Modification of the Metabolism and Cytotoxicity of Bioreductive Alkylating Agents by Dicoumarol in Aerobic and Hypoxic Murine Tumor Cells

Susan R. Keyes,2 Sara Rockwell, and Alan C. Sartorelli

Departments of Pharmacology [S. R. K., A. C. S.] and Therapeutic Radiology [S. R. J., Developmental Therapeutics Program, Comprehensive Cancer Center, Yale University School of Medicine, New Haven, Connecticut 06510

ABSTRACT

We have demonstrated previously that dicoumarol (DIC) increased the generation of reactive metabolites from mitomycin C (MC) in EMT6 cells under hypoxic conditions in vitro. This increased reaction rate was associated with an increased toxicity of MC to hypoxic EMT6 cells. In contrast, aerobic cells treated with DIC in vitro were protected from MC toxicity. We now demonstrate that DIC sensitizes EMT6 cells to two MC analogues, porfiromycin (POR) and the 7-N-dimethylaminomethylene analogue of mitomycin C (BMY-25282), in hypoxia and protects cells from these agents in air, despite the fact that POR is preferentially toxic to hypoxic cells and BMY-25282 is preferentially toxic to aerobic cells. In contrast, DIC increases menadione cytotoxicity in both air and hypoxia and has no effect on the cytotoxicity of Adriamycin. We have also shown previously that the preferential toxicity of POR to hypoxic cells is associated with an increased rate of drug uptake. In the present study, DIC had no measurable effect on the uptake of [3H]POR but increased the amount of efflux of this agent. MC-induced DNA cross-links, which have been proposed as the lesions responsible for the lethality of MC, are decreased by DIC in air and increased by DIC in hypoxia, in concert with the observed modifications of MC cytotoxicity by DIC. However, in aerobic cells treated with DIC and MC, the decrease in DNA interstrand cross-links is not directly associated with a decrease in cytotoxicity. L1210 cells, which have no measurable quinone reductase activity, demonstrate increased toxicity when treated with DIC and MC in hypoxia, as observed with EMT6 cells. Unlike EMT6 cells, however, L1210 cells are not protected by DIC from MC toxicity in air. Taken together, these findings suggest that DIC is altering the intracellular metabolism of MC and that quinone reductase or another, unidentified, enzyme sensitive to DIC may be involved in activating MC to a toxic product in aerobic EMT6 cells.

INTRODUCTION

MC,1 a bioreductive alkylating agent that is preferentially toxic to hypoxic cells (1-3), is bioactivated through a one- or two-electron reduction of its quinone moiety to a semiquinone and/or a hydroquinone. Two enzymes that have been extensively investigated as possible catalysts for the reductive activation of the quinone nucleus are NADPH-cytochrome c reductase (EC 1.6.99.2) and quinone reductase [NAD(P)H-quinone oxidoreductase, EC 1.6.99.2]; both have been shown to be capable of reducing MC (3-6). We have demonstrated previously that EMT6 mouse mammary tumor cells contain both of these enzymatic activities (3) and that concomitant treatment with DIC, an inhibitor of quinone reductase (7), increases the number of reactive metabolites of MC generated in these cells under hypoxic conditions (3). Treatment with DIC produces a corresponding increase in the toxicity of MC to hypoxic cells (8, 9). In contrast, DIC protects well oxygenated cells from MC toxicity (8, 9). These studies demonstrate that DIC increases the preferential toxicity of MC to hypoxic cells and led us to speculate that MC might undergo different metabolic alterations in air and in hypoxia. It appeared reasonable to speculate that quinone reductase might protect hypoxic cells from the toxicity of MC by metabolizing MC to a nontoxic product, in a process analogous to that demonstrated for other quinones in aerobic cells (10-12), and that some unidentified enzyme inhibited by DIC activates MC to a toxic product in air.

Recent studies conducted in our laboratories with POR (the N-methylaziridinyl analogue of MC) indicate that the different cytotoxicities of POR in air and in hypoxia are associated with differences in the rates of uptake of the drug under the different conditions of oxygenation (13), with a faster rate of uptake and greater cytotoxicity occurring in hypoxia. After entry of the drugs into cells, MC and POR are activated to reactive species capable of cross-linking DNA (14-17); DNA cross-links are believed to be the lesions causing cytotoxicity. Any of these processes (drug uptake, metabolism, or formation or processing of cross-links) could be modulated directly or indirectly by DIC. The data presented in this report may assist in defining the mechanism by which DIC influences the cytotoxicity of MC.

