In Vitro Selection and Characterization of a Bleomycin-resistant Subline of B16 Melanoma

Joan E. Zuckerman, Thomas A. Raffin, J. Martin Brown, Robert A. Newman, B. Bill Etit, and Branimir I. Sikic

Stanford University School of Medicine, Stanford, California 94305

ABSTRACT

A subline of B16 melanoma cells which is 10-fold resistant to bleomycin (BLM) was developed by exposure of the parental cell line to sequential increases in BLM concentration. This resistance to BLM is stable for over 30 passages in drug-free medium. Neither double minute chromosomes nor homogeneously staining regions were evident in karyotypes of the resistant cells. The subline, B16/BLM-R1, was slightly radioresistant, with a D0 ratio of 1.4 compared to the parental cells. No cross-resistance was observed to a number of cytotoxic drugs, including doxorubicin, melphalan, cisplatin, carmustine, daunorubicin, mitomycin C, and vinblastine. However, slight cross-resistance (2-fold) was noted with etoposide. Marked resistance to BLM was also demonstrated in vivo in mice bearing B16/BLM-R1 implanted s.c. Possible mechanisms of BLM resistance in these cells were explored through examination of the degree of drug inactivation by BLM hydrolysis and measurement of single- and double-strand DNA scission, as well as repair of single strand breaks by the alkaline elution technique. The specific activity of BLM hydrolyse was 70% higher in the resistant subline, commensurate with a 50% increase in protein content in these cells. Because this is insufficient to account for the 10-fold resistance, BLM hydrolyse activity does not appear to be a major determinant of resistance in B16/BLM-R1. The overall number of single and double strand breaks in DNA produced by bleomycin treatment did not differ in the sensitive and resistant cells. The cross-resistance with ionizing radiation and etoposide suggests an enhanced capability of B16/BLM-R1 cells to withstand or repair single strand breaks in DNA. However, this was not evident by measuring repair of single strand scission by alkaline elution.

INTRODUCTION

Biochemical drug resistance in tumor cells (relative to toxicities in normal tissues) is thought to be the major limiting factor in cancer chemotherapy (1). Understanding the various mechanisms of resistance to a drug may enhance knowledge of the action of the drug and lead to strategies to overcome or circumvent the resistance.

Relatively little information is available concerning the nature of resistance of eukaryotic cells to BLM, a mixture of glycopeptides whose mechanism of cytotoxicity is thought to involve the production of single- and double-strand breaks in DNA (2-6). Metabolic drug inactivation by a cytosolic aminopeptidase termed “bleomycin hydrolase” has been shown to be a possible mechanism of resistance to the drug in some tumor models (7-9). Other bleomycin-resistant cell lines do not show increased bleomycin hydrolyse activity (10, 11). Moreover, no correlation was observed between specific activities of BLM hydrolyse and in vitro drug sensitivities in human ovarian carcinoma specimens (12). Conversely, human fibroblasts from patients with ataxia telangiectasia, which exhibit deficient DNA repair mechanisms, have been observed to be unusually sensitive to BLM, as well as to ionizing radiation (13-15).

In this paper, we describe the in vitro selection and characterization of a BLM-resistant subline of B16 melanoma, a murine tumor which is relatively sensitive to BLM.

MATERIALS AND METHODS

Drugs and Chemicals

The chemotherapeutic agents BLM, daunorubicin, doxorubicin, vinblastine, etoposide, carmustine, 1-beta-d-arabinofuranosylcytosine (cispilatin), melphalan, and mitomycin C were provided by the Pharmaceutical Resources Branch of the National Cancer Institute. BLM was dissolved in HBSS or 0.9% NaCl solution and stored at -20°C for up to 90 days. Stock solutions of the other agents were diluted in HBSS and stored at -20°C in aliquots adequate for one assay. [3H]Thymidine (methyl-[3H]; 77 Ci/mmol) and [14C]thymidine (methyl-[14C]; 40-60 Ci/mmol) were purchased from New England Nuclear, Boston, MA.

