Generation of Reactive Oxygen Radicals through Bioactivation of Mitomycin Antibiotics

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ABSTRACT

Mitomycin C (MC) is a naturally occurring anticancer agent which has been shown to be more cytotoxic to hypoxic tumor cells than to their aerobic counterparts. The mechanism of action of this agent is thought to involve biological reductive activation, to a species that alkylates DNA. A comparison of the cytotoxicity of MC to EMT6 tumor cells with that of the structural analogues porfiromycin (PM), N-4(N', N'-dimethylnimino)methylene)amine analogue of mitomycin C (BMY-25282), and N-4(N', N'-dimethylaminomethylene)amine analogue of porfiromycin (BL-6783) has demonstrated that PM is considerably less cytotoxic to aerobic EMT6 cells than MC, whereas BMY-25282 and BL-6783 are significantly more toxic. The relative abilities of each of these compounds to generate oxygen free radicals following biological activation were measured. Tumor cell sonicates, reduced nicotinamide adenine dinucleotide phosphate-cytochrome c reductase, xanthine oxidase, and mitochondria were used as the biological reducing systems. All four mitomycin antibiotics produced oxygen radicals following biological reduction, a process that may account for the aerobic cytotoxicity of agents of this class. The generation of relative amounts of superoxide and hydroxyl radical were also measured in EMT6 cell sonicates. BMY-25282 and BL-6783 produced significantly greater quantities of oxygen free radicals with the EMT6 cell sonicate, reduced nicotinamide adenine dinucleotide phosphate-cytochrome c reductase, and mitochondria than did MC and PM. In contrast, BMY-25282 and BL-6783 did not generate detectable levels of free radicals in the presence of xanthine oxidase, whereas this enzyme was capable of generating free radicals with MC and PM as substrates. MC consistently produced greater amounts of free radicals than PM with all of the reducing systems. BMY-25282, BL-6783, and MC all generated hydroxyl radical, while PM did not appear to form these radicals. The findings indicate that a correlation exists between the ability of the mitomycin antibiotics to generate oxygen radicals and their cytotoxicity to aerobic EMT6 tumor cells.

INTRODUCTION

MC is an antibiotic isolated from Streptomyces caesipitosus which has been shown to be active against a broad spectrum of animal (1) and human (2) tumors. Furthermore, this agent has been shown to cause preferential cytotoxicity toward hypoxic tumor cells relative to their oxygenated counterparts both in vitro (3-5) and in vivo (6, 7). Studies on the molecular mechanism of action of MC have shown that biological activation to a reactive species is required to produce intrastrand cross-linking of DNA (8). While this appears to be the most plausible mechanism for the hypoxic cell toxicity of this agent, the futile oxygen cycling that occurs under aerobic conditions makes it likely that oxygenated cell toxicity involves reduction to the semiquinone rather than to the fully reduced species (9), and the subsequent reaction of the semiquinone with molecular oxygen to generate superoxide and hydroxyl radicals (10-14). PM, BMY-25282, and BL-6783, are all structural analogues of MC (Fig. 1). PM has greater preferential cytotoxicity to hypoxic EMT6 cells than MC; this phenomenon is due to the fact that although PM is equivalent to MC in producing cytotoxicity to hypoxic cells, it is significantly less toxic to aerobic cells (15). BMY-25282 and BL-6783, the porfiromycin counterpart of BMY-25282, have preferential cytotoxicity to aerobic EMT6 cells. BMY-25282 is 10 times more cytotoxic to aerobic EMT6 cells and 4 times more cytotoxic to their hypoxic counterparts than MC.

Because of the structural similarities of these 4 compounds, we have examined the relative abilities of various biological systems to reductively activate these antibiotics under aerobic conditions to generate oxygen free radicals and have compared these activities with their cytotoxicities to oxygenated EMT6 tumor cells. In addition, the relative quantities of superoxide and hydroxyl radical generated by each of the mitomycin antibiotics was measured using EMT6 cell sonicates. Diethylene-triaminepentaacetic acid was included in these reaction mixtures as an iron chelator to minimize iron-catalyzed Haber-Weiss-induced hydroxyl radical production. The findings demonstrated a correspondence between the toxicity of these agents to EMT6 tumor cells under aerobic conditions and their capacity to generate oxygen-containing radicals following reduction by sonicates of these cells.

