Effect of Treatment Duration and Glutathione Depletion on Mitomycin C Cytotoxicity in Vitro

Roger R. Perry, Bridget R. Greaves, Uli Rasberry, and Sam C. Barranco

Division of Surgical Oncology, Eastern Virginia Medical School, Norfolk, Virginia 23507 [R. R. P., B. R. G., U. R.], and Quest Biomedical Research Center, Virginia Beach, Virginia 23452 [S. C. B]

ABSTRACT
Glutathione (GSH) has been shown to modulate the cytotoxicity of a variety of chemotherapeutic agents. The effect of mitomycin C (MMC) treatment duration and the effect of GSH depletion on in vitro cytotoxicity against the human colon cancer cell line HT-29 was studied under aerobic conditions. Continuous-exposure experiments revealed that the cytotoxicity of 0.1 μM MMC, as measured by clonogenic cell survival, exhibited a shoulder until exposure time was at least 12 h, after which time exponential cytotoxicity was observed. Lowering GSH levels to <3% of control using buthionine sulfoximine (BSO) did not enhance cytotoxicity of MMC given for 1 h or continuously for <12 h. However, GSH depletion did enhance cytotoxicity of MMC given continuously for at least 12 h, with a dose-modifying factor at 1% survival of 1.4 for a 24-h treatment. GSH depletion under these conditions enhanced cytotoxicity of even minimally cytotoxic MMC concentrations (0.02 μM). Absolute levels of GSH-related enzymes, including glutathione-S-transferase, and the MMC-metabolizing enzyme DT-diaphorase did not change appreciably. A tetrazolium [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay was used to verify the results further and to determine the optimal sequence of BSO administration with a 24-h MMC treatment. BSO added simultaneously with MMC did not enhance cytotoxicity, compared to MMC alone. BSO added and then removed prior to MMC was effective (dose-modifying factor at 50% survival = 1.3), but the greatest cytotoxicity was noted when BSO was present before and during MMC treatment (dose-modifying factor = 1.5). GSH depletion in another cell line (SW480) showed similar enhancement of 24-h MMC cytotoxicity. These studies show that aerobic cytotoxicity of MMC is improved by administration of the drug in continuous fashion for at least 12 h, as opposed to continuous administration for shorter periods or 1-h bolus administration. Cytotoxicity of continuous (at least 12-h) MMC treatment can be modestly enhanced by GSH depletion, which must precede MMC exposure in order to be effective.

INTRODUCTION
MMC is one of the few agents with even marginal activity against gastrointestinal malignancy, such as colorectal cancer. MMC is a bioreductive alkylating agent, whose activity has been shown to be modulated by a number of factors. It appears to be more active under hypoxic as opposed to aerobic conditions (1), with oxygenation status at the time of MMC exposure being important (2). However, no difference in cytotoxicity is observed between plateau and exponential growth phase cells, and cytotoxicity is not pH dependent within the physiological range (2). Good correlation has been noted between the amount of DNA-DNA cross-linking and cytotoxicity (3), although other species, including DNA monoadducts, DNA-protein cross-links, and cytotoxic oxygen species (4), have been noted. Various resistance mechanisms have been described, including failure of MMC activation as a result of decreased ability to reduce the quinone structure to the active form (5), deficiency of DT-diaphorase (6), cell surface protein alterations (7), and increased efflux mechanisms mediated by P-glycoprotein (8).

MMC cytotoxicity is capable of being modulated by dicumarol, an inhibitor of DT-diaphorase. Under aerobic conditions dicumarol decreases MMC cytotoxicity, whereas under hypoxic conditions dicumarol increases MMC cytotoxicity (9). Surprisingly, little work has been done investigating the role of GSH in modulating MMC cytotoxicity. GSH has been shown to be important in modulating the cytotoxicity of a number of alkylating agents, including Melphalan (10) and doxorubicin (11), as well as in modulating the effect of radiation (10). Increased cytotoxicity by these agents is observed after depletion of GSH using BSO (11). Furthermore, in certain situations resistance to these agents can be effectively reversed by GSH depletion (12).

Thus far, the data are inconclusive regarding the influence of GSH depletion on MMC cytotoxicity. One study showed that GSH depletion enhanced the cytotoxicity of a 1-h MMC treatment under aerobic but not hypoxic conditions (13), whereas another study showed no effect under aerobic or hypoxic conditions (14).

This paper examines whether, and under what conditions, aerobic MMC cytotoxicity against the human colon cancer line HT-29 is influenced by GSH levels. We found MMC cytotoxicity, and its enhancement by GSH depletion using BSO, to be significantly influenced by treatment duration, as well as by drug sequence.