Cell Culture and In vitro Selection

Murine B16 melanoma cells were obtained from Mason Research Institute, Worcester, MA. Both parental (B16-WT) and BLM-resistant (B6/BLM-R1) cell lines were maintained in vitro in a mixture of Waymouth’s and McCoy’s media (1:1) (Grand Island Biological Co., Grand Island, NY), supplemented with insulin (5 µg/ml), penicillin (100 units/ml), streptomycin (100 µg/ml), and 15% NBCS, and designated as “W/M medium.”

Selection for BLM resistance was initiated by continuous exposure of the B16-WT parental cells to the IC50 (10-7 M) of BLM determined by a soft agar clonogenic assay. The BLM concentration used for selection was increased in stepwise fashion by 2-fold increments when the generation time of the cells approached that of the parent strain (17 h).

The generation time of the cells was measured after seeding 5 x 10⁴ cells into T-25 flasks (Corning Glassworks, Corning, NY). The number of cells per flask was counted in triplicate, daily for 4 days, using a hemocytometer and assessing cell viability by exclusion of trypan blue dye.

In vitro Drug Sensitivity Testing

Clonogenic Assay. The drug sensitivities of the cells were assessed both by a soft agar clonogenic assay and by [3H]thymidine incorporation. Clonogenic survival was determined after 1-h drug exposure (16). Cells growing in monolayer were harvested during mid-log phase from plastic tissue culture flasks (Corning Glassworks), using 0.06 M EDTA for 5 min and then washed with HBSS:10% NBCS prior to centrifugation at 200 x g for 10 min. Cell number and viability were determined by hemocytometer and trypan blue exclusion. Drugs to be tested were diluted in HBSS, placed into a series of plastic tissue culture tubes (Falcon Plastics, Division of Becton Dickinson and Co., Oxnard, CA), and stored frozen at -20°C. Cells (2 x 10⁴/ml) were incubated...
with or without drug for 1 h at 37°C in HBSS:10% NBCS in a shaking water bath. After incubation, cells were washed with HBSS:10% NBCS, centrifuged at 200 × g for 10 min, and resuspended in 2 ml of 0.3% agar in W/M media. One-half ml of this mixture was plated above a 0.5-ml lower layer containing 0.5% agar in W/M media. Cells aggregates of greater than 30 cells and at least 50 μm in diameter were scored as colonies. Plating efficiencies were calculated by dividing the mean colony count derived from triplicate wells by the number of cells originally plated. Drug effects are expressed as the percentage of control colony survival (equivalent to percentage of control plating efficiency).

Thymidine Incorporation Assay. Drug sensitivity also was assessed by quantitation of thymidine incorporation into TCA-insoluble cellular DNA after continuous drug exposure. Cells in the log phase of growth were harvested, washed, and counted as described above, and 4 × 10^4 cells in 0.2 ml W/M media were added to each of a 96-well Micro Test tissue culture plate (Falcon Plastics). Drugs to be tested were added to triplicate wells in a 0.02-ml volume and the plates were incubated for 48 h at 37°C in a humidified atmosphere of 5% CO₂ and air. [³H]Thymidine was then added in a volume of 0.02 ml containing 0.4 μCi/well at a final thymidine concentration of 10⁻⁴ M. After an additional 24 h of incubation, the cells were detached from the wells by treatment with 0.05% Pronase (Sigma Chemical Co., St. Louis, MO) in HBSS and harvested onto glass fiber filters with a 200μm pore size. The filter containing the cells was placed in a scintillation vial (Wheaton Scientific, Millville, NJ), 0.2 ml Protosol (New England Nuclear, Boston, MA) was added, and the vials were capped and heated at 52°C for 1 h. The vials were cooled, 0.02 ml of glacial acetic acid and 5 ml of Econofluor (New England Nuclear, Boston, MA) were added, and the radioactivity was counted in a LS 9000 liquid scintillation counter (Beckman Instruments Co., Palo Alto, CA).

