Effects of BMY 25282, a Mitomycin C Analogue, in Mitomycin C-resistant Human Colon Cancer Cells

James K. V. Willson, Byron H. Long, Subhas Chakrabarty, Diane E. Brattato, and Michael G. Brattain

ABSTRACT

BMY 25282, a newly designed analogue of mitomycin C (MMC), was assessed for its non-cross-resistant cytotoxic and biochemical action against MMC-resistant human colon carcinoma cells. The analogue has an amidine substituted at position 7 of MMC and has a more efficient intracellular activation to its active species than MMC. In this study we demonstrated that BMY 25282 can overcome MMC resistance in a series of previously described human colon carcinoma cells resistant to MMC (Cancer Res., 44: 5880, 1984). The non-cross-resistance of the analogue in the model was confirmed in vivo by treating tumor xenograft-bearing athymic mice with equitoxic doses of MMC or BMY 25282. We further investigated the formation of interstrand DNA cross-link (IDC) formation by BMY 25282 and MMC. MMC-sensitive cells contained 3 to 8 times as many IDCs as resistant colon carcinoma cells, while no significant differences in IDCs were found between the MMC-sensitive or -resistant cells incubated with BMY 25282. When MMC-sensitive or -resistant cells were exposed to the 70% inhibition concentration of either MMC or BMY 25282, no differences were seen with respect to IDC formation. These studies demonstrate that BMY 25282 is able to overcome MMC resistance in a series of human colon carcinoma cells and that IDC formation in the MMC-sensitive or -resistant cells parallels cytotoxicity for both MMC and the analogue.

INTRODUCTION

The BMY 25282 compound is one of a series of MMC analogues synthesized by medicinal chemists at the Bristol-Myers Company with an amidine group substituted at position 7 on MMC (Ref. 1; Chart 1). The quinone ring of the BMY 25282 analogue has a lower reduction potential than the parent compound. This lower reduction potential may be an advantage because the mechanism of action of MMC is thought to result from its intracellular metabolism to a reactive species that alkylates and cross-links DNA (2–4) and that the reduction of the quinone ring is the first step in the intracellular metabolism of MMC to the cytotoxic metabolite (5). Therefore, it is anticipated that the decreased reduction potential of BMY 25282 will result in a more efficient intracellular metabolism to the active species and perhaps a broader antitumor activity than the parent compound MMC.

A number of possible mechanisms have been suggested to explain human cancer cell resistance to MMC, including (a) reduced intracellular activation, (b) decreased intracellular drug accumulation, and (c) increased repair of interstrand DNA cross-links (6). Of these it is conceivable that the BMY 25282 compound might overcome MMC resistance due to the first two mechanisms cited. For example, if impaired intracellular activation accounts for cellular resistance to MMC, then the lower reduction potential of the analogue might result in sufficient activation in resistant cells to permit activity. On the other hand, if MMC resistance is a consequence of a rapid efflux of drug from resistant cells resulting in decreased intracellular accumulation, a mechanism of resistance to other naturally derived cytotoxic drugs in a number of cancer cell models (7–10), BMY 25282, might circumvent such resistance. This may occur, if intracellular activation were the rate-limiting step in the interaction of the active species with the DNA target, and then an increased activation rate might overcome such resistance by allowing for the intracellular accumulation of the active metabolite before elimination of drug by increased efflux.

In this study we evaluate the cytotoxic and biochemical effects of BMY 25282 in a series of previously described MMC-resistant human colon cancer cell lines, HCT 116R11 (R11), HCT 116R26 (R26), HCT 116R44 (R44) (11), and the MMC-sensitive parental cell, HCT 116 (12, 13). The MMC-resistant cells were selected from the parent cell HCT 116 by repeated exposure to MMC in culture (11). The in vitro sensitivity profiles of the HCT 116 cell line series have been verified in vivo by treating athymic mice bearing HCT 116 tumor xenografts grown from MMC-sensitive and MMC-resistant cells. We have also used alkali elution to analyze MMC-induced IDC formation and repair in the MMC-sensitive and MMC-resistant lines (11, 13). These studies revealed that MMC-induced IDC formation increased with increasing MMC concentration; resistant cells had fewer IDCs than sensitive cells; and repair of IDCs was not a mechanism for resistance to MMC in the resistant HCT 116 cell lines. Therefore, we have interpreted our previous studies to suggest that MMC resistance in this model is due to either reduced intracellular MMC activation or decreased intracellular accumulation of the active metabolite. Thus, these MMC-resistant human colon cancer cell lines are ideal models to evaluate the potential of BMY 25282 to overcome MMC resistance.

