Biochemical Pharmacology, Vol. 45, No. 11, pp. 2303–2309, 1993.
Printed in Great Britain.

0006-2952/93 $6.00 + 0.00

CONTRASTING MOLECULAR CYTOTOXIC MECHANISMS OF MITOMYCIN C AND ITS TWO ANALOGS, BMY 25282 AND BMY 25067, IN ISOLATED RAT HEPATOCYTES

JOSE M. SILVA, SUMSULLAH KHAN and PETER J. O'BRIEN*
Faculty of Pharmacy, University of Toronto, Toronto, Ontario, Canada

(*Received* 2 *November* 1992; *accepted* 3 *February* 1993)

Abstract—The molecular cytotoxic mechanisms of mitomycin C (MMC) and its analogs, BMY 25282 and BMY 25067, have been investigated using isolated hepatocytes as a model system for studying toxicity to nondividing tissues. These drugs have quinone and aziridine moieties, and tumor cell cytotoxicity has been attributed to DNA alkylation and cross-linking. By contrast, the following results suggest that these drugs cause oxidative stress in nondividing cells by different mechanisms. Both hepatocytes or hepatic microsomes and NADPH were able to catalyse oxygen activation by all three drugs, suggesting that enzymatic reduction results in the formation of auto-oxidizable species. Their relative effectiveness at activating oxygen was BMY 25282 ≫ BMY 25067 > MMC. However, their relative cytotoxic effectiveness was BMY 25067 ≫ BMY 25282 > MMC, and it was increased markedly if hepatocyte glutathione-reductase or catalase was inactivated. Furthermore, ascorbate increased the toxic potencies of both BMY 25282 and MMC in catalase-inactivated hepatocytes by as much as 60- and 40-fold, respectively. Hepatocyte glutathione (GSH) oxidation was also increased. The relative resistance of normal hepatocytes to MMC and BMY 25282 can be attributed therefore, to the high levels of enzymes in hepatocytes involved in hydrogen peroxide detoxification. BMY 25067 cytotoxicity unlike that of BMY 25282 or MMC was prevented by the addition of the thiol reductant dithiothreitol. BMY 25067 also differed in being much more toxic towards GSH-depleted hepatocytes. Furthermore, BMY 25067, unlike MMC and BMY 25282, caused a rapid decrease in hepatocyte ATP levels and inhibited mitochondrial respiration. This could be prevented by the addition of the thiol reductant dithiothreitol, which restored intracellular GSH levels. Its toxic potency to catalase- or glutathione reductase-inactivated hepatocytes also was not increased by ascorbate. Therefore, the cytotoxicity of BMY 25067 can probably be attributed to oxidative stress by the aminodisulfide moiety which causes GSH and mixed disulfide formation, resulting in mitochondrial toxicity.

Mitomycin C (MMC†) is an antibiotic, isolated from *Streptomyces caespitosus*, with a wide spectrum of antineoplastic activity [1]. It is used for the treatment of breast [2] and head and neck tumors [3]. MMC belongs to a class of bioreductive alkylating agents with selective toxicity towards hypoxic tumor cells [4–6]. Its mechanisms of toxicity is still unclear, but it has been associated with DNA–DNA cross-links, formation of MMC monoadducts with DNA, and free radical-induced DNA strand breaks [7, 8].

Activation of MMC is proposed to occur as a result of enzymatic reduction of the quinone moiety by either a one-electron pathway to a semiquinone or by a direct two-electron reduction pathway to a hydroquinone [8, 9]. Reduction of the quinone under anaerobic conditions is thought to activate the C-1 position of the aziridine ring resulting in binding to DNA [10]. The drug may then undergo further reductive activation to form a second alkylation site at the C-10 position after the loss of the carbamate side chain. However, in the presence of oxygen, the reductive species, semiquinone and hydroquinone, may be back oxidized to the parent quinone with a net generation of reactive oxygen species. Cardiotoxicity induced by Adriamycin® has been attributed to redox cycling and oxygen activation [10–12]. Previous work in this laboratory has demonstrated that hepatocyte cytotoxicity induced by the two aziridinylquinone agents, diaziquone and trenimon, also involves redox cycling and oxygen activation [12–14].

In an attempt to overcome human cancer cell resistance to MMC, several MMC analogs have been synthesized, including BMY 25282 and BMY 25067 (Fig. 1) [15]. BMY 25282 has an amidine group, while BMY 25067 had an aminodisulfide derivative substituted at position 7 on MMC. These analogs were developed to increase antitumor activity because they undergo bioreductive activation more readily than MMC [15, 16]. BMY 25282 was synthesized originally by the Antitumour Chemistry Department of Bristol-Meyers to overcome acquired MMC resistance by making the drug easier to reduce and thus activate [15]. BMY 25282 has been reported to have a greater potency than MMC in both animal tumor tests and in clonogenic assays and can overcome the acquired resistance of some neoplasms to MMC [15, 17]. However, more recent findings suggest that BMY 25282 is cardiotoxic [18–20].