MATERIALS AND METHODS

MC, PM, BMY-25282, and BL-6783 were provided by Drs. T. W. Doyle and D. M. Vyas of Bristol Laboratories (Syracuse, NY). Beef heart mitochondria were generously supplied by Dr. R. S. Pardini (University of Nevada, Reno, NV). NADPH-cytochrome c reductase was purified from phenobarbital-treated rabbit liver as previously described (16). All other materials were purchased from commercial sources: 5,5-dimethyl-1-pyrroline N-oxide and diethylene-triaminepentaacetic acid were purchased from Aldrich Chemical Co. (Milwaukee, WI); acetonitrile (HPLC grade) was from J. T. Baker Chemical Co. (Phillipsburg, NJ); succinic acid (disodium salt), NADH, NADPH, and xanthine oxidase (Grade I from buttermilk) were from Sigma Chemical Co. (St. Louis, MO); and fetal bovine serum and Waymouth's medium were from Grand Island Biological Co. (Grand Island, NY).

EMT6 mouse mammary tumor cells were grown at 37°C in Waymouth's medium supplemented with 15% fetal bovine serum and antibiotics in a humidified atmosphere of 95% air-5% CO₂. The characteristics of this cell line have previously been described (17). Cell sonicates were prepared from exponentially growing monolayer cultures by exposing them to 0.05% trypsin in PBS to remove cells from the flasks, washing with cold PBS, and subsequently resuspending them in the appropriate buffer system. The cells in an ice bath were disrupted by three 5-s bursts with a Branson Model 160 sonicator set at 25% of maximum intensity. Cellular disruption was verified microscopically.

Cytotoxicity studies were performed on EMT6 tumor cells growing exponentially in 25-cm² Corning plastic culture flasks under the conditions described above. Cells were exposed to drug and vehicle or

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2 To whom requests for reprints should be addressed.

3 The abbreviations used are: MC, mitomycin C; BL-6783, N-4(N'-N'-dimethylaminomethylene)amine analogue of porfiromycin; BMY-25282, N-4(N',N'-dimethylaminomethylene)amine analogue of mitomycin C; PBS, phosphate-buffered saline; PM, porfiromycin; HPLC, high performance liquid chromatography.

4 Unpublished results.
consumption was used for all of the enzyme assays. Control) were added and the new rate of oxygen consumption was either 1 or 5 mM; dilutions of these stock solutions resulted in no cells/ml, and subjecting them to sonication as described above. Final activation by EMT6 cell sonicates was carried out by the HPLC procedure described earlier (22). This procedure is a qualitative assay useful in identifying the oxygen radical species generated by biological systems and can be used to compare relative radical production by various agents in the same system. Prior to sonication, EMT6 cells were suspended in 50 mM Tris-HCl buffer (pH 7.8) with 1 mM diethylenetriaminepentaacetic acid and adjusted to a concentration of between 16 and 20 x 10⁶ cells/ml in the same buffer. The reaction mixture consisted of 200 µl of cell sonicate, 100 mM 5,5-dimethyl-1-pyrroline N-oxide, 0.5 mM NADH, 0.5 mM NADPH, and 4.0% ethanol or drug (in ethanol) in a final volume of 250 µl. The reaction mixture was incubated for 6 min at 37°C, at which time 25 µl of the solution were injected into the HPLC system which consisted of an Altex ultrasphere 3 µm octadecylsilane 75 x 4.6-mm inside diameter column attached to a Bioanalytical Systems LC4 A electrochemical detector (Bioanalytical Systems, Inc., West Lafayette, IN). The detector potential was set at +0.6 V versus an Ag⁺-AgCl reference electrode. A Rainin C-18 Microsorb Short-Ones column (Rainin Instrument Co., Woburn, MA) served as a guard column. The mobile phase consisted of 0.03 M citric acid (monohydrate), 0.05 M anhydrous sodium acetate, 0.05 M sodium hydroxide, and 8.5% acetonitrile, brought to pH 5.1 with glacial acetic acid; the flow rate was 1.0 ml/min. The relative peak areas of the radicals generated by each drug were determined by cutting the appropriate peaks from the HPLC tracings and weighing them on an analytical balance.

RESULTS

The data in Table 1 show the concentration of each of the mitomycin antibiotics that produced 50% kill of EMT6 mouse tumor cells under aerobic conditions. The order of decreasing potency of the compounds under these conditions was BMY-25282, BL-6783, MC, and PM. To determine whether this action correlated with the ability of these agents to generate oxygen radicals, the production of oxygen radicals was measured using different biological reducing systems by monitoring oxygen consumption, which is a convenient method of measuring oxygen radical formation (18).

