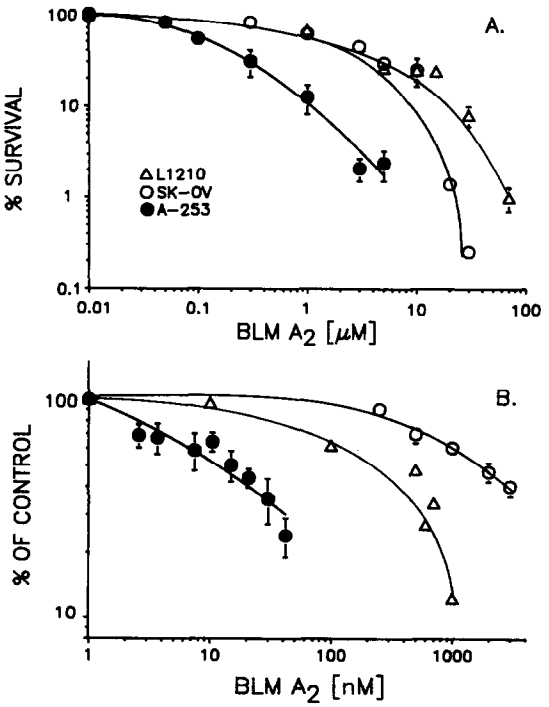

Fig. 1. Cellular sensitivity to BLM A_2-induced cytotoxicity. Panel A: Clonal growth after a 1-hr exposure to BLM A_2. Cells were incubated with various concentrations of BLM A_2 for 1 hr, washed, and plated in soft agar. The number of colonies formed was determined after 2 weeks. Panel B: Growth inhibition with continuous exposure to BLM A_2. Cells were incubated for 4 days in the presence or absence of BLM A_2 and the cell number was determined. In both panels each value is the mean ± SE from three or more determinations. Error bars are not shown when smaller than symbols for mean values. Key: A-253 (●), SK-OV (○), and L1210 (△).

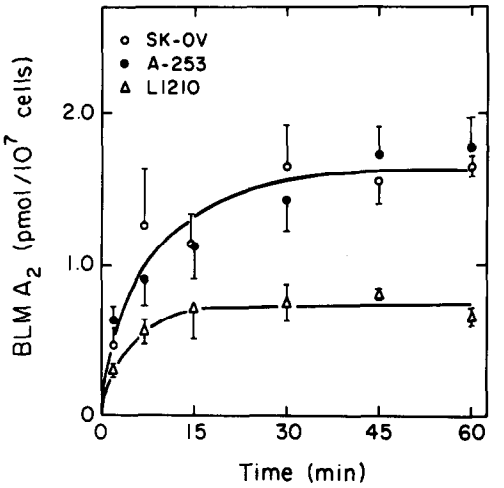

Fig. 2. Kinetics of BLM A_2 cell association. Exponentially growing A-253, SK-OV and L1210 cells were incubated in suspension at 37° with 1 μM [^{3}H]BLM A_2 for various times, washed, and centrifuged through oil, as described in Materials and Methods. The cell-associated radioactivity was measured by liquid scintillation techniques. Each value is the mean ± SE from a minimum of four determinations.

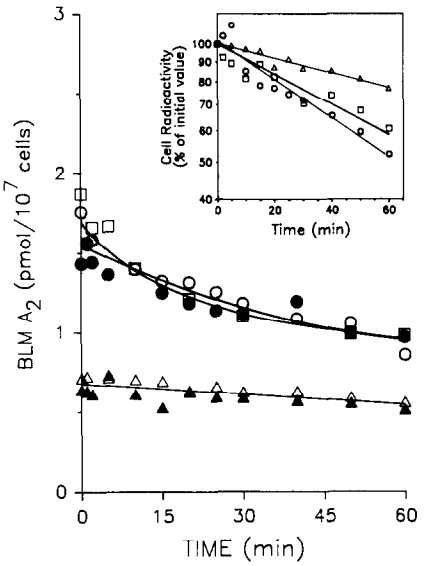

Fig. 3. Loss of cell-associated radioactivity. A-253 (○, ●), SK-OV (□), and L1210 (△, ▲) cells were preincubated with 1 μM [^{3}H]BLM A_2 at 37° for 1 hr. Cells were resuspended in radiolabel-free medium in the presence (open symbols) or absence (closed symbols) of 0.1 mM unlabeled BLM A_2 at 37° for various times. Cell-associated radioactivity was assayed as described in the legend of Fig. 2. Inset: Loss of radioactivity in A-253 (○), SK-OV (□), and L1210 (△) cells in the absence of unlabeled BLM A_2.