* Corresponding author: Dr. Peter J. O'Brien, Faculty of Pharmacy, University of Toronto, 19 Russell St., Toronto, Ontario, Canada M5S 2S2. Tel. (416) 978-2716; FAX (416) 978-8511.

† Abbreviations: MMC, mitomycin C; GSH, glutathione; GSSG, oxidized glutathione; HEPES, 4-(2-hydroxyethyl)-1-piperazineethane-sulfonic acid; DMSO dimethyl sulfoxide; BCNU, *N*,*N*-bis(2-chloroethyl)-*N*-nitrosourea; DTT, dithiothreitol; TMPD, *N*,*N*,*N*′,*N*′-tetramethylphenylenediamine.

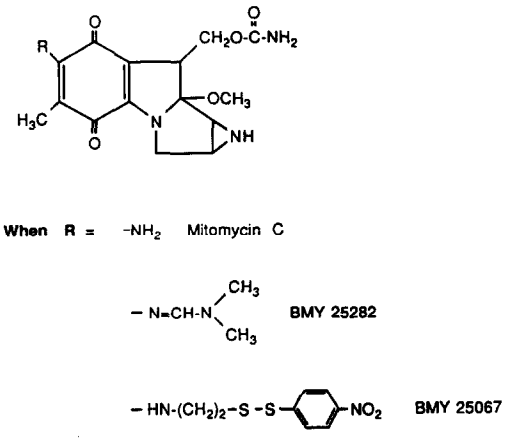

Fig. 1. Structures of MMC and its analogs, BMY 25282 and BMY 25067.

BMY 25067 has also been shown to be more effective than MMC against subcutaneously implanted B16 melanoma and causes less myelosuppression than MMC [20]. Other side effects of MMC include renal toxicity, interstitial pulmonary changes, cardiotoxicity, loss of hepatic function, and skin necrosis at the site of injection [21]. MMC has also been reported to increase Adriamycin-induced cardiomyopathy [11] and cardiopulmonary toxicity [10].

In this paper we have examined the mechanisms by which MMC and two of its analogs, BMY 25282 and BMY 25067, can cause cytotoxicity, using isolated hepatocytes as a model nondividing tissue cell. The cytotoxic mechanism of MMC and BMY 25282 involved redox cycling and oxygen activation. However, BMY 25067 was much more toxic, presumably as a result of mitochondrial oxidative stress caused by the aminosulfide moiety.

MATERIALS AND METHODS

Chemicals. *N*-*N*-Bis(2-chlorethyl)-*N*-nitrosourea (BCNU), MMC and its analogs, BMY 25282 and BMY 25067, were provided by Bristol-Myers Laboratories (Syracuse, NY). Trypan blue, glutathione (GSH), oxidized glutathione (GSSG), sodium azide, fluoro-2,4-dinitrobenzene, dithiothreitol (DTT) and iodoacetic acid were obtained from the Sigma Chemical Co. (St Louis, MO). Collagenase (from *Clostridium histoliticum*), NADPH, NADH, ATP, ADP, catalase and 4-(2-hydroxyethyl)-1-piperazineethane-sulfonic acid (HEPES) were purchased from Boehringer-Mannheim (Montreal, Canada). *N,N,N',N'*-Tetramethylphenylenediamine, metaphosphoric acid, and 1-bromoheptane were purchased from the Aldrich Chemical Co. (Milwaukee, WI). Other chemicals were of the highest grade available commercially.

Animals. Male Sprague–Dawley rats (body weight, 200–250 g) fed a standard chow diet and tap water *ad lib.* were used to prepare hepatocytes.

Hepatocyte isolation and incubation. Rat hepatocytes were prepared as previously described by Moldeus *et al.* [22] by collagenase perfusion of the liver. Cell viability at the start of the experiment was >85%. The hepatocytes (1×10^6 cells/mL) were preincubated at 37° in Krebs–Henseleit buffer (final volume 10 mL) containing 12.5 mM HEPES under an atmosphere of 10% O_2/5% CO_2/85% N_2 for 30 min in continuously rotating 50 mL round bottom flasks. To inactivate catalase and glutathione reductase, sodium azide (final concentration, 4 mM) and BCNU (final concentration, 50 μM) were added to the hepatocytes at 5 and 20 min, respectively, prior to the start of the experiment [13, 23]. To deplete GSH, the hepatocytes were incubated with bromoheptane (100 μM) for 20 min prior to the start of the experiment as previously described [24].

Microsomal and mitochondrial preparation. Microsomes and mitochondria were isolated from rat liver as previously described [25, 26] and kept cold on ice before use. Protein determination was measured by the method of Bradford [27].

Assays. Hepatocyte viability was assessed by the Trypan blue dye exclusion test in a Neubauer chamber, by light microscopy.

Total GSH and GSSG in the hepatocyte incubation mixture were measured by HPLC analysis in deproteinated samples (5% metaphosphoric acid) after derivatization with iodoacetic acid and fluoro-2,4-dinitrobenzene using a μ-Bondapak NH_2 column (Waters Associates, Milford, MA) [28]. GSH and GSSG were used as external standards. A Waters model 6000A solvent delivery system, equipped with a model 660 solvent programmer, a WISP 710A automatic injector, and a data module, was used for analysis.