In Vivo Antitumor Studies

Male C57BL/6 mice (Simonsen Laboratories, Gilroy, CA) approximately 2 months of age were inoculated with 3.5 × 10⁸ viable WT or R1 cells suspended in 0.1 ml HBSS:10% NBCS by s.c. injection into the right rear flank. When tumors became palpable, mice were random

RESULTS

B16/BLM-R1-resistant Subline. After approximately 20 passages in medium containing stepwise increasing concentrations of BLM, a melanoma cell line (B16/BLM-R1 or R1) was developed that could be continuously maintained in medium containing 10⁻⁴ M BLM. Cells were assayed for BLM sensitivity by clonogenic and thymidine incorporation assay methods after three passages in drug-free media.
medium. As shown in Fig. 1A, the resistant cells demonstrated a 10-fold shift in the IC₅₀ for BLM compared to the parental line, B16-WT (WT). The results of a continuous exposure assay utilizing thymidine incorporation are shown in Fig. 1B; this assay confirms that a 10-fold increase in the BLM IC₅₀ had occurred in the R₁ cells. This degree of resistance has remained stable during 50 passages in drug-free medium.

The generation time of the R₁ cells was 18.5 h, compared to 17 h for the WT cells (Table 1). The R₁ cells contained 15% more DNA and 53% more protein than the WT cells (Table 1). This increase in cell size of the R₁ cells also was evident on light microscopy and on light scatter measurements using a fluorescence-activated cell sorter (data not shown).

Cross-Resistance. Clonogenic assays were performed in order to assess whether B16/BLM-R₁ cells were cross-resistant to other chemotherapeutic agents. The data are summarized in Table 2. The coefficient of variation for this assay was 5 to 10% for both WT and R₁ cells. Thus, resistance values of greater than 1.5 should be considered significant. The ratio of IC₅₀ values (resistance ratio) was close to unity for all agents tested with the exception of etoposide. Even for etoposide, the resistance ratio was 2.1 compared to 10.0 for BLM, indicating only a minor degree of cross-resistance.

In Vivo Resistance to BLM. As shown in Fig. 2A, in vivo treatment with BLM produced a dose-dependent inhibition of tumor growth in mice inoculated s.c. with the parental line, B16-WT. Mice given the highest dose of BLM (25 mg/kg/day) demonstrated decreased body weight, inactivity, and signs of respiratory insufficiency as symptoms of BLM toxicity. The R₁ cells which had been selected for BLM resistance in vitro also manifested resistance to BLM in vivo (Fig. 2B).

Radiation Sensitivity. Fig. 3 illustrates that R₁ cells are significantly resistant to ionizing radiation compared to the WT cells with a D₀ ratio (R₁/WT) of 1.4.

Assessment of Bleomycin Metabolism. The cytosols of WT and R₁ cells were incubated with varying concentrations of BLM-B₂, and the extent of drug metabolism was measured by HPLC. The R₁ cells exhibit 70% more bleomycin hydrolase activity when expressed on a per cell basis, Fig. 4A. This demonstrated decreased body weight, inactivity, and signs of respiratory insufficiency as symptoms of BLM toxicity. The R₁ cells which had been selected for BLM resistance in vitro also manifested resistance to BLM in vivo (Fig. 2B).

Table 2 Drug sensitivities of B16 wild type (WT) and B16/BLM-R₁ (R₁) cells

<table>
<thead>
<tr>
<th>Drug</th>
<th>WT (µg/ml)</th>
<th>R₁ (µg/ml)</th>
<th>Resistance ratio (R₁/WT)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bleomycin</td>
<td>0.5</td>
<td>5.0</td>
<td>10.0</td>
</tr>
<tr>
<td>Etoposide</td>
<td>0.99</td>
<td>2.07</td>
<td>2.1</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>0.024</td>
<td>0.032</td>
<td>1.3</td>
</tr>
<tr>
<td>Vinblastine</td>
<td>0.59</td>
<td>0.65</td>
<td>1.1</td>
</tr>
<tr>
<td>Melphalan</td>
<td>0.38</td>
<td>0.34</td>
<td>0.9</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>3.2</td>
<td>3.8</td>
<td>1.2</td>
</tr>
<tr>
<td>Carmustine</td>
<td>1.9</td>
<td>1.85</td>
<td>1.0</td>
</tr>
<tr>
<td>Mitomycin C</td>
<td>1.2</td>
<td>1.75</td>
<td>1.5</td>
</tr>
<tr>
<td>Dactinomycin</td>
<td>0.014</td>
<td>0.014</td>
<td>1.0</td>
</tr>
</tbody>
</table>