reduce cell survival to 50% was 3 μM for both L1210 and SK-OV cells and 0.3 μM for A-253 cells (Fig. 1A). Using this criterion, A-253 cells were 10-fold more sensitive to BLM A_2 induced lethality than L1210 and SK-OV cells. When cell survival of 10% or less was examined, L1210 cells were slightly more resistant to BLM than the SK-OV cells but both cell lines remained significantly more resistant to BLM A_2 compared to A-253 cells. With continuous exposure to BLM A_2, L1210 and SK-OV cells were even more resistant to BLM A_2 compared to A-253 cells (Fig. 1B).

Cellular association and dissociation of [^{3}H]*BLM* A_2. Incubation of cells with 1 μM [^{3}H]BLM A_2 at 37° resulted in rapid cellular accumulation of radioactivity, with a steady-state plateau phase attained by 15–30 min in all three cell lines tested (Fig. 2). Both human cell lines displayed similar drug accumulation kinetics and steady-state levels of approximately 1.7 pmol/10^7 cells. This amount of radioactivity was also found with A-253 cells that had been incubated with [^{3}H]BLM A_2 for 2 hr. The steady-state level for murine L1210 cells was only half that of the human cell lines.

Dissociation of radioactivity from the three cell types, which were exposed to 1 μM [^{3}H]BLM A_2 for 1 hr, was studied by resuspending cells in medium lacking radiolabeled BLM A_2 (Fig. 3). A-253, SK-OV, and L1210 cells lost approximately 50, 40, and 20% of cell-associated radioactivity, respectively,

Table 1. Nuclear content of BLM A_2

Cell line	Radioactivity (pmol/10^8 cells)	% of Total cellular radioactivity
A-253	1.06 ± 0.16 (10)	11.07 ± 3.16
SK-OV	0.75 ± 0.12 (7)	10.48 ± 1.73
L1210	0.22 ± 0.04* (12)	15.64 ± 2.23

Suspended cells were incubated with 1 μM (30 μCi/ml) Cu(II)[^{3}H]BLM A_2 for 30 min at 37°. Cells were centrifuged and washed, and nuclei were isolated as described in Materials and Methods. Each value is the mean ± SE for the sample size given in parentheses.

* Using ANOVA and the least significant difference test [20], $P < 0.05$ compared to A-253 or SK-OV cells.

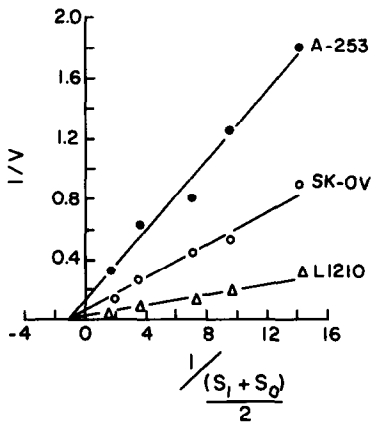

Fig. 4. Double-reciprocal plot of the rate of BLM dA_2 formation vs BLM A_2 concentration. The 105,000 *g* supernatant fractions of homogenates from L1210, SK-OV and A-253 (●) cells were used (800 μg protein/reaction). Each value is the mean of three or more determinations. Velocity is expressed as nanomoles BLM dA_2 formed per hour per milligram of protein; substrate concentration is millimolar.

during the 60-min incubation in drug-free medium. Dissociation of radiolabel from A-253 and L1210 cells was unaffected by incubation with 100-fold excess of unlabeled BLM A_2; SK-OV cells were not studied. The rate of loss of cellular radioactivity in drug-free medium was approximately first order for all cells with estimated $T_{1/2}$ values for A-253, SK-OV, and L1210 cells of 65, 115, and 155 min respectively ($r^2 > 0.92$ for all).

Nuclear content of [^{3}H]*BLM* A_2. The nuclear content of radiolabeled material ranged from 10 to 16% of the total drug associated with cells (Table 1). Consistent with their lower cellular level of drug, the amount of drug in the nuclei of L1210 cells was 5-fold less than that in A-253 cell nuclei. SK-OV nuclear content was not statistically different from that of A-253 cells.