MATERIALS AND METHODS
Human Colon Cancer Cell Lines. The HT-29 cell line was obtained from the American Type Culture Collection (Rockville, MD). Cells were maintained in Ham's F-12 medium supplemented with 20% newborn calf serum, glutamine, penicillin, and streptomycin. The cells were incubated at 37°C in a 95% air/5% CO2 environment. A few additional experiments were performed with the SW480 cell line, also obtained from the American Type Culture Collection and grown under identical conditions.

Drugs and Biochemicals. MMC was kindly provided by Daniel Elliott of Bristol-Myers Co. (Evansville, IN). Fresh solutions were prepared on the day of each experiment. NADPH, NADP+, aldehyde dehydrogenase, catalase, GR, and oxidized glutathione were obtained from Boehringer-Manheim Biochemicals (Indianapolis, IN). BSO was obtained from Schweizerhall, Inc. (South Plainfield, NJ). GSH and all of the other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO).

Clonogenic Cell Survival Assay. This assay was performed as previously described (15), with minor modifications. Briefly, 3 x 10⁴ cells in log phase growth were plated in 60-mm Petri dishes, 24 h prior to MMC exposure. After completion of drug treatment, the cells were trypsinized, washed, and counted. Serial dilutions were performed at
each experimental point, and each dilution was plated in triplicate. Ten days later, the plates were fixed and stained with ethanol and crystal violet. Macroscopic colonies of >50 cells were counted, and SF at each treatment point was determined by dividing the plating efficiency of treated cells by the plating efficiency of control (untreated) cells. Survival curves were constructed by plotting the surviving fractions versus drug concentrations or incubation times. Each survival curve experiment was performed at least three times.

DMF values were calculated by dividing the dose of MMC required to achieve 1% or 50% survival in non-BSO-treated cells by the dose of MMC required in BSO-treated cells.

MTT Assay. In order to determine whether similar results could be obtained using a different method of measuring cell survival, the MTT assay was performed, using a modification of a method previously described (16). Briefly, 2.5 × 10^4 cells in logarithmic phase growth were plated into 96-well plates, 24 h prior to MMC treatment. After completion of drug treatment, 50 μl of MTT (2 mg/ml) were added to each well. After incubation for 4 h at 37°C, the medium was removed and the plates were dried. The incorporated formazen crystals were solubilized with 150 μl of light mineral oil, by further incubation at 37°C. Cells for each experimental point were plated into at least six wells, and the absorbance of each well was read at 570 nm, using a Biokinetics EL340 microplate reader (Bio-Tek Instruments, Inc., Winooski, VT). Survival fraction was determined by dividing the absorbance of treated wells by the absorbance of control wells.

GSH Assay. GSH was extracted by adding 0.6% sulfosalicylic acid to culture dishes containing plated cells and then incubating the dishes at 4°C for 20 min (17). Supernatants were removed from the dishes, and measurements of total GSH were made using a modified Tietze assay (18). Absorbance was monitored at 412 nm, using a Hitachi U-2000 spectrophotometer (Hitachi Instruments, Inc., Danbury, CT). Glutathione levels were normalized to cell count.

Enzyme Assays. Cells were trypsinized, washed, and resuspended in 5 mM diethylenetriamine pentaacetic acid. Cells were disrupted using a Branson sonicator, and total protein was measured in the homogenates by using the method of Bradford (19). The homogenates were centrifuged at 16,000 g for 10 min, and the supernatants were stored at -155°C until assay. GST was measured by the method of Habig et al. (20), using 1-chloro-2,4-dinitrobenzene as a substrate. GR was measured using the method of Massey and Williams (21). DTD was measured by the method of Ernest (22), using menadione as the electron acceptor. The diconamol-sensitive part of the activity was taken as a measure of DTD. Xanthine oxidase was measured by the method of Heinz (23), using xanthine as a substrate.

Flow Cytometry. Cell cultures were washed with Puck’s solution A, and cells were detached with pepsin (0.5%) and fixed by addition of ethanol to a final concentration of 70%. Cells were treated with RNase (0.1%) and propidium iodide (0.1%) to identify cells with damaged DNA. Cell cycle analysis was performed using a Coulter Profile flow cytometer (laser tuned to an excitation wavelength of 488 nm). The fractions of cells in G1, S, and G2-M phases were calculated, with coefficients of variation generally ranging from 2 to 4%.