ATP in hepatocytes was extracted using an alkaline extraction procedure and quantified by HPLC, using a C18 μ-Bondapak reverse phase column (Waters Associates) as previously described by Stocchi *et al.* [29].

Oxygen consumption was measured by a Clark-type electrode (model 5300; Yellow Springs Instrument Co., Inc.) in a 2-mL incubation chamber maintained at 37°. Hepatocytes (10^6 cells/mL) were kept at 37° in Krebs–Henseleit buffer, plus HEPES (12.5 mM), pH 7.4, under a stream of 10% O_2, 85% N_2 and 5% CO_2. Potassium cyanide (KCN; 2 mM, neutralized with HCl) was added to inhibit mitochondrial respiration. Measurement of respiration was performed with rat liver mitochondria (1 mg/mL) suspended in a respiration buffer containing 0.25 mM sucrose, 5 mM KH_2PO_4, 10 mM KCl, 5 mM $MgCl_2$ and 10 mM Tris–HCl at pH 7.4. Mitochondrial respiration substrates consisted of a 5 mM concentration of either succinate, β-hydroxybutyrate or ascorbate/TMPD (*N,N,N',N'*-tetramethylphenylenediamine). The respiratory control ratio (RCR) defined as the ratio of oxygen consumption in the presence (state 3) and absence (state 4) of ADP (200 μM), when the substrate concentration is not limiting, was determined by dividing state 3 over state 4 respiration.

Statistical significance of differences between treatment groups in these studies was determined by Student's *t*-test. The minimal level of significance chosen was $P < 0.05$.

Table 1. MMC and analog-catalysed oxygen consumption by rat liver microsomes and cyanide-resistant respiration in isolated hepatocytes

	Oxygen consumption			
	Microsomes/NADPH		Hepatocytes	
Additions	K_m (μM)	V_{max} (nmol/mg/min)	K_m (μM)	V_{max} (nmol/10^6 cells/min)
MMC	200	11.8 ± 2.4	170	10.4 ± 2.2
BMY 25067	150	10.2 ± 2.1	155	8.9 ± 2.8
BMY 25282	12	12.3 ± 2.6	25	11.6 ± 3.0

Oxygen uptake was measured using a Clarke-type electrode as described in Materials and Methods. MMC and analogs were added to rat liver microsomes (1 mg/mL) in 0.1 M Tris–HCl, pH 7.4, in the presence of 1 mM NADPH and isolated rat hepatocytes (10^6 cells/mL) in Krebs–Henseleit buffer, containing HEPES (12.5 mM), pH 7.4 in the presence of KCN (2 mM) at 37°. Values are the means ± SEM of three separate experiments.

RESULTS

Redox cycling mediated oxygen activation to MMC and analogs. To investigate the ability of MMC and its analogs to activate oxygen by one-electron redox cycling, ascorbate was used as the reductant. The addition of 1 μM BMY 25282 to 10 mM ascorbate caused rapid and continuous oxygen consumption from the incubation medium (3.2 ± 0.5 μmol O_2/min). The rate of oxygen consumption was proportional to the concentration of BMY 25282 (results not shown). Addition of catalase to the incubation mixture at the end of the experiment caused a recovery of 42–45% of the oxygen consumed indicating that the product of oxygen activation was H_2O_2 (results not shown). By contrast, addition of either MMC or BMY 25067 at concentrations as high as 500 μM to ascorbate (10 mM) did not stimulate oxygen consumption during a 5-min incubation period and suggests that they are less readily reduced than BMY 25282.

The intracellular reductase catalysed oxygen activation resulting from the redox cycling of MMC and its analogs was then assessed. Table 1 shows that rat liver microsomes in the presence of NADPH catalysed oxygen activation by all three compounds. The V_{max} values for the enzymatic catalysed redox cycling for all three compounds were very similar. However, the apparent K_m value for BMY 25282 was markedly lower than those for BMY 25067 and MMC. Cyanide-resistant respiration was also induced in isolated hepatocytes upon addition of MMC and its two analogs (Table 1) with an apparent K_m for BMY 25282 also much lower than those for BMY 25067 and MMC.

Toxicity of MMC, BMY 25282 and BMY 25067 to isolated hepatocytes. The cytotoxicity of MMC, BMY 25282 or BMY 25067 as determined by Trypan blue uptake was concentration dependent (Table 2). The analog BMY 25067 was the most cytotoxic, causing 100% cell death at a concentration of 150 μM (Table 2). BMY 25282 and MMC were markedly less toxic requiring concentrations of 2 and 3 mM, respectively, for cell death to occur (Table 2). However, inactivation of hepatocyte catalase by azide increased cytotoxicity 2- to 3-fold for all three compounds (Table 2). The addition of ascorbic acid (5 mM) to catalase-inactivated hepatocytes, so as to increase MMC or analog reduction, caused a dramatic increase in BMY 25282-induced toxicity without affecting control hepatocytes. Concentrations of BMY 25282 that cause cytotoxicity were now 60-fold lower when ascorbate was present. MMC was also approximately 40-fold more effective, whereas BMY 25067 was not more effective if ascorbate was present (Table 2).