MATERIALS AND METHODS

Cell Culture

Cells were maintained in McCoy's 5A tissue culture medium supplemented with 10% heat-inactivated fetal bovine serum in 25-cm² flasks.

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2 Recipient of an American Cancer Society junior faculty fellowship. To whom requests for reprints should be addressed, at Division of Oncology, William S. Middleton Memorial Veterans Administration Hospital, 2500 Overlook Terrace, Madison, WI 53705.
3 The abbreviations used are: MMC, mitomycin C; IC50, 50% inhibition concentration; IC70, 70% inhibition concentration; IDC, interstrand DNA cross-link.
4 Received 3/25/85; revised 7/15/85; accepted 7/29/85.
Cells are free of Mycoplasma contamination as documented by (Coming, Corning, NY) and subcultured weekly as described previously (12). MMC-resistant phenotype selected from the parental HCT 116 cell line as previously described (11) was maintained in culture medium without MMC exposure; e.g., HCT 116R11 identifies resistant cells following 11 MMC treatment cycles prior to passage of the resistant cells to 10 days.

Cytotoxicity Assays

Cells were plated into 25-cm² flasks containing 5 ml of growth medium on Day 1 at concentrations of 1.4 x 10⁵ cells/ml. On Day 2, growth medium was removed and replaced with growth medium with drug concentrations of MMC (range, 0.1 to 10 µM) or BMY 25282 (range, 0.2 to 2 µM). After incubation for 1 h at 37°C in medium containing drug, cultures received a complete change of medium without drug. Growth inhibition was determined by hemocytometer counts on Day 7 after suspension of the cells by treatment with 0.2% trypsin and 0.01 M EDTA (Grand Island Biological Co., Grand Island, NY) for 5 min at 37°C. Control cultures were treated in an identical manner, except that they received a complete medium change on Day 2 without drug. Growth inhibition of drug-treated cells was expressed as the fraction of surviving cells on Day 7 relative to untreated control cultures x 100%.

MMC and BMY 25282 were kindly provided by Dr. W. Bradner, Pharmaceutical Research and Development Division, Bristol Myers Company, Syracuse, NY. Both were aliquoted and stored in dimethyl sulfoxide (DMSO). MMC and BMY 25282 were dissolved in DMSO and aqueous medium then diluted to the indicated concentrations in tissue culture medium. MMC stock solution was freshly prepared daily at 10 µM, and BMY 25282 stock solution was prepared at 100 µM in DMSO.

Establishment of Xenografts

Female 20-g (6 to 8 wk old) athymic mice of BALB/c parentage were obtained from Sprague-Dawley (Madison, WI). Experiments were performed in groups of 5 to 10 drug-treated tumor xenograft-bearing mice. Each HCT 116 subpopulation was suspended from tissue culture flasks with 0.2% trypsin and 0.01 M EDTA for 5 min at 37°C and resuspended in Eagles’ minimal essential medium (GIBCO, Grand Island, NY) at a concentration of 2.5 x 10⁶ cells/ml. Mice were given s.c. injections at the right anterior lateral trunk just behind the front leg with 0.2 ml of the cell suspension (5 x 10⁶ cells). Mice developed palpable xenografts in 5 to 10 days.

Tumor xenograft size was measured serially in individual animals with calipers, and tumor volumes were determined as previously described (11). Animals were weighed twice weekly during the course of in vivo studies. Tumor xenografts grown in mice which lost 25% of weight or died during the 25-day evaluation period were not included in antitumor evaluations.

Drug Treatment of Xenograft

When the maximum diameter of tumor xenografts was 5 to 8 mm (usually 10 days after cell inoculation), drug treatment was initiated. MMC, BMY 25282, saline, or vehicle was administered i.v. in 0.1 ml by tail vein on a Day-1, -5, and -9 schedule. BMY 25282 was administered in Lyophile 0.1% pluronic F-88 (pH 6.4) in 0.01 M citrate.

Alkaline Elution Assay for Interstrand DNA Cross-Links

Materials. Mitomycin C was dissolved in water as 1 mM solutions. Dilutions were made in tissue culture medium. [methyl-3H]thymidine (2 Ci/mmol) and [2-14C]thymidine (60 mCi/mmol) were all purchased from Schwarz-Mann, Spring Valley, NY.

Radioactive Labeling. Cultures of human colon carcinoma cells intended for alkaline elution were plated in 60-mm tissue culture dishes 3 days before the experiment, and 0.01 µCi of [3H]thymidine per ml was added 48 h prior to addition of drug. Reference cultures were also grown for 3 days in 75 cm² flasks and labeled with 0.1 µCi of [3H]thymidine per ml 2 days before the experiment.

