Lack of a Role of Glutathione in Cellular Nonenzymatic Activation of BMS-181174, a Novel Analogue of Mitomycin C

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ABSTRACT

Recent studies, using a cell-free system, have suggested that thiol-dependent nonenzymatic bioactivation may be responsible for the superior antitumor activity of the mitomycin C analogue BMS-181174 [N-7-[2-(4-nitrophenyl)dithio)ethyl]mitomycin C] when compared to the parent compound. If operational in tumor cells, this pathway could have enormous clinical implications since tumor cell resistance to a variety of anticancer agents is often associated with increased glutathione (GSH) levels and BMS-181174 may be used to reverse this mechanism of resistance. The present study was undertaken to determine the role of GSH in cellular activation of BMS-181174 using a pair of well-characterized human bladder cancer cells (J82 and SCaBER) as a model. A 20-h pretreatment of J82 and SCaBER cells with a nontoxic concentration of D,L-buthionine-S,R-sulfoximine (BSO) caused about 80—88% reduction in cellular GSH levels. Surprisingly, the sensitivity of both cells to BMS-181174 was increased, not reduced, by BSO-induced GSH depletion. On the other hand, the cytotoxicity of BMS-181174 was significantly reduced in both cells by a 4-h pretreatment with 1 mM GSH. Like BSO, a 4-h pretreatment with another thiol compound (cysteine) resulted in a statistically significant sensitization of both cells to BMS-181174. Cellular GSH levels were not affected in either of the cell lines by pretreatment with GSH or cysteine. In conclusion, the results of the present study argue against a role of GSH in cellular nonenzymatic activation of BMS-181174 in J82 and SCaBER cells.

INTRODUCTION

MMC has shown activity against various solid tumors, including bladder and colorectal carcinomas (1, 2). However, the clinical effectiveness of this anticancer agent is often restricted by its dose-limiting toxicity (2). At conventional doses, common side effects of MMC include delayed cumulative neutropenia and thrombocytopenia. Even with an intermittent dosing schedule, e.g., once every 6—8 weeks, the hematological toxicity of MMC remains dose limiting. This has led to the synthesis of MMC analogues in an attempt to improve the therapeutic potential of the parent drug. Chemical modifications of MMC have been attempted at positions 1a, 7, and 10. Although substitutions at positions 1a or 10 have often produced poorly active analogues, variations at position 7 have resulted in a series of compounds with reduced toxicity and/or superior antitumor activity (for review, see Ref. 3).

BMS-181174 [N-7-[2-(4-nitrophenyl)dithio)ethyl]mitomycin C; previously known as BMY 25067] is one such MMC analogue that has shown promise preclinically (3—7). Toxicological studies have revealed that relative to MMC, BMS-181174 is less myelosuppressive with more rapid recovery from the nadir for neutropenia and thrombocytopenia (4). Preclinical studies, including those from our laboratory, have also shown that BMS-181174 is significantly more cytotoxic (10-fold to >200-fold) than MMC against several different murine and human tumor cells (5—8). Furthermore, BMS-181174 has exhibited superior antitumor activity in vivo against B16 melanoma xenografts. A high percentage of cures resulted when both the tumor cells and the drug were administered i.v (4). In a separate experiment, BMS-181174 displayed superior antitumor activity when B16 cells were implanted s.c. and the drug was given i.v. (4). However, the mechanism of superior antitumor activity of BMS-181174 as compared to MMC remains to be elucidated.