To ascertain whether the mitomycin antibiotics were capable of being activated to a state that generates oxygen radicals in a complete tumor cell system, drug-induced oxygen consumption was measured in EMT6 cell sonicates supplemented with sufficient NADH and NADPH to ensure that these substrates were not rate limiting. All of the compounds tested gave a linear dose dependent rate of oxygen consumption in the presence of EMT6 cell sonicates and substrate (Fig. 2). BMY-25282 caused the greatest rate of oxygen consumption followed by BL-6783; the rate of oxygen consumption produced by MC and PM was approximately 10-fold lower.

To provide information on systems with the potential to activate these drugs to forms that generate oxygen radicals, enzymatic systems which have previously been shown to activate quinones to produce oxygen radicals were examined; these consisted of NADPH-cytochrome c reductase (9, 14, 18, 23), xanthine oxidase (20), and intact mitochondria (14, 24).

The rate of activated drug-induced oxygen consumption produced by NADPH-cytochrome c reductase is shown in Fig. 3.

<table>
<thead>
<tr>
<th>Compound</th>
<th>R₁</th>
<th>R₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mitomycin C</td>
<td>-NH₂</td>
<td>H</td>
</tr>
<tr>
<td>Porfiromycin</td>
<td>-NH₂</td>
<td>CH₃</td>
</tr>
</tbody>
</table>
| BMY-25282     | -N⁺⁺⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻~-~-

Fig. 1. Structural formulae of mitomycin antibiotics.
Fig. 2. Mitomycin antibiotic-induced stimulation of oxygen consumption catalyzed by EMT6 cell sonicates. Reaction mixtures consisted of 2 ml of EMT6 cell sonicates (3 to 5 x 10^6 cells/ml in 50 mM Tris-HCl buffer, pH 7.8), 1 mM diethylenetriaminepentaacetic acid, 0.5 mM NADPH, and 0.5 mM NADH. Drugs were added at the indicated final concentrations and the reactions were run at 37°C as previously described. O, mitomycin C; @, porfiromycin; □, BMY-25282; Δ, BL-6783. Points, average of at least 4 determinations; bars, SD.

Fig. 3. Mitomycin antibiotic-induced stimulation of oxygen consumption catalyzed by NADPH-cytochrome c reductase. Reaction mixtures consisted of 2 ml of 50 mM phosphate buffer (pH 7.4), 0.18 unit of NADPH-cytochrome c reductase, 1 mM diethylenetriaminepentaacetic acid, and 0.5 mM NADPH. Drugs were added at the indicated final concentrations and the reactions were run at 30°C as previously described. O, mitomycin C; @, porfiromycin; □, BMY-25282; Δ, BL-6783. Points, average of at least 4 determinations; bars, SD.

BMY-25282 and BL-6783 gave similar rates of oxygen consumption that were approximately 4 times those generated with PM and MC, which produced statistically similar rates of oxygen consumption. In contrast to the findings with cell sonicates and NADPH-cytochrome c reductase, xanthine oxidase-activated drug-induced oxygen consumption followed a different pattern (Fig. 4). With this enzyme, MC and PM produced relatively large amounts of oxygen radicals, whereas both BMY-25282 and BL-6783 did not. Since xanthine oxidase is capable of generating superoxide in the presence of substrate, we examined the possibility that superoxide formed from a reaction between xanthine oxidase and NADH might be involved in reducing both MC and PM, which in turn reduced oxygen; this mechanism contrasted with a direct reduction of MC and PM by xanthine oxidase in the presence of NADH. If the former mechanism was involved, the addition of superoxide dismutase would be expected to inhibit drug-induced oxygen consumption. The addition of superoxide dismutase to the reaction mixture, however, did not prevent drug-induced oxygen consumption, thereby eliminating this possibility, and supporting a direct reduction of MC and PM by NADH catalyzed by xanthine oxidase.

Mitochondrial-activated drug-induced oxygen consumption is shown in Fig. 5. Both NADH and succinate were added as substrates to ensure that both pathways of electron flow (i.e., NADH dehydrogenase and succinate dehydrogenase) were operative. The findings demonstrated that both BMY-25282 and BL-6783 were readily activated by bovine heart mitochondria to generate oxygen radicals, whereas MC and PM did not serve as effective substrates.

