Effect of Buthionine Sulfoximine and Ethacrynic Acid on Cytotoxic Activity of Mitomycin C Analogues BMY 25282 and BMY 25067

Bing H. Xu and Shivendra V. Singh

Cancer Research Laboratory, Mercy Hospital of Pittsburgh, Pittsburgh, PA 15219

ABSTRACT

BMY 25282 and BMY 25067, analogues of mitomycin C (MMC), were synthesized in an attempt to increase the therapeutic potential of the parent drug. The present studies were undertaken to determine if the cytotoxicity of MMC or its analogues is affected by cellular glutathione (GSH) and/or GSH transferase (GST) levels by using sensitive (P388/S) and multidrug resistant (P388/R-84) mouse leukemia cells as a model. P388/R-84 cells were cross-resistant to all three drugs. BMY 25067 was >100 times more cytotoxic than MMC in both the cells. MMC and BMY 25282 produced significantly lower DNA interstrand cross-links (ISC) in P388/R-84 cells, whereas BMY 25067 induced ISC formation was comparable in these cells. GSH depletion with \( \text{L-buthionine-S,R-sulfoximine} \) (BSO) increased sensitivity to MMC, BMY 25282, and BMY 25067 by 3.4-, 4.1-, and 1.8-fold, respectively, in the resistant cells. Pretreatment of P388/R-84 cells with a nontoxic concentration of ethacrynic acid (EA) (10 \( \mu \text{g/ml} \) for 1 h), an inhibitor of GST activity, also resulted in a significant increase in the cytotoxic activities of MMC and BMY 25282 (3.8- and 3.1-fold, respectively), but not of BMY 25067. Combined pretreatment of P388/R-84 cells with BSO and EA caused further increase in the cytotoxic activities of both MMC and BMY 25282. Potentiation of BMY 25067 cytotoxicity by combined BSO and EA pretreatments was similar to that observed by BSO pretreatment alone. The ISC formation by MMC and BMY 25282 were also increased significantly by BSO or EA pretreatment in these cells. Whereas BSO treatment increased BMY 25067 induced ISC formation, it was not affected by EA pretreatment. These results suggest that (a) a potentiation of the cytotoxic activity of MMC or BMY 25282 can be achieved by GSH depletion and/or GST inhibition, (b) the enhanced cytotoxicity may be caused at least in part by the increased formation of drug-DNA cross-links, and (c) the mechanism of BMY 25067 cytotoxicity may be different from the other two drugs. The results of the present study also suggest that BMY 25067 may be seriously considered for further clinical development because it is much more active than MMC, and unlike the parent drug cytotoxicity of BMY 25067 does not seem to be affected by GST levels, which have been suggested to play an important role in cellular resistance to several cancer chemotherapy drugs.

INTRODUCTION

MMC has shown activity against various solid tumors (1–3). However, the clinical effectiveness of MMC is often limited by its dose-limiting toxicity (delayed cumulative myelosuppression) and emergence of drug resistant tumor cells. This has led to the synthesis of analogues in an attempt to improve the therapeutic potential of the parent drug (4). Substitutions at positions 1a or 10 have produced poorly active analogues, whereas variations at position 7 (Fig. 1) have resulted in a series of compounds with reduced toxicity and/or superior antitumor activity as compared to MMC (see review, Ref. 4).

Inability of the resistant cells to bioactivate MMC, due to the suppression of the bioactivation enzyme(s), appears to be the most important factor in cellular resistance to MMC (5–7). Several other mechanisms have also been proposed to account for MMC resistance, including decreased intracellular drug accumulation (8), altered expression of cell surface proteins (9), reduced formation of MMC stimulated oxygen radicals (10), and increased repair of interstrand DNA cross-links (11). While many of these factors may contribute to MMC resistance, the major mechanism of MMC cytotoxicity appears to be the formation of DNA cross-links (12–15). However, in some systems cytotoxicity of MMC has been suggested to be mediated by drug stimulated oxygen radicals (10, 13, 16–18). Increased metabolic drug inactivation, mediated by GSH and/or GST, has also been implicated recently in cellular resistance to MMC (8, 9). Elevated levels of GSH or GST have been noted in certain MMC resistant cancer cells (8, 10).