Alkaline Elution Assay for Interstrand DNA Cross-Links. The assays for IDCs were conducted as detailed elsewhere (13). In summary, drug-treated cells (5 x 10⁶ cells) containing [3H]DNA were exposed to 600 rads of γ-radiation on ice from a 57Co source in a GammaCell 200 irradiator (Atomic Energy of Canada, Ottawa, Canada) and then layered over polyvinyl chloride filters (2.0-µm pores, 25-mm diameter from Nucleopore, Pleasanton, CA). The cells were washed twice by gravity filtration at 4°C with 10 ml of cold phosphate-buffered saline (0.15 M NaCl-0.01 M NaH2PO4), pH 7.5. Suspended cells containing [3H]DNA were irradiated on ice with 300 rads of γ-radiation to introduce random single-strand DNA breaks then distributed among the filters in aliquots of 5 x 10⁶ cells to provide an internal elution standard. The cells were again washed with phosphate-buffered saline using gravity filtration and then lysed on the filters by addition of 3 ml of sodium dodecyl sulfate-EDTA lysis solution, which was followed by addition of sodium dodecyl sulfate-EDTA lysis solution containing proteinase K (0.5 mg/ml; E. Merck, Darmstadt, Germany) that had been heated at 56°C for 1 h. DNA was eluted from the filters with 30 ml of tetrapropylammonium hydroxide-EDTA elution buffer, pH 12.1, at a rate of 40 µl/min. Fractions were collected in scintillation vials every 90 min. Filters, tubing, lysis samples, and eluted fractions were processed for radioactive counting as previously described. The resulting 14C and 3H cpm of each eluted fraction were expressed as percentages of total cpm obtained from the sum of the respective radioactivities in the filter, tubing, lysis, and eluted fractions. The log of percentages for 14C cpm of each eluted fraction was plotted against the log of percentages of 3H cpm of the same fraction (13).

MMC and BMY 25282 Activation by Microsomal Enzymes

The method of Kennedy et al. (14) was used to assess activation activity of MMC or BMY 25282 in microsomes of rat hepatocytes. Microsomes from hepatocytes were prepared by the rapid calcium precipitation method of Schenkman and Cinti (15).

RESULTS AND DISCUSSION

In Vitro Activation of BMY 25282 and MMC. Activation of MMC and BMY 25282 by rat hepatocytes in vitro under anaerobic conditions is shown in Chart 2. While both drugs are activated in a concentration-dependent fashion, the activation of BMY 25282 is greater at all drug concentrations tested. Thus, the lower reduction potential of the analogue has apparently
resulted in an enhanced cellular activation of BMY 25282 to its alkylating and cytotoxic metabolite.

As discussed above, the more efficient cellular activation of BMY 25282 could possibly help to circumvent potential mechanisms of MMC resistance, such as resistance resulting from altered drug transport and/or impaired intracellular activation. Therefore, we evaluated the effect of BMY 25282 in HCT 116R cell lines.

In Vitro Response of HCT 116 and MMC-resistant Variants to BMY 25282. Growth inhibition curves as a function of concentration of MMC (Chart 3A) or BMY 25282 (Chart 3B) in HCT 116 and the MMC-resistant subpopulations are shown in Chart 3. A total of 3 survival curves were generated for each cell line, and the resulting IC50 and IC70 values are presented in Table 1. Resistance to MMC increased with continued exposures to MMC as presented previously (11), and this is reflected by the progressive increase in IC50 and IC70 values for R11, R26, and R44 (Table 1). In contrast, the in vitro response to BMY 25282 (Chart 3B) was equivalent in the parental cell, HCT 116, and MMC-resistant cells, R11 and R26.

While the IC50 values for BMY 25282 in each of the MMC-resistant cells were not significantly different from the parental line, HCT 116, the IC70 value for R44 was greater than in all other cell lines tested (Table 1). The increased resistance to BMY 25282 in the R44 cell is also evident in the dose-response curve for this cell (Chart 3B). The increased resistance to higher concentrations of BMY 25282 in R44 may indicate that collateral resistance to the analogue has developed with increasing MMC resistance. A second possible explanation is that a subpopulation of BMY 25282-resistant cells has appeared, and its presence accounts for the plateau observed in the dose-response curve (Chart 3B) in R44. Clonal studies of the R44 line, however, have not demonstrated a subpopulation with higher resistance to BMY 25282 (data not shown); therefore, the former postulate is a more likely explanation for the differences in the response curves.