*IC₅₀ values for WT and R₁ cells determined by clonogenic assay in soft agar after a 1-h drug exposure.
* The resistance ratio is the ratio of IC₅₀ for each drug in the R₁ and WT cells.

```
Fig. 1. BLM sensitivity of wild type (WT) and resistant (R₁) cell lines as assessed by soft agar clonogenic (A) and thymidine incorporation (B) assays. ○, B16-WT cells; ◦, B16/BLM-R₁ cells.

Table 1 Alterations in total DNA, protein content, and generation time in BLM-R₁ cells

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>R₁</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytosolic protein (µg/10⁶ cells)</td>
<td>226 ± 24*</td>
<td>359 ± 13*</td>
</tr>
<tr>
<td>Total protein (µg/10⁶ cells)</td>
<td>390 ± 30</td>
<td>597 ± 28*</td>
</tr>
<tr>
<td>DNA (µg/10⁶ cells)</td>
<td>19.3 ± 1.2</td>
<td>20.3 ± 0.9*</td>
</tr>
<tr>
<td>Generation time (h)</td>
<td>17</td>
<td>18.5</td>
</tr>
</tbody>
</table>

* Mean ± SD.
* P < 0.001, Student's t test.
* P < 0.01, Student's t test.
```

Fig. 2. In vivo tumor growth of WT (A) and R₁ (B) cells after s.c. inoculation in C57BL/6 mice. ○, saline control; ◦, BLM, 5 units/kg/day; □, BLM, 15 units/kg/day; ■, BLM 25 units/kg/day. Treatments were administered daily for 9 days after the appearance of measurable tumors.

Fig. 3. Relative radioresistance of R₁ (○) cells as compared to WT (■) cells.

Fig. 4. BLM metabolism profile of WT (A) and R₁ (B) cells.
difference in enzyme activity depicted in Fig. 4A is largely accounted for by a 50% greater protein content in R1 cells compared to WT cells. When BLM hydrolase activity is expressed on the basis of cellular protein content (Fig. 4B), both cell lines appear to have similar enzyme activity.

DNA Damage and Repair. Fig. 5 displays the dose-dependent single strand DNA scission produced in WT and R1 cells by a 1-h exposure to BLM. Similar elution profiles were obtained for both cell lines over a wide range of BLM concentrations. BLM can, however, also produce double-strand DNA scission as a result of proximal single-strand scissions. This type of lesion can be detected by DNA elution at neutral pH. Fig. 6 shows the results of a neutral elution of BLM-treated R1 and WT cells. DNA elution patterns in both cell lines are similar, suggesting that resistance is not the result of an alteration in the degree of BLM-induced DNA double-strand scission.

To evaluate DNA repair, cells were treated with BLM for 1 h, washed, and either immediately prepared for alkaline elution or allowed to incubate in drug-free media for 1, 2, or 4 h to assess time-dependent repair of DNA single-strand breaks. As shown in Fig. 7, repair profiles for the two cell lines are similar, although there is a suggestion of a somewhat faster repair rate in the R1 cells. However, these data show no major alteration in the kinetics of repair of single strand lesions in the R1 cell line.

Karyotypic Analysis. The karyotypes of both the WT and R1 cells displayed considerable aneuploidy and cell-to-cell variation. Neither double minute chromosomes nor homogeneously staining regions were observed in the resistant cells. Thus, direct
karyotypic evidence of gene amplification was not observed in the BLM-resistant cells.