Recently, we have utilized a multidrug resistant P388 cell line, exhibiting significant cross-resistance to MMC, to elucidate the mechanisms of MMC resistance (19). The cellular accumulation as well as the bioactivation of MMC appeared to be similar in P388/S and P388/R-84 cells (19), suggesting that MMC resistance in P388/R-84 cell line must depend on other factors. Even though GSH levels were comparable in these cell lines (20), GST activity was significantly higher in the P388/R-84 cells (21). Treatment of P388/R-84 cells with a combination of BSO and EA increased MMC cytotoxicity by 2-fold as measured by continuous drug exposure colony formation assay (19), providing further evidence for the involvement of GSH/GST system in cellular resistance to MMC.

BMY 25282 and BMY 25067 are MMC analogues and contain substitutions at position 7 (4). BMY 25282 has a much lower quinone reduction potential as compared to MMC (4–6) and because of its easy bioactivation, BMY 25282 has shown superior antitumor activity when compared to the parent drug (5, 6).

This study was undertaken to determine if the sensitivity to BMY 25282 or BMY 25067 is affected by GSH and/or GST levels. We have, therefore, determined the effect of BSO (GSH depletor) and EA (GST inhibitor) pretreatments on the cytotoxicities of BMY 25282 and BMY 25067 in the P388/R-84 cells. In addition, we have compared drug induced DNA crosslink formation in P388/S and P388/R-84 cells and determined the effect of BSO and EA pretreatments on ISC formation in the resistant cells.

MATERIALS AND METHODS

Chemicals. MMC, BMY 25282, and BMY 25067 were a generous gift from Bristol Myers Squibb Company. Stock solution of MMC was prepared in PBS, whereas MMC analogues, BMY 25282 and BMY 25067, were dissolved in dimethyl sulfoxide. Sources of other chemicals were the same as described previously (19, 20).
Cell Lines. P388/S and P388/R-84 cell lines were kindly provided by Dr. A. Krishan, University of Miami School of Medicine, Miami, FL. Suspension cultures of these cell lines were maintained as described previously (21). It is important to mention that multidrug resistance transporter P-glycoprotein is expressed only in the P388/R-84 cells (22), as determined by flow cytometry with the use of C-219 monoclonal antibody.

Cytotoxicity Assay. Cytotoxicity was assessed by soft agar colony formation assay. Approximately 1 x 10^6 cells were exposed to the drug for 1 h at 37°C, washed twice with PBS, suspended in fresh medium, and plated for colony formation assay. Colonies of greater than 60 μm were counted after 96 h of incubation at 37°C in an atmosphere of 95% air and 5% CO2. To study the effect of BSO induced GSH depletion on drug cytotoxicity, approximately 4 x 10^6 cells (0.4 x 10^6 cells/ml in 10.0 ml complete medium) were first incubated with 100 μM BSO (final concentration) for 24 h at 37°C. Subsequently, the cells were washed twice with PBS and counted. Approximately 1 x 10^6 BSO treated cells were then exposed to different concentrations of the drug for 1 h at 37°C and processed for colony formation assay as described above. To determine the effect of EA on cytotoxicity, 1 x 10^6 cells were incubated with 10 μg/ml EA for 1 h at 37°C, washed twice with PBS, exposed to various concentrations of the drug, and processed for colony formation assay. To determine the combined effects of BSO and EA pretreatments, 4 x 10^6 cells were first incubated with 100 μM BSO for 24 h at 37°C and washed twice with PBS. The cells (1 x 10^6) were then treated with 10 μg/ml EA for 1 h at 37°C, washed twice with PBS, exposed to the drug, and processed for colony formation assay as described above.