These in vitro data suggest that the BMY 25282 compound has overcome a mechanism of MMC resistance in the HCT116R cell lines. It might be argued, however, that this apparent non-cross-resistance is simply a result of a greater, yet nonspecific, potency of the analogue. To further evaluate the non-cross-resistant action of BMY 25282, we compared in vivo the activity of equitoxic doses of analogue and MMC against HCT 116 MMC-resistant cells grown as xenografts. We also assessed the formation of IDCs at comparable concentrations of each drug in vitro.

Response to BMY 25282 In Vivo. Athymic mice bearing tumor xenografts grown from the parental HCT 116 cell and the MMC-resistant cells R11, R26, and R44 were treated as described with vehicle (saline or the Lyophile vehicle), MMC, or BMY 25282. Tumor growth in mice treated with either saline or Lyophile did not differ (Student's t test, two tailed), and, therefore, results for the two vehicle-treated groups are combined in subsequent analyses. In preliminary dose-finding studies, a range of BMY 25282 and MMC doses was evaluated in xenograft-bearing athymic mice treated on Days 1, 5, and 9 by tail vein injection (Table 2). A maximum tolerable dosage for each drug, 1.0 mg/kg/injection of MMC and 0.36 mg/kg/injection of BMY 25282,
was used for in vivo comparison studies. In nontumorous normal female BALB/c mice, BMY 25282 was 4-fold more potent than MMC. The tolerable MMC dose for tumor-bearing athymic mice used in these studies is lower than the optimal MMC dose in strains of normal mice treated on a similar schedule; however, the optimal MMC dose has been found to vary among different strains of normal mice (16), and the optimal dose of cytotoxic agents differs between athymic mice and normal mice of common parentage (17). Thus the optimal dose should be determined for the individual in vivo model used.

Detailed analyses of the course of treatment for each HCT 116 subpopulation grown as xenografts in vehicle or drug-treated mice are shown in Chart 4. The toxicity observed during these comparative efficacy trials was similar for both drugs, data shown in legend of Chart 4. On Day 25, HCT 116 xenografts were significantly (Student’s t tests, two tailed) smaller in BMY 25282-treated mice and in MMC-treated mice (P < 0.001) than tumor xenografts grown in vehicle-treated mice on Day 25, but the difference was not significant. The influence of the slower growth rate of the R44 tumor xenograft suggests that the slightly higher in vitro IC70 value for MMC than in HCT 116 cells (A), an observation consistent with the MMC-resistant nature of R26 cells. However, the incubation of R44 with MMC (D) resulted in similar IDC formation at comparable drug concentrations. The different sensitivities of these two cell populations to MMC and BMY 25282 are further illustrated in Table 3 when known; however, the apparent in vivo resistance of the R44 xenograft suggests that the slightly higher in vitro IC70 value for BMY 25282 is evidence of the development of collateral resistance to BMY 25282 in R44.

Formation of Interstrand DNA Cross-Links. Chart 5 presents typical alkaline elution curves resulting from 1-h exposure of HCT 116 cells and R26 cells to different concentrations of MMC and BMY 25282. A comparison of A and B reveals that the same concentration of MMC produced fewer IDCs in R26 cells (B) than in HCT 116 cells (A), an observation consistent with the MMC-resistant nature of R26 cells. However, the incubation of HCT 116 cells and R26 cells with BMY 25282 (Chart 5, C and D) resulted in similar IDC formation at comparable drug concentrations. The different sensitivities of these two cell populations to MMC and BMY 25282 are further illustrated in Table 3 when apparent IDCs are calculated from the elution curves presented in Chart 5. HCT 116 cells incubated with MMC contained 3 to 8 times as many IDCs as HCT R26 cells, whereas almost no differences in IDCs were found between HCT 116 and R26 cells incubated with BMY 25282.

These in vivo studies demonstrate that BMY 25282 was effective against MMC-resistant tumor xenografts carried in mice treated with an equitoxic dose of MMC. Tumor xenografts from the R44 cell were smaller in BMY 25282-treated mice than in vehicle-treated mice on Day 25, but the difference was not significant. The influence of the slower growth rate of the R44 tumor xenograft on the response to BMY 25282 in vivo is not