Bioreductive activation of MMC is a prerequisite for its antitumor activity (9—11). Elucidation of the mechanisms of bioactivation of MMC has been the subject of intense research for more than a decade. Several different enzymes, including NADPH:cytochrome P450 reductase, NAD(P)H:quinone oxidoreductase (DT-diaphorase), NADH:cytochrome b5 reductase, xanthine oxidase, and xanthine dehydrogenase are capable of activating MMC (12—15). Deficient drug activation, due to the down-regulation of one or more MMC bioactivation enzymes, has been suggested to be the primary mechanism of tumor cell resistance to this drug (16—19). Although the enzymes responsible for activating BMS-181174 remain to be identified, He et al. (20) have recently proposed a novel nonenzymatic mechanism of activation for this MMC analogue. This study showed that BMS-181174, but not MMC, can be activated to DNA cross-linking species by thiols including GSH in a cell-free system. These investigators postulated that the superior antitumor activity of BMS-181174 may be due to this self-reductive activation capability. However, the significance of this pathway in tumor cell killing activity of BMS-181174 is not clear. If thiol-dependent activation of BMS-181174 plays an important role in its overall cytotoxicity, tumor cell sensitivity to this MMC analogue must be reduced by GSH depletion.

In the present study, we have investigated the role of thiols in cellular nonenzymatic activation of BMS-181174 by determining the effects of BSO (a specific inhibitor of the rate-limiting enzyme of GSH synthesis, γ-glutamylcysteine synthetase), GSH, and cysteine pretreatments on both cellular GSH levels and BMS-181174 cytotoxicity in a pair of well-characterized human bladder cancer cell lines, J82 and SCaBER.

MATERIALS AND METHODS

Chemicals. BMS-181174 was a generous gift from Bristol-Myers Squibb (Evansville, IN). BSO, GSH, and cysteine were purchased from Sigma (St. Louis, MO). All other reagents were of the highest purity available.

Cell Lines and Culture. Human bladder cancer cell lines J82 and SCaBER were obtained from American Type Culture Collection (Rockville, MD). Monolayer cultures were maintained in Eagle’s MEM supplemented with 100 μM nonessential amino acids, 1 mM sodium pyruvate, 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin.

Colony Formation Assays. Effects of BSO, GSH, and cysteine treatments on the sensitivities of J82 and SCaBER cells to BMS-181174 were determined using a colony formation assay. Stock solutions of BMS-181174 were prepared in DMSO immediately before use. The final concentration of DMSO was <0.025%, which did not affect the colony-forming efficiencies of J82 and SCaBER cells. To determine the effect of BSO treatment on BMS-181174 cytotoxicity, exponentially growing cells (2 × 10⁶) were plated in 75-cm² flasks in 1 ml of complete medium and then exposed to 200 μM BSO for 20 h at 37°C. Subsequently, different concentrations of BMS-181174 were added to
the flasks, and incubation was resumed for an additional 60 min in the presence of BSO. Following incubation, the cells were washed twice with PBS, trypsinized, and counted. Cells (2 × 10⁶) from each treatment group were plated in 25-cm² flasks in 4 ml of complete medium. The flasks were incubated for 7 days at 37°C in an atmosphere of 95% air and 5% CO₂. Colonies were fixed with 10% buffered formalin, stained with 0.5% methylene blue, and counted under an inverted microscope. Colonies containing ≥50 cells were counted as survivors. The IC₅₀ value was determined from a plot of the percentage of survival versus drug concentration. To determine the effect of GSH or cysteine on BMS-181174 cytotoxicity, 2 × 10⁶ cells were plated in 25-cm² flasks and exposed to either 1 mM neutralized GSH or cysteine for 4 h at 37°C prior to the addition of BMS-181174. Subsequently, the cells were washed twice with PBS and processed for a colony formation assay.