Inactivation of hepatocyte glutathione reductase by BCNU (50 μM) also increased the toxicity of MMC and its two analogs. Under these conditions, the toxic potency of BMY 25282 was increased 10-fold, whereas the potencies of MMC and BMY 25067 were increased 2- and 3-fold, respectively (Table 2).

Effects of MMC and analogs on hepatocyte GSH levels. Addition of toxic doses of either MMC, BMY 25282 or BMY 25067 to hepatocytes caused intracellular GSH depletion prior to cell death (Fig. 2A). GSH depletion occurred gradually over time as a result of exposing the cells to toxic concentrations of either MMC or BMY 25282 (Fig. 2A). Part of the GSH depletion was attributed to GSSG formation (Fig. 2B). Much more GSH oxidation was observed with catalase or GSH reductase-inactivated cells (results not shown). Addition of a toxic dose of BMY 25067 to normal hepatocytes, however, caused much more rapid GSH depletion, and within the first 10 min of incubation most of the depleted GSH was accounted for by GSSG formation (Fig. 2B). BMY 25067 but not MMC or BMY 25282 was also much more cytotoxic to GSH-depleted cells than to normal cells (Table 2).

Addition of the thiol reductant DTT 10 min after BMY 25067 addition caused an immediate GSH recovery (Fig. 3) and prevented cell death (Table 2). However, MMC and BMY 25282 cytotoxicity was not prevented by DTT addition.

Effects of MMC and analogs on intracellular levels of ATP. Isolated rat hepatocytes maintain their intracellular ATP levels during a 3-hr incubation period. Addition of toxic doses of either MMC or

Table 2. Comparative cytotoxicity of MMC analogs to isolated hepatocytes

Treatment	Cytotoxicity (% Trypan blue uptake) 60 min	120 min	180 min
Normal hepatocytes			
Control	16 ± 2	18 ± 2	19 ± 2
MMC 2 mM	17 ± 2	21 ± 3	24 ± 3
3 mM	17 ± 2	35 ± 4	78 ± 7*
BMY 25282 1 mM	17 ± 2	21 ± 3	23 ± 3
2 mM	17 ± 2	41 ± 4	88 ± 7*
BMY 25067 0.10 mM	18 ± 2	21 ± 3	26 ± 4
0.15 mM	24 ± 3	65 ± 6	100*
Normal hepatocytes + DTT (5 mM)			
MMC 3 mM	17 ± 2	45 ± 4	83 ± 7*
BMY 25282 2 mM	17 ± 2	36 ± 4	79 ± 6
BMY 25067 0.15 mM	17 ± 2	22 ± 3	28 ± 3
Catalase-inactivated hepatocytes†			
Control	16 ± 2	20 ± 3	22 ± 3
MMC 2 mM	17 ± 2	39 ± 3	70 ± 6*
BMY 25282 1 mM	21 ± 3	56 ± 6	84 ± 8*
BMY 25067 0.05 mM	23 ± 3	49 ± 6	100
Ascorbate 5 mM	17 ± 2	21 ± 3	23 ± 3
Ascorbate + MMC 0.1 mM	24 ± 3	47 ± 5	76 ± 6*
Ascorbate + BMY 25282 0.01 mM	39 ± 4	100*	
Ascorbate + BMY 25067 0.05 mM	21 ± 2	45 ± 4	79 ± 6*
Glutathione reductase-inactivated hepatocytes†			
Control	16 ± 2	20 ± 2	19 ± 2
MMC 1 mM	18 ± 2	36 ± 4	84 ± 7
BMY 25282 0.20 mM	18 ± 2	32 ± 4	74 ± 7
BMY 25067 0.05 mM	22 ± 2	57 ± 5	100
GSH-depleted hepatocytes†			
Control	16 ± 2	18 ± 2	22 ± 2
MMC 3 mM	17 ± 2	32 ± 3	73 ± 7
BMY 25282 2 mM	17 ± 2	43 ± 3	87 ± 3
BMY 25067 0.05 mM	19 ± 3	43 ± 5	86 ± 7

Hepatocytes (10^6 cells/mL) were preincubated at 37° in Krebs–Henseleit buffer, pH 7.4. MMC drugs were then added to the cell incubation mixture. Cell toxicity was determined as the percentage of cells taking up Trypan blue. Values are the means ± SEM of at least three separate experiments.

* Significantly different from untreated cells ($P < 0.001$).

† Compromised hepatocytes were prepared as described under Materials and Methods.

its two analogs perturbed the ability of the cells to maintain constant ATP levels (Fig. 4). Incubation of either MMC or BMY 25282 with hepatocytes markedly decreased their intracellular ATP concentration by approximately 50% after a 1-hr incubation period at which time cytotoxicity was not significantly different from control cells. Addition of toxic concentration of BMY 25067, however, decreased ATP levels by 90% in the first 15 min of incubation and no ATP was observed in the hepatocytes after 60 min, well before cytotoxicity ensued (Fig. 4). Furthermore, addition of the thiol reductant DTT to the BMY 25067-treated cells prevented ATP depletion (Fig. 4).