Alkaline Elution Assay. For the determination of DNA cross-links, cells (4 x 10^6 in 10.0 ml) were labeled with [14C]thymidine (0.1 μCi/ml; specific activity, 56 mCi/mmol) for 20 h. The medium was removed and the radioactivity was chased by a 4-h postincubation at 37°C in fresh medium containing 10 μM nonradioactive thymidine. The labeled cells were washed twice with ice-cold PBS, incubated with the drug for 1 h at 37°C, and washed twice again with ice-cold PBS. Aliquots containing 1 x 10^6 cells were irradiated with 1500 rad of γ-radiation on ice. DNA cross-link formation was determined by using the alkaline elution technique as described previously (23). Interstrand cross-link frequency was calculated by using the equation:

\[ ISC \text{ (rad eq)} = \left[ \left( R_0 - R_1 \right) / \left( 1 - R_1 \right) \right]^{1/0} \times 1500 \]

where \( R_0 \) and \( R_1 \) represent the fraction of DNA retained from control and drug treated cells, respectively. To determine the effect of GSH depletion on ISC formation, 4 x 10^6 P388/R-84 cells in 10.0 ml medium were coincubated with 100 μM BSO and 0.1 μCi/ml [14C]thymidine for 20 h at 37°C. Alkaline elution on BSO treated cells was then performed as described above. To determine the effect of EA on ISC formation, 4 x 10^6 P388/R-84 cells were first labeled with [14C]-thymidine as described above. The labeled cells were then incubated with 10 μg/ml EA for 1 h at 37°C and processed for alkaline elution assay.

RESULTS

The in vitro cytotoxicity of MMC, BMY 25282, and BMY 25067 as measured by 1-h drug exposure clonogenic assay in P388/S and P388/R-84 cells is shown in Fig. 2. The P388/R-84 cells, with IC_{50} of 883 ± 74 ng/ml, were about 6.2-fold more resistant to MMC (Fig. 2A) when compared to the sensitive cell line (IC_{50} = 143 ± 20 ng/ml; P < 0.0001, sensitive versus resistant). BMY 25282 and BMY 25067 appeared to be much more active than the parent drug in both these cells (Fig. 2, B and C). The IC_{50} values for BMY 25282 in P388/S and P388/R-84 cells were 3.6 ± 0.3 and 36.2 ± 5.6 ng/ml, respectively (P < 0.0001, sensitive versus resistant). Clonogenic assays revealed that BMY 25282 was 39.7- and 24.4-fold more active than MMC in sensitive and resistant P388 cells, respectively (Fig. 2B). On the other hand, BMY 25067 was 143- and 130-fold more cytotoxic than the parent drug in P388/S and P388/R-84 cells, respectively (Fig. 2C). The IC_{50} values for BMY 25067 in sensitive and resistant P388 cells were 1.0 ± 0.2 and 6.8 ± 0.7 ng/ml, respectively (P < 0.0005, sensitive versus resistant). The P388/R-84 cells displayed 10- and 6.8-fold resistance to BMY 25282 and BMY 25067, respectively, when compared to the P388/S cells.

Table 1 summarizes the effect of BSO (GSH depletor) and EA (GST inhibitor) pretreatments on drug sensitivity in P388/R-84 cells. BSO pretreatment (100 μM for 24 h at 37°C) increased sensitivity to MMC by 3.4-fold in these cells (Fig. 3A). The IC_{50} value for MMC was reduced from 883 ± 74 to 263 ± 67 ng/ml by BSO pretreatment. We have reported earlier that MMC cytotoxicity is increased by only 13% by a similar BSO treatment when the colony formation assays are performed following continuous drug exposure (19). Lack of significant potentiation of MMC cytotoxicity by BSO treatment measured by continuous drug exposure clonogenic assay may be attributed to the rapid regeneration of GSH after removal of BSO. We have...
shown previously that more than 60% of the GSH is regenerated within 8 h after the removal of BSO from the culture medium (20). It may be argued that a greater potentiation of MMC cytotoxicity by BSO treatment can be achieved if GSH depletion is maintained until cytotoxic lesions have fully formed. However, when P388/R-84 cells were treated with 100 µM BSO for 24 h and incubated further with 50 µM BSO during the duration of cytotoxicity assay, the colony formation ability of these cells was inhibited by 47.58% (data not shown). These results suggest that prolonged exposure of GSH-depleted cells to BSO increases the toxicity of this agent.