DISCUSSION

The structure and mechanism of action of BLM are unique among clinically useful anticancer drugs. The BLM glycopeptides bind to DNA via partial intercalation of the bithiazole groups, and produce single- and double-strand breaks with the release of free bases (2–5, 23–28). The DNA scission reaction in vitro is dependent on both ferrous iron and molecular oxygen and appears to involve the generation of free radical species from the complex of BLM-Fe²⁺-O₂ (4, 5, 29–31).

This mechanism of action suggests the possibility that the cellular capacity to repair DNA may be an important determinant of BLM cytotoxicity. The increased sensitivity to BLM of fibroblasts from patients with ataxia telangiectasia supports this concept (13–15). Details of the repair process of DNA injury from BLM are not well understood. Cells from most complementation groups of xeroderma pigmentosum, involving defects in excision of thymine dimers from UV irradiation, are not more sensitive to BLM (32, 33). However, cells from several genetic disorders which are thought to involve DNA repair (Fanconi’s anemia, Bloom’s syndrome, and Cockayne’s syndrome, in addition to ataxia telangiectasia), display increased sensitivity to both BLM and ionizing radiation (33). The lesion in ataxia telangiectasia is thought to be a defect in recognition of DNA strand breaks (34). A strong relationship between DNA repair capacity and bleomycin sensitivity has also been demonstrated in both eukaryotic and prokaryotic cells with a variety of DNA repair defects (35, 36).

The cross-resistance between BLM and ionizing radiation in our B16/BLM-R1 cells is therefore indirect evidence of an increased capacity to repair or withstand DNA strand breakage. The alkaline elution method did not demonstrate differences in the formation of single strand breaks between the two cell lines, nor did it show any major differences in the rate of repair of single-strand breaks. However, it is possible that double-strand breaks are more important and we are at present investigating the possibility of differences in the rate and extent of repair of double-strand breaks following BLM treatment.

The effect of BLM is more pronounced on linker DNA in eukaryotic cells, with release of nucleosomes which is distinguishable from the actions of micrococcal or endogenous nucleases (37). This raises the possibilities that damage or repair at discrete sites of DNA, or alterations in chromatin structure not detectable by alkaline elution, might be responsible for the BLM resistance in the R1 subline.

Metabolic inactivation of bleomycin by the aminopeptidase “bleomycin hydrolase” has been implicated as a mechanism of resistance in some cell lines (7–9), but not in others (10, 11). The mechanism of resistance to BLM in the latter models was not elucidated (10, 11). In contrast to some of these studies (9, 11), we did not utilize preliminary mutagenesis to increase the mutation frequency prior to or during BLM selection. Our studies of bleomycin hydrolase indicate that the activity of this enzyme is not correlated with the 10-fold resistance to BLM in the B16/BLM-R1 subline. These results are consistent with the report of a lack of correlation between BLM sensitivity and BLM hydrolase activity in primary human tumor specimens (12).

Other possible mechanisms of resistance to BLM include alterations in free radical scavenging pathways and decreased drug entry into cells. BLM resistance in Escherichia coli has been developed by induction of increased levels of superoxide dismutase with exposure to paraquat (38). This enzyme was not measured in our cells, nor have differences in antioxidant capacity been investigated for the other published eukaryotic models of BLM resistance. However, in nucleated organisms the cytotoxic action of BLM is thought to involve local generation of a free radical complex at the site of DNA binding, rather than diffuse superoxide radicals, so that overall antioxidant activity is not likely to serve a major protective function against BLM-mediated DNA scission (28–31). We attempted to measure BLM uptake in our sensitive and resistant cells using both ⁵⁷Co and ¹⁴H-labeled BLM (data not shown). Very little BLM was taken up by either the WT or R1 cells, which is consistent with the recent finding that only 0.1% of extracellular BLM was accumulated by HeLa cells (39). It is likely that BLM accumulation did not differ in our sensitive and resistant cells, since there were no differences in either metabolic degradation of drug or DNA breakage as measured by alkaline and neutral elutions. Since bleomycin hydrolase activity was similar between WT and R1 cells, less strand breakage should have been found in the R1 cells if the amount of drug entering the cells was decreased.