Effect of BSO, GSH, or Cysteine on Cellular GSH Levels. Cells were plated in 75-cm² flasks at a density of 2 × 10⁶ cells/flask in 13 ml of complete medium and exposed to 200 μM BSO for 20 h at 37°C. The cells were then trypsinized, washed thoroughly with PBS, suspended in 0.5 ml PBS, and sonicated for 30 s three times with a 5-min interval between each sonication. The cell extract was centrifuged at 14,000 × g for 30 min, and the supernatant fraction was used for GSH estimation. Some cells were pretreated with 200 μM BSO for 20 h and then exposed to 0.46 or 0.91 μM BMS-181174 for 1 h in the presence of BSO. Some cells were treated only with 0.46 or 0.91 μM BMS-181174 for 1 h. To determine the effect of thiols on cellular GSH levels, 2 × 10⁶ cells were plated in 75-cm² flasks and exposed to 1 or 5 mM neutralized GSH or cysteine for 4 h at 37°C. Subsequently, cells were trypsinized and processed for GSH estimation according to the procedure described by Beutler (21). Protein content was measured according to the method of Bradford (22).

RESULTS

Effects of BSO and/or BMS-181174 Treatments on Cellular GSH Levels. Table 1 summarizes the effects of BSO and/or BMS-181174 treatments on GSH levels in J82 and SCaBER cell lines. Cellular GSH levels did not differ significantly between J82 and SCaBER cells. A 1-h exposure of cells to 0.46 or 0.91 μM BMS-181174 alone did not significantly affect GSH levels in either of the cell lines. However, BSO treatment caused a significant reduction in the GSH levels of both cell lines. GSH levels in BSO-treated J82 and SCaBER cells were 20% and 12%, respectively, of those in controls. On the other hand, GSH levels were comparable in cells exposed to BSO alone or treated with BSO plus BMS-181174 (Table 1).

Effect of BSO on BMS-181174 Cytotoxicity. Fig. 1 shows the effect of BSO treatment on the cytotoxicity of BMS-181174 in J82 and SCaBER cells. The colony-forming efficiencies of control and BSO-treated J82 cells did not differ significantly (32 ± 2% and 29 ± 3%, respectively). Thus, a 20-h treatment of J82 cells with 200 μM BSO was nontoxic. Higher concentrations of BSO, however, were extremely toxic to these cells (data not shown). BSO treatment resulted in a statistically significant sensitization (about 1.46-fold) of J82 cells to BMS-181174 (Fig. 1a). The IC₅₀ values for BMS-181174 in control and BSO-treated J82 cells were 0.35 ± 0.05 and 0.24 ± 0.06 μM, respectively (P < 0.05 using Student’s t test).

Table 1 Effects of BSO and/or BMS-181174 treatments on cellular GSH levels in J82 and SCaBER cell lines

<table>
<thead>
<tr>
<th>Treatment</th>
<th>BMS-181174 (μM)</th>
<th>GSH levels (nmol/mg protein)</th>
<th>J82</th>
<th>SCaBER</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control cells</td>
<td>0.0</td>
<td>114 ± 13³</td>
<td>113 ± 13</td>
<td></td>
</tr>
<tr>
<td>0.46</td>
<td></td>
<td>122 ± 24 (107)⁶</td>
<td>103 ± 8.91</td>
<td></td>
</tr>
<tr>
<td>0.91</td>
<td></td>
<td>117 ± 22 (103)</td>
<td>120 ± 18 (106)</td>
<td></td>
</tr>
<tr>
<td>BSO-treated cells</td>
<td>0.0</td>
<td>23 ± 4 (20)</td>
<td>14 ± 5 (12)</td>
<td></td>
</tr>
<tr>
<td>0.46</td>
<td></td>
<td>19 ± 6 (17)</td>
<td>14 ± 6 (12)</td>
<td></td>
</tr>
<tr>
<td>0.91</td>
<td></td>
<td>22 ± 6 (19)</td>
<td>13 ± 5 (11)</td>
<td></td>
</tr>
</tbody>
</table>

³ Values represent mean ± SD of three independent experiments.