Metabolism of BLM A_2. BLM hydrolase activity was assayed in cytosolic fractions prepared from L1210, SK-OV and A-253 cells. All cytosolic fractions had considerable amounts of enzyme activity, with the same apparent K_m value of 0.9 mM and differing apparent V_{max} values of 70, 20, and 8 nmol·hr^{-1}·(mg protein)$^{-1}$ for L1210, SK-OV, and A-253 cells respectively (Fig. 4). To examine the metabolism of BLM A_2 in intact cells, we incubated L1210, SK-OV and A-253 cells with [^{3}H]BLM A_2 for 60 min. More than 95% of the total cellular radioactivity was recovered from the HPLC column with greater than 90% present as the parent compound in all three cell lines (Fig. 5). In addition, L1210 cells were incubated with 1 μM [^{3}H]BLM A_2 for 1 hr, washed free of drug, and resuspended in medium lacking BLM A_2 for an additional 1 or 3 hr, and no BLM dA_2 was detected associated with the cells (data not shown). The HPLC profile obtained from cells incubated with metal-free [^{3}H]BLM A_2 was identical to that seen with [^{3}H]Cu(II)BLM A_2 (data not shown). Additionally, the growth medium of A-253 cells was analyzed by HPLC after 4 days of continuous cell exposure to [^{3}H]BLM A_2, and only BLM A_2 was detected (data not shown). To ensure that the [^{3}H]BLM A_2 was a substrate for BLM hydrolase, we incubated metal-free [^{3}H]BLM A_2 *in vitro* with BLM hydrolase from the postmicrosomal supernatant fraction of L1210 cell homogenates and found the rate of [^{3}H]BLM dA_2 formation similar to that observed with unlabeled BLM A_2 (data not shown). Thus, despite *in vitro* evidence of BLM hydrolase activity, no evidence for extensive formation of BLM dA_2 was detected with intact cells.

DNA damage and repair following BLM A_2 *treatment*. Initial single-strand DNA damage was assayed in A-253, SK-OV, and L1210 cells following a 1-hr treatment with 5 μM BLM A_2 (Table 2; Fig. 6). L1210 cells had no detectable DNA damage after a 1-hr exposure to 5 μM BLM A_2 whereas exposure to the same level of drug produced 321 rad equivalents of initial DNA damage in A-253 cells and 776 rad equivalents of single-strand DNA damage in SK-OV cells. Incubation of L1210 cells at a 5-fold higher concentration of BLM A_2 (25 μM) resulted in a level of initial single-strand DNA damage that was still 4-fold lower (80 rad equivalents) than that produced in A-253 cells at 5 μM BLM A_2.

The rate of the repair of the initial single-strand DNA damage in A-235 cells after a 1-hr exposure to 5 μM BLM A_2 was 2.4-fold greater than that seen in SK-OV cells (Table 2). The rate of repair in L1210 cells exposed to 25 μM BLM was intermediate to that seen with the human cells exposed to 5 μM BLM A_2.

DISCUSSION

It is well established that the antineoplastic agent

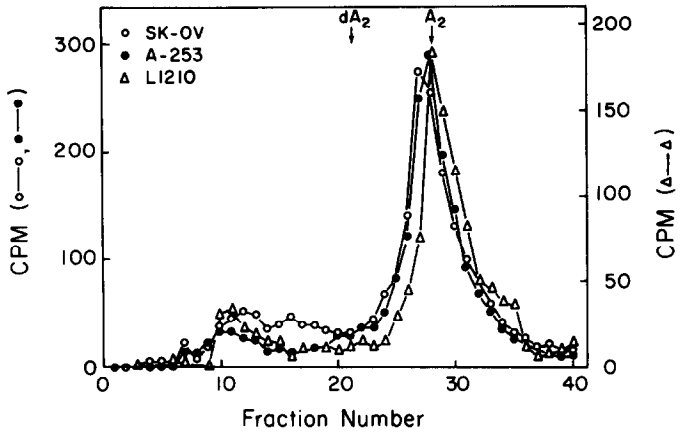

Fig. 5. HPLC profile of the cell-associated radioactivity. SK-OV, A-253 and L1210 (△) cells were incubated with 1 μM [^{3}H]BLM A_2 for 60 min, then washed twice with ice-cold PBS, and lysed by sonication. The sonicates were centrifuged at 15,600 *g* for 5 min, and the supernatant fractions were filtered before applying to the HPLC. The fractions where unlabeled BLM dA_2 (dA_2) and BLM A_2 (A_2) standards eluted are as indicated. Each fraction was counted in a liquid scintillation counter for 5 min at least three times.