The slight degree of cross-resistance of etoposide in the R1 cells (2.1-fold; Table 2) is of interest in that the cytotoxicity of etoposide also involves single-strand scission of DNA, although by a different mechanism from BLM, involving interaction with topoisomerase II (40, 41). In contrast to the slight cross-resistance between BLM and etoposide observed in our BLM-selected R1 cells, a human epithelial line selected for resistance to etoposide did not display cross-resistance with BLM (42).

Doxorubicin can also produce some DNA strand breakage, probably as a consequence of its intercalating capability and not to the same extent as bleomycin (43). The degree of cross-resistance to doxorubicin in the R1 cells [1.3-fold compared to 10-fold for BLM in R1 cells (Table 2)] is probably not significant. The 1.5-fold cross-resistance with mitomycin C, a DNA cross-linking agent, is also quite minor.

The BLM resistance in our B16 cells is clearly not related to the phenotype of pleiotropic drug resistance which has been reported for various antitumor antibiotics and plant alkaloids (44, 45). Bleomycin is usually non-cross-resistant in the models of pleiotropic or multidrug resistance, which are associated with decreased accumulation of the resistant drugs and various plasma membrane alterations (44, 45). The R1 cells do not exhibit cross-resistance with vinblastine or daunomycin, drugs which are characteristically involved in pleiotropic resistance (Table 2).

It is of interest that 100- to 1000-fold degrees of resistance are commonly reported with the pleiotropic resistance phenotype. In contrast, we were not able to step up the degree of resistance to BLM in B16 cells beyond 10–20-fold, and the other reports of BLM resistance in the literature also describe 10-fold or lower resistance (7–11). In parallel experiments to our in vitro BLM selection, we also treated mice bearing solid B16 melanoma tumors s.c. with continuous s.c. infusions of BLM at therapeutic doses for eight months and were unable to develop a BLM-resistant strain (data not presented). The reasons for these difficulties in further stepping up in vitro resistance and failure to develop a resistant strain in vivo are obscure. One possible explanation is the cell cycle phase specificity of BLM (6). In vivo treatments may result in a high surviving fraction of cells which have not progressed through the vulnerable G₂ and M phases. These cells would retain bleomycin sensitivity but survive on a cytokinetic basis, thus obscuring the selection of stable biochemically resistant mutants. Another possibility involves the induction of epigenetic resistance to
BLM. Decreasing BLM toxicity with increasing time of exposure has been observed in vitro and was postulated to be due to induced epigenetic resistance to BLM (46).

An alternative explanation for our observations is the possibility that DNA may not be the only, or even the primary, target of BLM-mediated cytotoxicity. A recent study has shown that DNA may not be as highly interrelated as previously thought (47). Bleomycin cytotoxicity might instead be correlated with damage to specific elements of DNA, or perhaps with toxic effects on another, either nuclear or extranuclear, cellular target. This other possible site of drug action may be altered in the resistant R1 cells and account for some of the observed resistance. As discussed previously, however, DNA scission has been regarded historically as the major mechanism of action of BLM.

In summary, we have developed a 10-fold BLM-resistant strain of B16 melanoma by in vitro stepwise selection with BLM. Metabolic inactivation of BLM by BLM hydrolyase was not responsible for this resistance, nor is it likely that BLM uptake and accumulation differed in the R1 cells. Overall repair of single-strand DNA breaks as assessed by alkaline elution was not markedly different between the WT and R1 cells. However, these cells are cross-resistant to ionizing radiation and epipospide, implicating a role for the capacity to repair or withstand DNA strand breakage as at least one of the mechanisms of resistance to BLM in the B16/R1 subline.

ACKNOWLEDGMENTS

We thank Dr. Jeffrey Sawyer for the preparation and interpretation of the karyotypes of the B16-WT and B16/BLM-R1 cells and Sarah Potter for her assistance in typing and preparation of the manuscript.

REFERENCES

In Vitro Selection and Characterization of a Bleomycin-resistant Subline of B16 Melanoma


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/46/4_Part_1/1748

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.