Effects of MMC and analogs on mitochondrial respiration. Since intracellular ATP levels were affected by MMC and its analogs, we investigated their abilities to impair mitochondrial respiration. As shown in Table 3, incubation of isolated mitochondria with either MMC or BMY 25282 at concentrations as high as 500 μM did not affect significantly state 4 or state 3 mitochondrial respiration (supported by substrates succinate or β-hydroxybutyrate). However, BMY 25067 (200 μM) inhibited state 3 and 4 respiration supported by either succinate or β-hydroxybutyrate but not by ascorbate/TMPD. β-Hydroxybutyrate-induced respiration was more sensitive to BMY 25067-induced inhibition of state 3 and state 4 respiration. Furthermore, addition of DTT (2 mM) to the mitochondrial incubation mixture reversed the BMY 25067-induced inhibition of state 3 and state 4 respiration. DTT alone did not induce mitochondrial respiration.

DISCUSSION

The studies presented in this paper investigated the molecular mechanisms of toxicity of MMC and its analogs, BMY 25282 and BMY 25067, using isolated rat hepatocytes as a model cell system for studying the toxicity of antitumor drugs to normal nondividing tissues.

MMC is a bioreductively activated drug which has

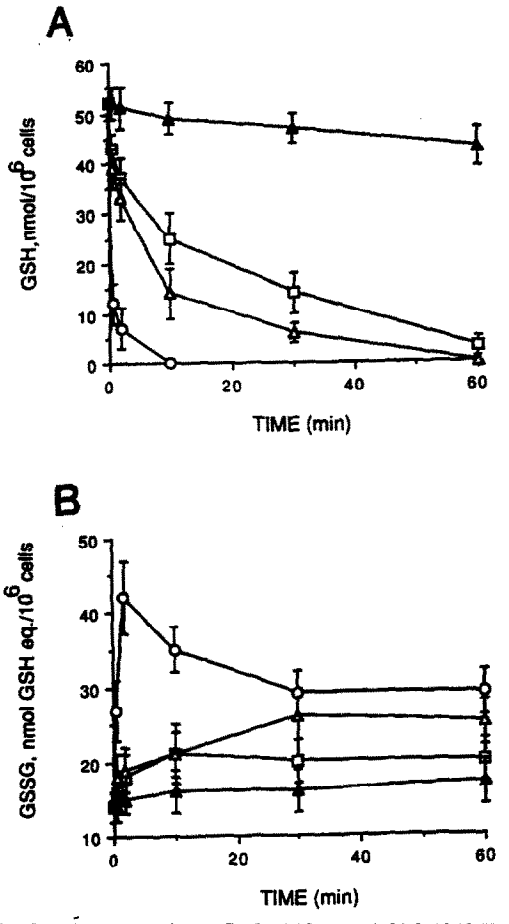

Fig. 2. Effects of MMC, BMY 25282 and BMY 25067 on hepatocyte GSH depletion (A) and GSSG formation (B). Hepatocytes (10^6 cells/mL) were incubated with the following: 3 mM MMC (□), 2 mM BMY 25282 (△), 150 μM BMY 25067 (○) and no additions (▲). GSH and GSSG levels were determined as described under Materials and Methods. Values are the means ± SEM of at least three separate experiments.

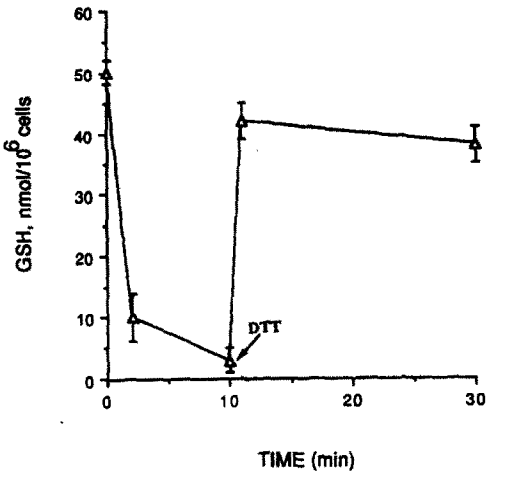

Fig. 3. Recovery of intracellular hepatocyte GSH by DTT. Hepatocytes (10^6 cells/mL) were incubated with 150 μM BMY 25067; DTT (5 mM) was added 10 min after BMY 25067 addition. GSH and GSSG levels were determined by the method of Reed *et al.* [28], as described in Materials and Methods. Values are the means ± SEM of at least three separate experiments.