Statistics. Results are expressed as mean ± SEM. Differences between means were examined using the Wilcoxon rank-sum test. All P values reported are two sided.

RESULTS

In BSO treatment groups, HT-29 cells were incubated with 0.5 mM BSO beginning 24 h prior to MMC treatment and continued during MMC treatment. This BSO concentration was chosen because it caused maximal GSH depletion (to levels <3% of control at 24 h) without cytotoxicity. Higher doses of BSO were cytotoxic. In spite of this degree of GSH depletion, no enhancement of MMC cytotoxicity was noted during a 1-h MMC treatment (Fig. 1A). However, GSH depletion did enhance cytotoxicity of a 24-h MMC treatment (Fig. 1B). The dose for 1% survival for MMC was 0.085 ± 0.008 μM and for MMC and BSO was 0.061 ± 0.006 μM (P = 0.032), with a DMF of 1.4. Use of a lower concentration of BSO was also effective in enhancing cytotoxicity of a 24-h MMC treatment. In a separate series of experiments, HT-29 cells were exposed to 0.05 mM BSO for 24 h, with resulting depletion of GSH to levels <8% of control. Virtually identical enhancement of 24-h MMC cytotoxicity was observed with this lower dose of BSO (Fig. 1C).

To see if this BSO enhancement of MMC cytotoxicity was limited to the HT-29 cell line, the 24-h experiments were repeated in a different colon cancer cell line. SW480 cells were exposed to 0.01 mM BSO for 24 h, with resulting depletion of GSH to levels <2% of control. Again, this dose was chosen because of excellent GSH depletion without cytotoxicity. Significant, albeit modest, enhancement of 24-h MMC cytotoxicity was observed (Fig. 1D).

To further investigate the time dependence of MMC cytotoxicity in the absence or presence of BSO, the effect of 0.5 mM BSO administration started 24 h prior to and continued during MMC treatment was examined for continuous MMC treatment periods from 1 h to 24 h. As shown in Fig. 2A, the cytotoxicity of a concentration of MMC (0.02 μM) that, when given alone, was only minimally cytotoxic to HT-29 cells was significantly enhanced in GSH-depleted cells. However, few cells were actually killed (50% survival at 24 h). This enhancement was time dependent and was seen only with MMC treatment times of at least 12 h (Fig. 2A). When a higher concentration of MMC (0.1 μM) was given continuously, a proportional
The effect of MMC, with and without BSO, on GSH and enzyme levels was examined for various MMC treatment times (1, 4, 12, and 24 h). GSH levels did not change appreciably during the duration of the experiment in either control or drug-treated cells (results not shown). Excellent GSH depletion in BSO-treated cells was maintained during the entire experiment. No significant change was observed during the experiment in levels of total GST, GR, or DTD, and xanthine oxidase levels were not detectable (results at 24 h shown in Table 1).

Continuous exposure of HT-29 cells to 0.5 mM BSO for 48 h resulted in an approximately 10% increase of cells in S phase and an approximately 10% decrease of cells in G1 phase. This change resolved within 24 h after BSO exposure (data not shown). Previous work by us failed to show any effects of 0.05 mM BSO on cell cycle kinetics for periods of BSO treatment of up to 48 h (12).

A series of experiments using the MTT assay were performed to see if results similar to the clonogenic assay could be obtained using this technique and to determine the optimal sequence of BSO with a 24-h MMC treatment (Fig. 4). BSO added simultaneously with MMC did not increase cytotoxicity, compared to MMC alone (DMF at 50% survival = 1.0). BSO added and then removed prior to MMC was effective (DMF = 1.3), but the greatest cytotoxicity was noted when BSO was present before and during MMC (DMF = 1.5).

DISCUSSION

This work has demonstrated the following points. (a) MMC is effective when cells are treated for extended periods of time. Because of the shoulder demonstrated on the survival curves, the drug is more effective if given for at least 12 h. (b) GSH levels appear to have a role in modulating MMC cytotoxicity, but only when MMC treatment is for at least 12 h. (c) The sequence of MMC administration and glutathione depletion appears critical, because GSH depletion must occur prior to MMC exposure for increased cytotoxicity to be observed.

Such continuous exposure is possible, because MMC has been shown to maintain biological activity even after 48 h in cell culture (25). The demonstration of a shoulder on the survival curve has previously been demonstrated in LoVo colon cancer cells, in which a "plateau" was demonstrated in 50% inhibitory concentration values of MMC with 6-24 h of drug exposure (27). Taken together, the results suggest that MMC may have cellular effects beyond those of a pure DNA-alkylating agent and may also be acting upon other cellular targets.