Table 2. BLM-induced DNA damage and repair

Hours after BLM	A-253	L1210	SK-OV
	Rad equivalents of damage		
0	321	0 (80)	776
1	113	0 (48)	567
	% Repair at 1 hr		
	65%	ND (40%)	27%

Cells were incubated for 1 hr in suspension with 5 μM or 25 μM (values in parentheses) BLM A_2. Cells were washed rapidly and assayed by alkaline elution [5, 7] either immediately for initial single-strand DNA damage in drug-free medium or after 1 hr for DNA repair. Data are the average of two determinations, and rad equivalent values are calculated as previously described [5, 7]. ND = not determined.

bleomycin can cleave isolated DNA [1, 2, 21, 22]. Less clear is the relationship between cellular or nuclear drug content and initial DNA damage. The cleavage of DNA requires not only BLM but also a coordinated metal, oxygen and, for catalytic damage, readily available reducing equivalents. Several studies suggest that the DNA damaging effects of bleomycin do not necessarily correlate with cellular sensitivity to the drug [2, 3, 8] and that other non-nuclear sites of damage may exist [10, 11]. Little is known concerning the cellular pharmacokinetics, nuclear translocation, and metabolic fate of BLM. Understanding these processes is a critical precursor for defining the lethal site of action of this drug. Thus, in the present study, we have examined the initial cellular pharmacokinetics, nuclear drug content, drug metabolism, DNA damage and repair of DNA damage resulting from BLM exposure in a limited number of naturally sensitive and resistant cell lines.

Three cell lines were chosen: A-253 cells were relatively sensitive to the toxic effects of BLM A_2 (Fig. 1), whereas SK-OV and L1210 cells showed a non-acquired, 10-fold or greater resistance to the drug. Cellular content of [^{3}H]BLM A_2 by all three cell types reached a steady state by 15–30 min (Fig. 2), similar to results obtained with HeLa [12] and KB cells [8]. Our results indicate, therefore, that the rapid achievement of steady-state levels of BLM A_2 is a general phenomenon seen with cells in culture but with no evidence for the concentration of drug within cells despite the affinity of BLM for DNA. Both human cells displayed similar initial drug association rates and steady-state drug levels, and these were approximately twice the corresponding values observed for murine L1210 cells. In contrast to the relatively rapid uptake of [^{3}H]BLM A_2 by all cell lines, efflux of the drug was relatively slow and was unaffected by addition of unlabeled drug to the incubation medium (Fig. 3). This lack of exchange is similar to results obtained by Lyman *et al.* [23] for Ehrlich cells and may reflect inaccessibility of BLM A_2 due to localization in subcellular compartments. The proposition of Lyman *et al.* [23] that metabolic inactivation of the drug in cells prevents drug exchange does not appear tenable because HPLC analysis of cell homogenates showed 90% of the radioactivity as unaltered drug (Fig. 5). In contrast to our results and those of Lyman *et al.* [23], Roy and Horwitz [12] found a rapid exchange of labeled BLM A_2 when HeLa cells were incubated with an excess of non-labeled compound. This suggests the possibility that cell type differences in the subcellular localization of BLM may exist.

L1210 cells accumulated only one-half the [^{3}H]BLM A_2 found with A-253 and SK-OV cells. This may reflect the smaller volume of L1210 cell (0.39 pl/cell) compared to SK-OV (2.1 pl/cell) and A-253 (2.4 pl/cell). The cellular concentration of BLM A_2 in L1210 cells, if it is assumed BLM A_2 is distributed uniformly in cellular water, would be