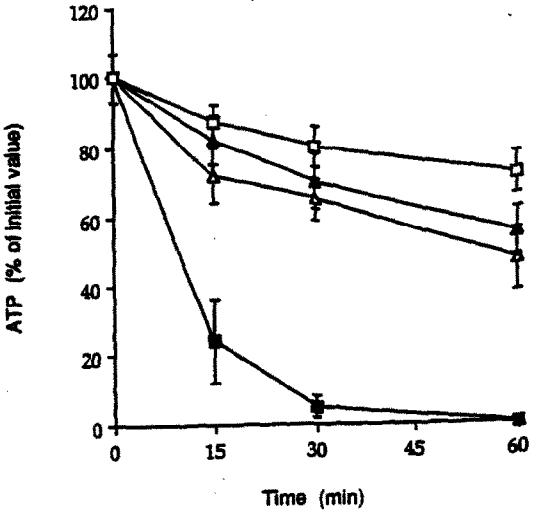

Fig. 4. Effects of MMC, BMY 25282 and BMY 25067 on cellular ATP levels. Hepatocytes (10^6 cells/mL) were incubated with the following: 3 mM MMC (▲), 2 mM BMY 25282 (△), 150 μM BMY 25067 (■) and 5 mM DTT + 150 μM BMY 25067 (□). ATP levels were measured as described in Materials and Methods. Values are the means ± SEM of least three separate experiments. The ATP level in normal (unheated) hepatocytes was 60 nmol/10^6 cells.

been reported to show specific toxicity towards tumor cells [1]. Reduction has been proposed to form alkylating agents that can bind monofunctionally to DNA, RNA and proteins [7], or bind bifunctionally thereby cross-linking complementary strands of DNA [8]. Along with DNA alkylation it has been argued that MMC-induced toxicity also involves the generation of reactive oxygen species including superoxide and hydroxyl radicals caused by redox cycling of the one-electron reduced product, semiquinone, with oxygen [8].

To further determine the involvement of oxidative stress in the toxic mechanism of MMC and its two derivatives, the abilities of the individual compounds to undergo redox cycling with oxygen were investigated in the presence of ascorbate. Ascorbic acid has been shown previously to catalyse one-electron reduction of benzoquinones to free radical semiquinones [13, 14, 30]. Ascorbate was also very efficient at stimulating oxygen consumption by BMY 25282. However, no oxygen consumption was detected with either MMC or BMY 25067 in the presence of ascorbate. The ability of NADPH:cytochrome P450 reductase to catalyse redox cycling and oxygen activation of MMC and its

Table 3. Effects of MMC, BMY 25282 and BMY 25067 on mitochondrial respiration

Treatment	Substrate	State 3	State 4	RCR*
Control	β-OH butyrate	20.1 ± 2.5†	3.1 ± 0.4†	6.5
MMC (500 μM)	β-OH butyrate	18.5 ± 2.6	3.2 ± 0.5	5.8
BMY 25282 (500 μM)	β-OH butyrate	19.2 ± 2.2	3.2 ± 0.5	6.0
BMY 25067 (200 μM)	β-OH butyrate	0.9 ± 0.2‡	0.7 ± 0.2‡	1.3
+ DTT (5 mM)	β-OH butyrate	17.8 ± 2.8	3.3 ± 0.5	5.4
Control	Succinate	92.6 ± 5.9	25.7 ± 2.2	3.6
MMC (500 μM)	Succinate	83.4 ± 5.7	22.9 ± 2.4	3.6
BMY 25282 (500 μM)	Succinate	80.4 ± 6.2	22.1 ± 2.0	3.6
BMY 25067 (200 μM)	Succinate	3.2 ± 1.4†	2.3 ± 0.5‡	1.4
+ DTT (5 mM)	Succinate	69.6 ± 2.1	21.2 ± 1.9	3.3
Control	Ascorbate-TMPD	55.8 ± 5.1	26.7 ± 2.5	2.1
BMY 25067 (200 μM)	Ascorbate-TMPD	52.5 ± 4.9	24.9 ± 3.2	2.1

Experiments were performed as described in Materials and Methods. Values are the means ± SD of three separate experiments.

* RCR, respiratory control ratio.

† Values are given in nmol oxygen uptake/min/mg protein.

‡ Significantly different from control, $P < 0.001$.

analogs was studied by following the oxygen uptake catalysed by microsomes/NADPH. A kinetic study revealed that the V_{max} values for MMC, BMY 25282 and BMY 25067 were similar. However, the K_m values differed considerably, indicating that BMY 25282 had a 12- to 17-fold higher affinity for the reductase than MMC or BMY 25067.

The present studies show that MMC and its novel analogs, BMY 25282 and BMY 25067, caused hepatocyte cytotoxicity in a concentration-dependent manner. However, the cytotoxic mechanisms differ. BMY 25067 was shown to be markedly more toxic to hepatocytes than either BMY 25282 or MMC. Intracellular GSH was depleted over time and prior to cell death in hepatocytes incubated with either BMY 25282 or MMC. A fraction of the depleted GSH was accounted for by GSSG accumulation, suggesting that both alkylating species and oxidative stress occurred. By contrast, incubation of a toxic dose of BMY 25067 caused complete intracellular GSH depletion within the first 10 min with most of the GSH being oxidized to GSSG.

Addition of ascorbic acid to catalase-inactivated hepatocytes increased both BMY 25282 and MCC toxicity by approximately 60- and 40-fold, respectively. In contrast, BMY 25067 toxicity under these conditions was not increased. Ascorbic acid has also been reported to increase aerobic MMC toxicity to Chinese hamster ovary cells in a clonogenic assay [6].