The effect of GSH depletion on MMC cytotoxicity has been unclear. One group has shown that GSH depletion sensitizes EMT6/SF cells to a 1-h MMC treatment, but only under aerobic conditions (DMF = 2.1). No further sensitization of hypoxic cells was noted (13). Other groups failed to notice any

Figure 2. Clonogenic cell survival curves for continuous-exposure MMC. A, 0.02 μM MMC; B, 0.1 μM MMC; C, cells treated with 0.5 mM BSO for 24 h prior to and during MMC treatment. Even a minimally cytotoxic concentration of MMC (0.02 μM) showed increased cytotoxicity in GSH-depleted cells (A). MMC, given alone or with BSO, showed cytotoxicity which was significantly time dependent.

Figure 3. Clonogenic cell survival curves investigating the effect of integral dose (concentration × time) on surviving fraction, using the data from continuous-exposure experiments in Fig. 2. A, cells treated with MMC; B, cells treated with 0.5 mM BSO for 24 h prior to and during MMC treatment; C, cells treated with BSO only. A shoulder is still demonstrated and the advantage of GSH depletion using BSO remains apparent.

An increase in kill was not seen with increasing exposure time (Fig. 2B). Rather, there was a "shoulder" before 12 h, after which time there was an exponential increase in kill. Even in this situation, GSH depletion by BSO enhanced cytotoxicity but, again, only when MMC treatment time was at least 12 h (Fig. 2B). Upon examination of surviving fraction versus the integral dose of MMC (concentration × time), a shoulder was still seen (Fig. 3). The advantage of GSH depletion remained apparent, but only at higher integral doses. Note that high integral dose alone was not sufficient, however, because the cytotoxicity of high doses of MMC given for 1 h was not enhanced by GSH depletion (Fig. 14).
Dose-Modifying Factor (DMF)

sequence of BSO and MMC. Control, cells treated with MMC for 24 h; BSO and 0.5 mM BSO simultaneously for 24 h. GSH depletion must occur prior to BSO. Before + During, cells treated with 0.5 mM BSO for 24 h prior to MMC. Before MMC, cells treated with 0.5 mM BSO for 24 h prior to MMC for 24 h; BSO effect of GSH depletion in EMT6 cells with a 1-h MMC treatment. The dose required in BSO-treated cells. *, P = 0.02 versus Control; +, P = 0.03 by dividing the dose of MMC for 50% survival in control (non-BSO-treated) cells enhanced in vitro cytotoxicity of MMC given for at least 12 h. Another group, using EMT6/SF cells in an in vivo/in vitro model demonstrated that BSO pretreatment of the tumors enhanced cytotoxicity (DMF = 1.4–1.8), but only when tumor excision and plating were delayed for 24 h after MMC treatment. The hypothesized mechanism was a decrease in tumor excision and plating were delayed for 24 h after MMC treatment. The mechanism of BSO enhancement of MMC cytotoxicity remains to be elucidated. Although it is tempting to attribute this effect to depletion of GSH levels alone, the response may, instead, be due to other cellular systems, such as the cell membrane or protein synthesis, that may be indirectly affected by GSH perturbation (30). The observed effect does not appear to be due to changes in DTD or total GST, because these did not change during the experiments, or to xanthine oxidase levels, which were not detectable in HT-29 cells. Although DTD appears to be important in aerobic activation of MMC when present (31), other means of aerobic activation, including NADPH:cytochrome c reductase or xanthine oxidase, may be important when DTD is absent. DTD appears to have little role in drug activation or protection from lethal effects in cells under hypoxic conditions (31).

The time dependence of the enhancement of cytotoxicity by BSO demonstrated in our study is not due to further depletion of GSH, because the cells were already maximally depleted prior to MMC treatment. It is not due to total levels of GST, although that does not exclude a time-dependent change in one of the GST isoenzymes. We are currently investigating this possibility.

The time dependence of BSO enhancement of MMC cytotoxicity might be due to changes induced in cell cycle kinetics by BSO. However, the cell cycle changes we observed that were caused by 0.5 mM BSO have not been associated with increased sensitivity to MMC (32). Use of 0.05 mM BSO, which does not affect cell cycle kinetics, as previously shown (12), still caused identical enhancement of MMC cytotoxicity. Thus, cell cycle kinetics alone cannot be implicated in BSO enhancement of MMC cytotoxicity.