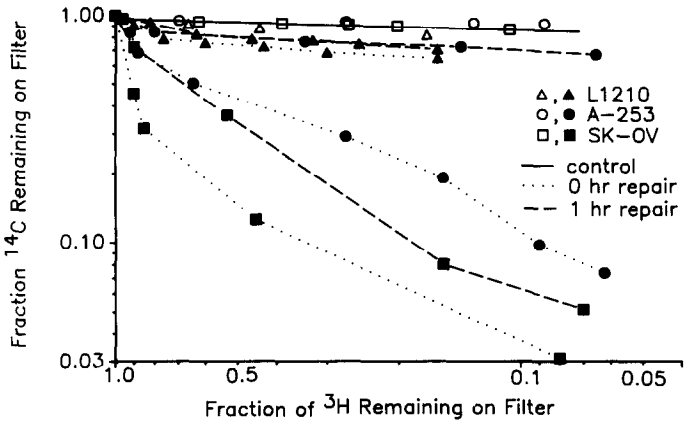

Fig. 6. DNA damage after BLM treatment. Cells were treated for 1 hr with BLM A_2 (solid symbols). Single-strand DNA damage was assayed by alkaline elution methods immediately after the 1-hr exposure to BLM A_2 (dotted lines) or 1 hr after incubation in drug-free medium (dashed lines). A-253; 5 μM BLM A_2 (●). SK-OV; 5 μM BLM A_2 (■). No significant damage was detected with L1210 cells incubated with 5 μM BLM A_2 and, thus, 25 μM BLM A_2 was used (▲). The integrity of DNA was also measured in untreated cells (open symbols; solid line).

approximately 2.5-fold greater than that found in A-253 and SK-OV cells. It is of interest, therefore, that the nuclear level of radiolabel in L1210 cells was much lower than that in A-253 and SK-OV cells (Table 1) and the initial single-strand DNA damage (Table 2) in L1210 cells also was lower and was, in fact, undetectable at 5 μM BLM A_2. The initial single-strand DNA damage seen with A-253 and SK-OV cells, however, did not correlate with either nuclear radioactivity content nor the sensitivity of the human cells to BLM A_2. Repair of BLM-induced DNA damage (Fig. 6; Table 2) also did not correlate with sensitivity in any of the cell lines tested.

BLM hydrolase is believed to be an important factor in controlling BLM-induced lung toxicity [15], and enzyme activity is found in homogenates of human tumors [16]. There still remains a controversy about the importance of this enzyme in tumor sensitivity to BLM A_2. Some authors have found increased BLM hydrolase activity in homogenates of resistant cells [1, 24, 25], while others observed no difference in the homogenates of sensitive and resistant cells [26–28]. There has been no extensive examination of the metabolic fate of BLM with intact cells. In our studies with homogenates from three tumor cell types, BLM hydrolase activity has kinetic parameters similar to those of the enzyme in rabbit pulmonary fibroblasts [29]. Based upon our calculations of cellular drug association at steady state (Fig. 2; approximately 0.5 to 2 pmol/10^7 cells) and the cellular water content, the estimated BLM A_2 concentration, if distributed uniformly in cellular water, is 10^{-3} to 10^{-4} the K_m for BLM hydrolase. Thus, it is unlikely that the enzyme is saturated by BLM within the cell. Considering the estimated velocity of the deamidation for BLM hydrolase, it was surprising that we found no evidence for metabolism of [^{3}H]BLM A_2 by this enzyme with intact cells. These results, however, confirm the preliminary findings of Roy and Horwitz [12] that cell-associated BLM A_2 is unmetabolized. This apparent latency could reflect an inaccessible cellular location of this enzyme or the presence of endogenous inhibitors, such as glutathione or cations [19], in intact cells. Alternatively, the latent nature of BLM hydrolase could reflect the presence of significant quantities of metal-bound BLM A_2 within cells, since metal-bound BLM is not a substrate for BLM hydrolase [19]. This seems unlikely because the cytotoxicities seen with both metal-free and Cu(II)BLM A_2 are similar in these and other cells [6] and the metal-bound BLM requires nonphysiological conditions to cleave DNA [30]. Furthermore, intracellular metal binding proteins have been described [31] that can potentially remove metals from BLM, and the HPLC profiles obtained with cells incubated with radiolabeled metal-free BLM A_2 and Cu(II)BLM A_2 were identical. Nevertheless, the observation that Cu(II)BLM A_2 can participate in nondestructive interactions with DNA *in vitro* [32] suggests that further attention should be directed toward determining the metal-ligand nature of BLM within cells.

In summary, we found, using pharmacologically relevant drug concentrations, that cultured cells rapidly achieved steady-state levels of BLM A_2 and that drug efflux was much slower. Second, we saw no evidence for inactivation of BLM by BLM hydrolase in intact cells despite considerable amounts of BLM hydrolase activity in all three cell types. Third, cellular or nuclear factors, in addition to BLM A_2 content, affected initial single-strand DNA damage in the human cells. Moreover, cell survival was not readily related to the extent of initial single-strand DNA damage, as assessed by alkaline elution, or its repair rate. Thus, our data support the notion [2, 8–10] that factors other than single-strand damage of chromosomal DNA may contribute to BLM-induced cell death.

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