Inhibition of GSH reductase by BCNU was shown to greatly enhance BMY 25282 toxicity against isolated hepatocytes (approximately 10-fold) while increasing both MMC and BMY 25067 toxicity by a lesser amount (approximately 2-fold). These data suggest that BMY 25067 cytotoxicity involves much less redox cycling mediated oxygen activation than the cytotoxicity of MMC and BMY 25282. Dusre *et al.* [31] also found that a positive correlation existed between cytotoxicity and free radical formation and interstrand DNA cross-links for both MMC and BMY 25282 in human breast tumor cell lines (MCF-7) sensitive and resistant to Adriamycin. However, BMY 25067 formed very little interstrand DNA cross-links in either cell line yet showed cross-resistance to the Adriamycin-resistant cell line and was the most toxic analog [31].

Incubation of hepatocytes with BMY 25067 caused a rapid decrease in intracellular ATP levels within 15 min of incubation possibly resulting from its ability to completely inhibit ADP-induced state 3 mitochondrial respiration supported by either succinate or β-hydroxybutyrate but not by ascorbate/TMPD. This indicates that Complexes I and II were much more susceptible than Complex IV. β-Hydroxybutyrate-induced respiration was more sensitive, indicating that the thiol-dependent NADH dehydrogenase is a target for BMY 25067. Both of these effects were prevented by the presence of the thiol reducing agent DTT. In contrast, mitochondrial respiration was not affected by either MMC or BMY 25282, and intracellular ATP levels were decreased slowly over time in the presence of either of these two compounds.

The data presented in this study appear to support a role for oxidative stress, as a result of redox cycling, in the toxic mechanisms of both MMC and BMY 25282. The toxic potency of both of these compounds was increased dramatically in cells which catalase and glutathione reductase had been inactivated, indicating that oxidative stress is involved in both MMC- and BMY 25282-mediated hepatocyte cytotoxicity.

By contrast, BMY 25067 cytotoxicity towards hepatocytes increased only slightly if hepatocyte catalase or glutathione reductase was inactivated beforehand and presumably reflects that the molecular cytotoxic mechanism does not involve futile redox cycling and oxygen activation. The hepatocyte GSSG formation induced with BMY 25067 likely results from the oxidation of GSH by the aminodisulfide substituent. The GSSG formed and/or the aminodisulfide moiety of BMY 25067 may then form mixed disulfides with critical protein

thiols and initiate the cytotoxic process. In support of this hypothesis, depletion of hepatocyte GSH beforehand potentiated toxicity by as much as 3-fold. Furthermore, the thiol reductant, DTT, caused partial recovery of intracellular GSH, restored ATP levels, and prevented cytotoxicity. DTT also restored mitochondrial respiration supported by either succinate or β-hydroxybutyrate which had been inhibited with BMY 25067. Previously, we found that the marked cytotoxicity of disulfide metabolites could be prevented with DTT or markedly enhanced by prior GSH depletion [32]. BMY 25067 cytotoxicity can, therefore, be attributed to protein thiol oxidation by the aminodisulfide moiety of BMY 25067.