Although it may be possible that the effects of BSO are mediated through the mechanism of PLD repair (33), whether or not such repair occurs in MMC-treated cells is controversial. PLD repair was implicated in an in vivo/in vitro model using EMT6/SF tumors pretreated with BSO prior to MMC treatment (29). However, others have failed to demonstrate the occurrence of PLD repair in EMT6/RW cells treated in vitro with MMC (2). We have not seen PLD repair in the HT-29 cell line for up to 24 h after a 1-h MMC treatment (data not shown).

Although BSO concentrations as low as 0.05 mM were effective in the HT-29 cell line, and 0.01 mM in the SW480 cell line, the lowest concentration of BSO that enhances MMC cytotoxicity under the conditions studied has yet to be determined. It is certainly possible that lesser degrees of GSH depletion will prove effective, and this is an active area of investigation in our laboratory. We are also studying other model systems to see if these results can be generalized. Continuous infusion of MMC for periods of at least 12 h might prove more effective than bolus administration, the technique which is currently used clinically. The effects of such continuous infusion on normal tissue toxicity need to be determined, but continuous infusion may decrease normal tissue toxicity, similar to the reduced toxicity observed when 5-fluorouracil is given continuously rather than by bolus (34). Furthermore, this work suggests that aerobic cytotoxicity of MMC given by continuous infusion for at least 12 h may be modestly increased by GSH depletion.

It is difficult to predict whether GSH depletion by BSO in vivo will increase or change the pattern of MMC normal tissue toxicity. GSH depletion has been shown to increase the cardiac toxicity of cyclophosphamide in mice, as well as to cause skeletal muscle toxicity (35), and GSH appears to be protective of bone marrow (36). On the other hand, multiple doses of i.v. BSO did not potentiate Melphalan cytotoxicity in mice as long as the BSO doses were kept below 800 mg/kg/dose (37). Also, it appears that GSH depletion can be achieved in tumors with BSO doses lower than those required for normal tissues (38). Much work will be necessary to establish the schedule and

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**Table 1 GSH levels and enzyme activities after 24-h MMC treatment**

No significant differences were noted at 1, 4, 12, or 24 h. Xanthine oxidase levels were not detectable.

<table>
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<th>Control</th>
<th>BSO</th>
<th>0.02 μM MMC</th>
<th>0.1 μM MMC</th>
<th>0.02 μM MMC and BSO</th>
<th>0.1 μM MMC and BSO</th>
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<tbody>
<tr>
<td>GSH (nmol/10^6 cells)</td>
<td>25.8 ± 4.6</td>
<td>25.8 ± 5.4</td>
<td>25.8 ± 4.4</td>
<td>20.9 ± 38</td>
<td>0.2 ± 0.1</td>
<td></td>
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<tr>
<td>GST (nmol/min/mg protein)</td>
<td>203 ± 29</td>
<td>191 ± 12</td>
<td>182 ± 11</td>
<td>199 ± 30</td>
<td>0.2 ± 0.1</td>
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<tr>
<td>GR (nmol/min/mg protein)</td>
<td>26 ± 8</td>
<td>29 ± 11</td>
<td>28 ± 10</td>
<td>31 ± 11</td>
<td>0.2 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>DTD (μmol/min/mg protein)</td>
<td>1.3 ± 0.3</td>
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<td>1.1 ± 0.2</td>
<td>1.5 ± 0.3</td>
<td>1.3 ± 0.4</td>
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* Represents > 95% of total diaphorase activity detected.

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**Fig. 4. DMF obtained from MTT assay results and used to determine optimal sequence of BSO and MMC. Control, cells treated with MMC for 24 h; BSO Before MMC, cells treated with 0.5 mM BSO for 24 h prior to MMC for 24 h; BSO During MMC, cells treated with 0.5 mM BSO and MMC simultaneously for 24 h; BSO Both Before + During, cells treated with 0.5 mM BSO for 24 h prior to MMC and 0.5 mM BSO simultaneously for 24 h. GSH depletion must occur prior to MMC exposure if enhanced cytotoxicity is to be observed. DMF was determined by dividing the dose of MMC for 50% survival in control (non-BSO-treated) cells by the dose required in BSO-treated cells. *, P = 0.02 versus Control; +, P = 0.03 versus BSO Before MMC group.**
dosages in vivo which will maximize MMC cytotoxicity without increasing normal tissue toxicity. Thus, assuming that increased normal tissue toxicity can be avoided, depletion of GSH prior to continuous infusion of MMC may enhance the effectiveness of an already useful antineoplastic agent.

REFERENCES


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