Oxygen radical species such as the hydroxyl radical are thought to be more cytotoxic than other species such as superoxide. Since differences in the species of oxygen radicals gen-
OXYGEN RADICALS GENERATED BY MC ANTIBIOTICS

The findings from these studies demonstrate that the enzymatic composition of target cell types can affect the reactivity of various mitomycin antibiotics in that system. Although the present studies were performed with enzymes from various mammalian sources, the results are probably representative of the general interaction of these compounds with the enzyme systems used. However, direct comparisons to in vivo drug activation must be made with caution since relative enzyme concentrations vary between biological systems, and in the present studies different sources of the enzymes were used and each assay was optimized for the particular enzyme being assayed.

BMY-25282 and BL-6783 generated more hydroxyl and superoxide radicals than either PM or MC. MC in turn produced more superoxide and hydroxyl radicals than PM. The markedly greater production of superoxide and hydroxyl radicals by BMY-25282 and BL-6783 may well contribute to the greatly enhanced aerobic cytotoxicity of these derivatives, over that produced by PM and MC, to EMT6 tumor cells.

In summary, the mitomycin antibiotics tested generate various amounts of oxygen free radicals with EMT6 cell sonicates. The relative quantities of oxygen radicals produced by these compounds in this system correspond to their relative aerobic cytotoxicities to EMT6 tumor cells. This relationship suggests a possible contribution of oxygen radicals to the aerobic cytotoxicity of these compounds. NADPH-cytochrome c reductase and xanthine oxidase were the most effective activators of PM and MC, whereas NADPH-cytochrome c reductase and mitochondrial reductase(s) were the most efficacious systems in catalyzing the reductive activation of BMY-25282 and BL-6783.

DISCUSSION

Quinone-containing compounds are capable of undergoing one electron reduction and subsequent reaction with molecular oxygen to generate various oxygen species such as superoxide, hydrogen peroxide, and hydroxyl radical (25, 26). The intracellular production of these toxic oxygen species by compounds of this type have the potential to produce cytotoxicity. Thus, Adriamycin (27, 28), paraquat (29), menadione (30), and bleomycin (31) are some of the compounds whose cytotoxic actions have been attributed to the generation of oxygen radicals. The finding that the reduction of MC leads to the generation of oxygen radicals (10–14) raises the possibility that these radicals may be involved in the aerobic cytotoxicity of this agent. The differences in the cytotoxicities of the 4 mitomycin analogues shown in Fig. 1, together with their structural similarities, provided a good series for evaluating the possible role of oxygen radicals in aerobic toxicity. The substitution of a methyl group on the aziridine ring portion of the molecule (the R1 position in Fig. 1) resulted in a decreased rate of oxygen radical generation. The substitution of an N-((N',N'-dimethylaminomethylene)amine for the amine group on the quinone ring (R1 in Fig. 1) caused a greatly enhanced rate of oxygen radical generation. Since the substitution at the R1 position results in a change in the redox potential of MC and PM from an E00 of 0.35 V to 0.16 V (32), BMY-25282 and BL-6783 are more readily reduced, a property that presumably accounts for their increased capacity to generate oxygen radicals. BMY-25282 and BL-6783, however, do not appear to be activated by xanthine oxidase, while both PM and MC are activated by this enzyme to generate oxygen radicals. No information is available to explain these observations, although one reasonable possibility is that steric hindrance caused by the bulkiness of the substituted amine group at the R1 position prevents activation by xanthine oxidase.

Table 2 Relative quantities of drug-induced oxygen radicals formed in EMT6 cell sonicates containing mitomycin antibiotics

<table>
<thead>
<tr>
<th>Compound</th>
<th>Superoxide</th>
<th>Hydroxyl radical</th>
</tr>
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<tbody>
<tr>
<td>Mitomycin C</td>
<td>1.29 ± 0.03</td>
<td>1.18 ± 0.10</td>
</tr>
<tr>
<td>Porfirofomycin</td>
<td>0.97 ± 0.20</td>
<td>1.06 ± 0.10</td>
</tr>
<tr>
<td>BMY-25282</td>
<td>2.12 ± 0.20</td>
<td>1.35 ± 0.16</td>
</tr>
<tr>
<td>BL-6783</td>
<td>1.68 ± 0.30</td>
<td>1.52 ± 0.12</td>
</tr>
</tbody>
</table>

Values with a significant difference of at least P < 0.05 (by the paired Student's t-test) when compared to the control samples.

REFERENCES

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