BNO-mediatated sensitization of J82 cells to BMS-181174 was more pronounced at higher drug concentrations. For example, survival of control J82 cells exposed to 0.46 and 0.91 μM BMS-181174 was higher by about 1.7- and 1.8-fold, respectively, as compared to BSO-treated cells. The SCaBER cell line was about 2.5-fold more resistant (P < 0.05) to BMS-181174 as compared to J82. The cytotoxic activity of BMS-181174 was also increased in SCaBER cells by BSO-induced GSH depletion (Fig. 1b). The IC₅₀ values for BMS-181174 in control and BSO-treated SCaBER cells were 0.88 ± 0.22 and 0.65 ± 0.14 μM, respectively. Like the J82 cell line, the effect of BSO on BMS-181174 cytotoxicity in SCaBER cells was more pronounced at higher drug concentrations. The colony-forming efficiencies of control and BSO-treated SCaBER cells did not differ significantly (42 ± 5% and 39 ± 3%, respectively).

Effect of GSH on BMS-181174 Cytotoxicity and Cellular GSH Levels. As shown in Fig. 2, the cytotoxicity of BMS-181174 was significantly reduced in both cell lines by a 4-h pretreatment with 1 mM GSH. The colony-forming efficiencies of control and GSH-treated J82 (33 ± 4% and 32 ± 4%, respectively) and SCaBER cells (37 ± 9% and 36 ± 10%, respectively) were comparable. The IC₅₀ value for BMS-181174 in J82 cells was increased from 0.12 ± 0.01 to 0.29 ± 0.05 μM by GSH treatment (P < 0.05). Thus, GSH treatment reduced the cytotoxicity of BMS-181174 by about 2.4-fold in the J82 cell line (Fig. 2a). The IC₅₀ values for BMS-181174 in control and GSH-pretreated SCaBER cells were 0.62 ± 0.05 and 1.15 ± 0.18 μM, respectively (P < 0.05). The cytotoxicity of BMS-181174 in these cells appeared to be cell density dependent because the IC₅₀ values were lower when...
ROLE OF OSH IN BIOACTIVATION OF BMS-181174

be due to a novel thiol-dependent nonenzymatic mechanism of its activation. These observations may have enormous clinical implications provided GSH-dependent reduction of BMS-181174 occurs in tumor cells as well. Since tumor cell resistance to a variety of anticancer agents is often associated with increased levels of GSH (25, 26), inclusion of BMS-181174 in combination chemotherapy protocols could overcome this mechanism of drug resistance.

The major objective of this study, therefore, was to determine whether GSH-dependent nonenzymatic activation of BMS-181174 contributes to its cytotoxic activity. We argued that if GSH plays a major role in the cellular activation of BMS-181174, cytotoxicity of this agent must be reduced by GSH depletion. The results of the present study showed that even though treatment of J82 and SCaBER cells with a nontoxic concentration of BSO produced approximately an 80–88% reduction in cellular GSH levels, cytotoxicity of BMS-181174 was increased, especially at higher drug concentrations, not reduced by GSH depletion. These results are similar to our previous studies with the P388/R-84 cell line (a multidrug-resistant variant of P388 mouse leukemia), which also showed a significant sensitization of these cells to BMS-181174 by BSO-induced GSH depletion (6). Potentiation of BMS-181174 cytotoxicity by BSO-induced GSH depletion in P388/R-84 cells was associated with an increase in DNA interstrand cross-link formation (6). However, the effect of GSH depletion on both cytotoxicity and DNA interstrand cross-linking was more pronounced for MMC than BMS-181174 (6). Collectively, these observations not only argue against a role of GSH-dependent reductive activation in tumor cell kill by BMS-181174, but also suggest that BMS-181174 and/or its metabolite(s) may be inactivated through conjugation with GSH. GSH/GST-mediated conjugation of MMC

Effect of GSH pretreatment on cellular GSH levels was also determined. A 4-h pretreatment with 1 or 5 mM GSH did not alter cellular GSH levels in either of the cell lines (data not shown). Prolonged exposure to 1 mM GSH (20 h) was also ineffective in increasing cellular GSH levels in these cells (data not shown).