Prior to investigating the effect of EA on MMC cytotoxicity, colony formation assays were performed to determine a nontoxic concentration of this agent. Treatment of P388/R-84 cells with 100 µg/ml EA for 1 h inhibited the colony formation ability of these cells by 53.8% (data not shown). On the other hand, 10 µg/ml EA pretreatment for 1 h had no significant effect on the colony formation ability of these cells (data not shown). Thus, 1-h pretreatment with 10 µg/ml EA was used in the subsequent experiments. EA pretreatment enhanced the MMC cytotoxicity by 3.8-fold (Fig. 3A) and the IC_{50} values for MMC in control and EA pretreated cells were 883 ± 74 and 231 ± 44 ng/ml, respectively (Table 1). Combined pretreatment of these cells with BSO and EA revealed a 5.3-fold increase in the MMC cytotoxicity (Table 1).

Similarly, BSO and EA pretreatments increased BMY 25282 cytotoxicity by 4.1- and 3.1-fold, respectively, in P388/R-84 cells (Fig. 3B). Combined pretreatment of P388/R-84 cells with BSO and EA resulted in an 8.0-fold increase in the BMY 25282 cytotoxicity (Table 1). The IC_{50} values for BMY 25282 in the control P388/R-84 cells and those treated with BSO plus EA were 36.2 ± 5.6 and 4.5 ± 1.4 ng/ml, respectively (Table 1).

BSO pretreatment produced a much lower effect on BMY 25067 cytotoxicity when compared to either MMC or BMY 25282 (Fig. 3C). The IC_{50} values for BMY 25067 in control and BSO treated P388/R-84 cells were 6.8 ± 0.7 and 3.8 ± 0.7 ng/ml, respectively (Table 1). Interestingly, EA pretreatment did not affect sensitivity to BMY 25067 (Fig. 3C). Potentiation of BMY 25067 cytotoxicity by combined BSO plus EA pretreatments was similar to that observed by BSO treatment alone (Table 1).

Since DNA-ISC formation is believed to be important in the cytotoxic activity of MMC and its analogues (10, 14, 15), ISC formation was compared in sensitive and resistant P388 cells (Table 2). The alkaline elution experiments were performed with the use of a single drug concentration, which represented the concentration between IC_{50} values for sensitive and resistant cells. ISC frequency induced by 0.6 µg/ml MMC was significantly lower in the resistant cells than in the P388/S cells (Table 2). Similarly, BMY 25282 (18 ng/ml) induced ISC frequency was significantly lower in the P388/R-84 cells as compared to the sensitive cells (Table 2). On the other hand, BMY 25067 induced ISC formation was comparable in P388/S and P388/R-84 cells.

Table 2 shows the effect of BSO and EA pretreatments on ISC formation in P388/R-84 cells. Pretreatment of these cells with BSO and EA increased MMC induced ISC formation by 1.24- and 2.06-fold, respectively (Table 3). Similarly, BMY 25282 induced ISC formation was also increased significantly by BSO or EA pretreatments. Whereas BSO pretreatment of these cells revealed a 1.52-fold increase in the BMY 25067 induced ISC formation, the ISC frequency remained unaffected.
by the EA pretreatment (Table 3). Although the combined effects of BSO and EA pretreatments on ISC formation were not investigated, results obtained from the cell survival experiments would suggest that such a treatment may produce higher ISC with MMC and BMY 25282 when compared to the single treatments of BSO or EA.