REFERENCES

1. Crooke ST and Bradner WT, Mitomycin C: A review. *Cancer Treat Rev* **3**: 121–139, 1976.
2. Randford J, Knight R and Rubens R, Mitomycin C and vinblastine in the treatment of advanced breast cancer. *Eur J Cancer Clin Oncol* **21**: 1475–1477, 1985.
3. Vogl SE, Lerner H, Kaplan BH, Camacho F, Cinberg J and Schoenfeld D, Mitomycin-C, methotrexate, bleomycin and cis-diaminedichloroplatinum II in the chemotherapy of advanced squamous cancer of the head and neck. *Cancer* **50**: 6–9, 1982.
4. Kennedy KA, Rockwell S and Sartorelli AC, Preferential activation of mitomycin C to cytotoxic metabolite by hypoxic tumour cells. *Cancer Res* **40**: 2356–2360, 1980.
5. Keyes SR, Fracasso PM, Heimbrook DC, Rockwell SG and Sartorelli AC, Role of NADPH-cytochrome *c*-reductase and DT-diaphorase in the biotransformation of mitomycin C. *Cancer Res* **44**: 5638–5643, 1984.
6. Marshall RS and Rauth AM, Modification of the cytotoxic activity of mitomycin C by oxygen and ascorbic acid in Chinese hamster ovary cells and a repair-deficient mutant. *Cancer Res* **46**: 2709–2713, 1986.
7. Iyer VN and Szybalski W, A molecular mechanism of mitomycin action: Linking of complementary DNA strants. *Proc Natl Acad Sci USA* **50**: 355–362, 1963.
8. Siegel D, Beall H, Senekowitsch C, Kasai M, Arai H, Gibson NW and Ross D, Bioreductive activation of mitomycin C by DT-diaphorase. *Biochemistry* **31**: 7879–7885, 1992.
9. Pan SS, Andrews PA, Glover CJ and Bachur NR, Reductive activation of mitomycin C and mitomycin C metabolites catalyzed by NADPH-cytochrome P-450 reductase and xanthine oxidase. *J Biol Chem* **259**: 959–966, 1984.
10. Buzdar AU, Legha SS, Tashima CK, Hortobagyi, GN, Yap HY, Krutchick AN, Luna MA and Blumenschein GR, Adriamycin and mitomycin C: Possible synergistic cardiotoxicity. *Cancer Treat Rep* **62**: 1005–1009, 1978.
11. Doyle LA, Ihde DC, Carney DN, Bunn PA, Cohen MH, Matthews MJ, Puffenbarger R, Cordes RS and Minna JD, Combination therapy with doxorubicin and mitomycin C in non-small cell bronchogenic carcinoma. Severe pulmonary toxicity from q 3 weekly mitomycin C. *Am J Clin Oncol* **7**: 719–724, 1984.
12. O'Brien PJ, Molecular mechanisms of quinone cytotoxicity. *Chem Biol Interact* **80**: 1–41, 1991.
13. Silva JM and O'Brien PJ, Diazequone-induced cytotoxicity in isolated rat hepatocytes. *Cancer Res* **49**: 5550–5554, 1989.
14. Silva JM, Rao DR and O'Brien PJ, Modulation of trenimon-induced cytotoxicity by DT-diaphorase in isolated rat hepatocytes under aerobic versus hypoxic conditions. *Cancer Res* **52**: 3015–3023, 1992.
15. Wilson JK, Long BH, Chakrabarty S, Brattain DE and Brattain MG, Effects of BMY 25282, a mitomycin C analogue, in mitomycin C-resistant human colon cancer cells. *Cancer Res* **45**: 5281–5286, 1985.
16. Bradner WT, Rose WC, Schurig JE, Florczyk AP, Huftalen JB and Catino JJ, Antitumor activity and toxicity in animals of BMY 25282, a new mitomycin derivative. *Cancer Res* **45**: 6475–6481, 1985.
17. Pristos CA and Sartorelli AC, Generation of reactive oxygen radicals through bioactivation of mitomycin antibiotics. *Cancer Res* **46**: 3528–3532, 1986.
18. Bregman C, Comereski CR, Buroker RA, Hirth R, Madissoo H and Hottendorf G, Single-dose and multiple-dose intravenous toxicity studies of BMY 25282 in rats. *Fundam Appl Toxicol* **9**: 90–109, 1987.
19. Radner WT, Bregman CL, Buroker RA, Pritsos CA and Sartorelli AC, Cardiotoxicity of mitomycin derivatives. *Proc Am Assoc Cancer Res* **29**: 267, 1988.
20. Politi PM, Rajagopalan S and Sinha BK, Free-radical formation by mitomycin C and its novel analogs in cardiac microsomes and the perfused rat heart. *Biochim Biophys Acta* **992**: 341–348, 1989.
21. Perry MC, Toxicity of chemotherapy. *Semin Oncol* **19**: 453–647, 1992.
22. Moldeus P, Hogberg J and Orrenius S, Isolation and use of liver cells. *Methods Enzymol* **52**: 60–71, 1978.
23. Babson JR and Reed DJ, Inactivation of glutathione reductase by 2-chloroethyl nitrosourea-derived isocyanates. *Biochem Biophys Res Commun* **83**: 754–762, 1978.
24. Khan S and O'Brien PJ, 1-Bromoalkanes as new nontoxic glutathione depletors in isolated rat hepatocytes. *Biochem Biophys Res Commun* **179**: 436–441, 1991.
25. Ernester L, Sukevitz P and Palade GE, Enzyme relationships in the endoplasmic reticulum of rat liver. *J Cell Biol* **15**: 541–562, 1962.
26. Cain K and Skilleter DN, Preparation and use of mitochondria in toxicological research. In: *Biochemical Toxicology: A Practical Approach* (Eds. Snell K and Mullock B), pp. 217–223. IRL Press, Washington, 1987.
27. Bradford MM, A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein dye binding. *Anal Biochem* **72**: 248–256, 1976.
28. Reed DJ, Babson JR, Brodie AE, Ellis WW and Potter DW, High performance liquid chromatography analysis of nanomole levels of glutathione, glutathione disulfide and related thiols and disulfides. *Anal Biochem* **106**: 55–62, 1980.
29. Stocchi V, Cucchiarini L, Magnani M, Chiarantini L, Palma P and Crescentini G, Simultaneous extraction and reverse-phase high-performance liquid chromatographic determination of adenine and pyridine nucleotides in human red blood cells. *Anal Biochem* **146**: 118–124, 1985.
30. Gutierrez PL, Egorin MJ, Davis TA and Bachur NR, Effects of ascorbic acid on biologically obtained diaziquone free radicals. *Biochem Pharmacol* **34**: 2394–2397, 1985.
31. Dusre L, Rajagopalan S, Eliot HM, Covey JM and Sinha BK, DNA interstrand cross-link and free radical formation in a human multidrug-resistant cell line from mitomycin C and its analogues. *Cancer Res* **50**: 648–652, 1990.
32. Jatoe SD, Lauriault V, McGirr LG and O'Brien PJ, The toxicity of disulphides to isolated hepatocytes and mitochondria. *Drug Metabol Drug Interact* **6**: 395–411, 1988.