Effect of Cysteine on BMS-181174 Cytotoxicity and Cellular GSH Levels. Unlike GSH, a 4-h pretreatment with 1 mM cysteine caused a significant increase in the cytotoxicity of BMS-181174 in both cell lines (Fig. 3). The IC_{50} value for BMS-181174 in J82 cells was reduced from 0.12 ± 0.02 µM to 0.09 ± 0.01 µM by cysteine treatment (P < 0.05; Fig. 3a). A similar cysteine treatment reduced the IC_{50} value for BMS-181174 from 0.58 ± 0.06 to 0.39 ± 0.10 µM (P < 0.05) in SCaBER cells. Colony-forming efficiencies of control and cysteine-treated J82 and SCaBER cells were comparable. To determine whether cysteine-mediated potentiation of BMS-181174 cytotoxicity resulted from an increase in GSH levels, cellular levels of this thiol were compared in control and cysteine-treated cells. Interestingly, GSH levels were not increased by cysteine treatment in either of the cell lines (data not shown).

DISCUSSION

BMS-181174 has shown superior antitumor activity as compared to MMC preclinically (4–8), and is currently in clinical trials as an anticancer agent (23, 24). However, the mechanism of superior antitumor activity of BMS-181174 is not fully understood. Recently, He et al. (20) have suggested that superior activity of BMS-181174 may

2 × 10^3 cells were exposed to BMS-181174 than in the BSO experiment (Fig. 1).

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metabolites has been suggested to affect antitumor activity of MMC (27–29).

It is worthwhile to mention that despite lower GSH levels in P388/R-84 cells (14.4 ± 0.02 nmol/mg protein; Ref. 30) than in J82 or ScABER cells, cytotoxicity of BMS-181174 is significantly higher in this mouse leukemia cell line (IC$_{50}$ for 1-h exposure = 0.012 μM; Ref. 6) as compared to human bladder cancer cells. A lack of correlation between cellular GSH levels and tumor cell sensitivity to BMS-181174 suggests further that nonenzymatic activation may not be important in the cytotoxic activity of this analogue. It is possible that in cellular systems, the enzymatic conversion of BMS-181174 to the DNA-alkylating species is more efficient than thiol-dependent nonenzymatic reduction. Also, the possibility that BMS-181174 is a better substrate for bioreductive enzymes than MMC cannot be ruled out, which may explain its superior antitumor activity.

KW-2149 is another MMC analogue which contains a disulfide function at the seven-amino group of the quinone ring. Recent studies by Lee et al. (31) have provided convincing evidence for GSH-dependent cellular activation of KW-2149. The cytotoxicity of KW-2149, but not MMC, was significantly increased by a 4-h pretreatment with 1 mM GSH or cysteine in HT-29 cells. Potentiation of KW-2149 cytotoxicity by pretreatment with GSH was associated with a 1.5-fold increase in cellular GSH levels (31). In the present study, however, cytotoxic activity of BMS-181174 was significantly reduced, not increased, in both cells by a 4-h pretreatment with 1 mM GSH. Also, cellular GSH levels were not altered by pretreatment with this thiol in either of the cell lines. Interestingly, despite a lack of effect on cellular GSH levels in either of the cells by a 4-h pretreatment with 1 mM cysteine, the cytotoxicity of BMS-181174 was increased significantly in both cell lines. Although the reasons for these discrepancies remain to be understood, certain explanations can be offered for these observations. For example, GSH-dependent chemical reduction of BMS-181174 extracellularly may produce metabolite(s) which is/are unable to enter the cells. Potentiation of BMS-181174 cytotoxicity by cysteine pretreatment may be due to mechanisms other than thiol-dependent nonenzymatic activation. Additional studies are, however, needed to test these hypotheses. Nonetheless, the results of the present study suggest that GSH-dependent activation of BMS-181174 may not play an important role in its cytotoxicity in J82 and ScABER cells.

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