**DISCUSSION**

As a single agent, MMC is one of the few cancer chemotherapy drugs to show activity against colorectal (1, 2, 5, 24), lung, and breast carcinomas (3, 25). Even though several different mechanisms have been proposed to account for MMC resistance, reduced bioactivation of the drug in the resistant cells appears to be the most frequently encountered mechanism (5–7). This has led to the synthesis of MMC analogues, such as BMY 25282, with lower quinone reduction potential than the parent drug (4). Since BMY 25282 is easily bioactivated, several MMC resistant cells with reduced drug activation capability show increased sensitivity to BMY 25282 (5, 6). It is not surprising to note that P388/R-84 cells are cross-resistant to both MMC and BMY 25282 when compared to the synthesis of MMC analogues with low quinone reduction potential, because MMC resistance appears to be multifactorial and such analogues may not always display superior antitumor activity.

Since MMC stimulated oxygen radicals have been implicated in its antitumor activity (10, 16, 17), reduced formation of such radicals in the resistant cells may represent another important mechanism of MMC resistance. Even though we have not compared the free radical formation in P388 cells, MMC, BMY 25282, or BMY 25067 stimulated OH radical formation was found to be significantly lower in MCF-7 ADR cells than in the wild type MCF-7 cells (10). Keeping in mind the importance of oxygen radicals in the cytotoxicities of MMC or its analogues, it is logical to suspect that depletion of the cellular GSH level may increase the sensitivities to these drugs. In this report, we show that GSH depletion significantly increases the cytotoxic activities of MMC and BMY 25282 in the P388/R-84 cells. On the other hand, potentiation of BMY 25067 cytotoxicity by GSH depletion was found to be much lower than those observed for MMC or BMY 25282. This is interesting because among these agents, BMY 25067 has been shown to be the most effective one in stimulating oxygen radical formation (10), and thus, a greater potentiation of its cytotoxic activity by GSH depletion may be anticipated. Collectively, these results suggest that whereas drug stimulated oxygen radicals may contribute to the cytotoxicities of MMC and BMY 25282, such radicals may not play an important role in BMY 25067 antitumor activity.

GST is a family of isoenzymes involved in the detoxification of a wide variety of electrophilic xenobiotics (26), including many cancer chemotherapy drugs (27–29). Recent studies suggest that GST mediated drug inactivation may also play an important role in anticancer drug resistance (30–32). Involvement of GST system in cellular resistance to melphan, hepsulfam, and chlorambucil is clearly established now as the GST mediated conjugation of these drugs to GSH has been demonstrated (27–29). Even though GST catalyzed MMC-GSH conjugation has not been demonstrated yet, we have shown previously that simultaneous continuous exposure to MMC and 1 µg/ml (nontoxic concentration) EA increases the MMC cytotoxicity by 36% in P388/R-84 cells (19). When P388/R-84 cells were treated with 1 µg/ml EA, an inhibition of 21.7% in GST activity was observed. Treatment of these cells with 2 µg/ml EA resulted in an 28.7% inhibition of GST activity. The inhibition of GST activity was 25.0% when P388/R-84 cells were cotreated with 1 µg/ml EA and 0.03 µg/ml MMC (the IC50 concentration of MMC for P388/R-84 cells in continuous exposure colony formation assay). The most likely explanation for the potentiation of MMC cytotoxicity by EA would be that inhibition of GST alters the potential of the cell to detoxify MMC. In the present study, cells were exposed to 10 µg/ml EA for 1 h, washed twice with PBS, and then treated with MMC for 1 h before plating for colony formation assay. Under these conditions, EA pretreatment produced a significantly higher potentiation of MMC cytotoxicity (3.8-fold). It is possible that an 1-h pretreatment with 10 µg/ml EA produces greater GSH depletion and/or GST inhibition in P388/R-84 cells.