Table 2

<table>
<thead>
<tr>
<th>Druga</th>
<th>No. of survivors on Day 25</th>
<th>Wt changeb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>52/53 (99)</td>
<td>+1.6</td>
</tr>
<tr>
<td>MMC (mg/kg/injection)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.0</td>
<td>1/8 (13)</td>
<td>-24</td>
</tr>
<tr>
<td>2.25</td>
<td>4/11 (36)</td>
<td>-4</td>
</tr>
<tr>
<td>1.5</td>
<td>8/12 (60)</td>
<td>-2.3</td>
</tr>
<tr>
<td>1.0</td>
<td>39/42 (93)</td>
<td>-3.1</td>
</tr>
<tr>
<td>BMY 25282 (mg/kg/injection)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.48</td>
<td>12/16 (75)</td>
<td>-5.5</td>
</tr>
<tr>
<td>0.36</td>
<td>39/43 (91)</td>
<td>-3.8</td>
</tr>
<tr>
<td>0.24</td>
<td>20/20 (100)</td>
<td>0</td>
</tr>
</tbody>
</table>

a Drug administered by tail vein on a Day-1, -5, -9 schedule.
b Mean percentage of change from Day 1 to Day 25.
c Numbers in parentheses, percentage of survivors.
**BMY 25282 EFFECTS IN MMC-RESISTANT CELLS**

![Alkaline elution curves from HCT 116 and HCT 116R26 cells as a function of MMC concentration. Cells containing [14C]DNA were incubated with different concentrations of either MMC or BMY 25282 for 1 h. Following drug treatment, the cells were exposed to 600 rads of γ-radiation on ice prior to lysis with detergent, protease K treatment, and elution at pH 12.1. Elution of 14C cpm is plotted against the elution of 3H cpm from an internal standard provided by the addition of an equal number of cells exposed only to 300 rads of γ-radiation. A, HCT 116; B, HCT 116R26; C, no MMC, no rads; D, 2 μM MMC, 600 rads; E, 10 μM MMC, 600 rads; F, 50 μM MMC, 600 rads; G, HCT 116R44; H, no BMY 25282, no rads; I, 0.2 μM BMY 25282, 600 rads; J, 1.0 μM BMY 25282, 600 rads.**

**Table 3**

<table>
<thead>
<tr>
<th>Apparent interstrand DNA cross-link formation in MMC-sensitive and MMC selected resistant cell populations incubated with IC70 concentrations of either mitomycin C or BMY 25282 at different concentrations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell population</td>
</tr>
<tr>
<td>HCT 116</td>
</tr>
<tr>
<td>HCT 116R26</td>
</tr>
<tr>
<td>HCT 116R44</td>
</tr>
</tbody>
</table>

| Cell population | Concentration ([μM]) | Apparent IDCs |
| BMY 25282 | 0.16 | 7.9 ± 2.8 |

*Mean ± SD.

MMC increase from 1.5 and for HCT 116 to 11.4 μM for R44 cells, whereas the IC70 concentrations for BMY 25282 are essentially the same for HCT 116 and HCT 116R26 and increase only slightly for HCT 116R44 cells (Table 4). HCT 116 cells incubated with IC70 concentrations of either MMC or BMY 25282 contained 8.9 and 9.9 apparent IDCs per 10^6 nucleotides. No significant differences were observed between HCT 116 cells and R26 and R44 cells with respect to IDC formation. Also, no differences were seen between IDC formation by MMC and BMY 25282 in any of the three cell populations presented in Table 4. These studies demonstrate that, not only is BMY 25282 able to overcome MMC resistance in selected resistant human colon carcinoma cells, but IDC formation in MMC-sensitive and MMC-resistant cells parallels cytotoxicity for both MMC and BMY 25282.

**Use of In Vitro and Tumor Xenograft Models of MMC Resistance for the Future Development of MMC-based Analogues.** A large number of MMC-based analogues have been developed in hopes of discovering a less toxic and more active drug than the parent compound (16, 18-21). In this paper, we have described one of these analogues, BMY 25282, and have shown its potential to overcome MMC resistance exhibited by a specific human colon cancer model. Further, we have presented evidence which suggests that the superior activity of BMY 25282 in the HCT 116R cells results from its greater intracellular activation to the active metabolite as compared to MMC. It is unlikely, however, that a single MMC-based analogue, such as BMY 25282, will prove universally superior in the clinic in a disease as heterogeneous as colon cancer (22, 23). Therefore, we propose that the approach taken in this study be used to identify additional MMC analogues, each with activity against a specific mechanism of MMC resistance in colon cancer. These analogues would be selected by *in vitro* and tumor xenograft models which exhibit unique mechanisms for MMC resistance. A large bank of human colon cancer cell lines exists from which these models could be selected (12, 24, 25) and characterized in a manner similar to that used for the HCT 116R model. MMC analogues would be selected for their ability to overcome a specific mechanism of MMC resistance. Combinations of these analogues would then be evaluated in models established from mixtures of the MMC-resistant cells. This approach may have important implications for the design and interpretation of future clinical trials of new analogues.

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5285
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