Use of EA as a modifier of alkylator sensitivity was first described by Tew et al. (33, 34). The results of Tew et al. were particularly encouraging because they showed a selective effect of EA in the resistant cells (33). Another interesting observation by these investigators was the lowering of cellular GSH levels following exposure to either EA alone or in combination with chlorambucil (33). It was proposed that in addition to GST activity inhibition, GSH depletion also contributed to the substantial increase in chlorambucil cytotoxicity. On the other hand, piriprost (an inhibitor of lipoygenase and GST activities) also potentiated chlorambucil cytotoxicity without causing GSH depletion (33). We have also shown previously (19) that EA treatment produces GSH depletion in P388/R-84 cells, which is increased further by exposing cells to a combination of EA and MMC (19). Even though the effect of EA alone or in combination with MMC and its analogues on GSH or GST levels were not assessed under present experimental conditions, it seems likely that the observed potentiation of MMC or BMY 25282 cytotoxicities by BSO or EA may be associated with GSH depletion and/or GST inhibition. However, the possibility that EA increases the cytotoxic activity of MMC or BMY 25282 through mechanisms other than GSH depletion and/or GST inhibition cannot be ruled out at this time. Potentiation by EA of melphalan and Adriamycin cytotoxicities have also been documented (35, 36). Interestingly, pretreatment of a number of drug sensitive and resistant human lung cancer cells with 3 µg/ml EA did not increase the cytotoxicity of either cisplatin or melphan (37). The reasons for this discrepancy remains to be determined.

BMY 25282 cytotoxicity was also increased significantly by EA, whereas EA pretreatment did not affect BMY 25067 cytotoxicity. These results suggest that BMY 25282 (or metabolites) may be inactivated by a GST catalyzed reaction, whereas a conjugation of BMY 25067 with GSH may not occur.

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**Table 3** Effect of BSO and ethacrynic acid pretreatments on MMC, BMY 25282, and BMY 25067 induced ISC frequencies in P388/R-84 cells

<table>
<thead>
<tr>
<th>Drug</th>
<th>Control +BSO</th>
<th>+EA</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMC*</td>
<td>35.4 ± 13.6</td>
<td>43.9 ± 6.6</td>
</tr>
<tr>
<td>BMY 25282*</td>
<td>44.4 ± 12.6</td>
<td>68.4 ± 6.5</td>
</tr>
<tr>
<td>BMY 25067*</td>
<td>24.4 ± 15.0</td>
<td>37.2 ± 9.8</td>
</tr>
</tbody>
</table>

* Cells were exposed to 0.6 µg/ml MMC.
* Mean ± SD of three experiments.
* P < 0.05, control versus treated P388/R-84 cells.
* Cells were exposed to 10 ng/ml BMY 25282.
* Cells were exposed to 3.5 ng/ml BMY 25067.

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At an equimolar concentration, MMC and BMY 25282 produced a significantly higher ISC in sensitive cells when compared to the P388/R-84 cells. These results suggest that cellular resistance to MMC and BMY 25282 may, at least in part, be due to the reduced ISC formation. BSO or EA treatments (both of which will affect GST activity) increased ISC formation induced by MMC and BMY 25282. Whereas BSO treatment slightly increased ISC frequency, EA treatment had no significant effect on the BMY 25067 induced ISC formation. These results are in agreement with the cell survival data where potentiation of BMY 25067 cytotoxicity by EA treatment was not observed.

In summary, these results suggest that (a) BMY 25282 and BMY 25067 are significantly more active in vitro than the parent compound, (b) BSO and EA may be useful in modulating the antitumor activity of MMC and BMY 25282 in resistant tumors, and (c) the mechanism of cytotoxicity as well as resistance for BMY 25067 may be different from those of MMC or BMY 25282.

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