MATERIALS AND METHODS

MC, POR, BMY-25282, and [3H]POR (36 mCi/mmol) were gifts from Dr. T. W. Doyle and Dr. D. M. Vyas of the Bristol-Myers Company (Wallington, CT). Adriamycin was provided by the Division of Cancer Treatment of the National Cancer Institute (Bethesda, MD). All other materials were purchased from commercial sources: DIC and menadione (Sigma Chemical Company, St. Louis, MO), Ultrafluor (National Diagnostics, Manville, NJ), proteinase K (E. Merck, Darmstadt, West Germany), [3-14C]thymidine and [5-3H]thymidine (55 Ci/mmol, respectively; Amersham Corporation, Arlington Heights, IL). EMT6 murine mammary tumor cells (subline EMT6-Rw) and L1210 murine leukemia cells were maintained as monolayers in Waymouth's medium plus 15% FBS and as suspension cultures in Fisher's medium plus 2 mM glutamine and 10% FBS, respectively, at 37°C in an atmosphere of 95% air/5% CO2 (18, 19). Cytotoxicity studies were performed using exponentially growing cells in glass bottles. Hypoxia was produced by gassing the cultures with 95% N2/5% CO2 for 2 h before addition of drug, as described previously (3). Aerobic cultures were gassed similarly with 95% air/5% CO2. Cultures were treated for 1 h with drug, drug plus DIC, or appropriate vehicle (0.5 M NaOH for DIC, dimethyl sulfoxide for menadione, or 70% ethanol for all other drugs) without interruption of the gas flow. EMT6 cell survival was assayed by colony formation on tissue culture dishes (18). L1210 cell survival was measured by colony formation in 0.3% agar (20). For both tumor lines, cells were incubated at 37°C for 10 days in 93% air/7% CO2. Colonies of L1210 cells were counted without staining, using a dissecting microscope.

The uptake and efflux of [3H]POR were measured as described in detail elsewhere (13), using suspensions of EMT6 cells in Waymouth's medium plus 15% FBS. Cells were prevented from attaching to the glass surface of the bottles used in these experiments by vigorous shaking. Cells were gassed and treated with drug or vehicle exactly as described for cell survival studies. At various times thereafter, aliquots

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3 The abbreviations used are: MC, mitomycin C; POR, porfiromycin; DIC, dicoumarol; FBS, fetal bovine serum.
Table 1 Effect of DIC on the toxicity of quinones to aerobic and hypoxic EMT6 cells

<table>
<thead>
<tr>
<th></th>
<th>MC*</th>
<th>POR</th>
<th>BMY-25282</th>
<th>Menadione</th>
<th>Adriamycin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Air</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Drug concentration (µM)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Drug alone</td>
<td>1.5</td>
<td>5</td>
<td>0.15</td>
<td>0.1</td>
<td>8</td>
</tr>
<tr>
<td>Drug + 100 µM DIC</td>
<td>0.095</td>
<td>0.18</td>
<td>0.017</td>
<td>0.33</td>
<td>0.067</td>
</tr>
<tr>
<td></td>
<td>(0.13-0.25)</td>
<td>(0.0073-0.038)</td>
<td>(0.29-0.37)</td>
<td>(0.035-0.13)</td>
<td></td>
</tr>
<tr>
<td>Hypoxia</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Drug concentration (µM)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Drug alone</td>
<td>1.5</td>
<td>1.5</td>
<td>0.4</td>
<td>0.5</td>
<td>8</td>
</tr>
<tr>
<td>Drug + 100 µM DIC</td>
<td>0.02</td>
<td>0.089</td>
<td>0.24</td>
<td>0.89</td>
<td>0.18</td>
</tr>
<tr>
<td></td>
<td>(0.060-0.13)</td>
<td>(0.19-0.30)</td>
<td>(0.83-0.97)</td>
<td>(0.13-0.24)</td>
<td></td>
</tr>
</tbody>
</table>

* Data in this column are reprinted from Ref. 8.

of the cell suspension were removed and cell-associated drug was separated from the drug remaining in the external medium by centrifugation through oil (silicone:mineral oil, 84:16, v/v) into 0.5 N perchloric acid. The amount of radiolabel in the pelleted cells was assessed by counting the perchloric acid layer in Ultrafluor. For technical reasons, uptake and efflux were measured using cultured cells in suspension. We have shown previously that the cytotoxicity of POR was similar for EMT6 cells in suspension and in monolayer cultures (13).

For measurements of drug-induced DNA cross-links and single-strand breaks, EMT6 cells were seeded at 5 x 10^5 cells/bottle in glass bottles that contained 10 ml of Waymouth's medium plus 15% FBS. After 24 h of incubation at 37°C in 95% air/5% CO_2, cells were labeled overnight with [1^4C]thymidine or [3H]thymidine and were then gassed and treated with drug or vehicle as described for cell survival studies. Total DNA cross-links and single-strand breaks were measured by alkaline elution, following exactly the procedure of Kohn et al. (21).

RESULTS

DIC increased the toxicity of MC and MC analogues (POR and BMY-25282) to EMT6 cells in hypoxia and decreased the toxicity of MC, POR, and BMY-25282 to these cells in air (Table 1). In contrast, DIC increased the cytotoxicity of menadione both in air and in hypoxia and had no effect on the cytotoxicity of Adriamycin under either condition of oxygenation (Table 1). DIC, therefore, had very different effects on the cytotoxicities of these different classes of quinones. Concentrations of the quinones were selected initially to give surviving fractions of approximately 0.1. It was not possible to use these levels in all cases. For instance, the surviving fraction of cells treated with DIC and an IC_50 concentration of menadione was immeasurably low. The data summarized in Table 1 are the partial results of many studies using different concentrations of quinones and/or DIC. The results of these other studies differ only quantitatively from those presented in Table 1.

We have shown previously that the preferential toxicity of POR to hypoxic cells is associated with an increased rate and extent of drug uptake (13). This can also be seen in the present experiments, by comparing the results shown in Fig. 1 (which were performed using 20 µM POR in air) with those shown in Fig. 2 (performed using 3 µM POR in hypoxia). These concentrations were chosen because they produce a similar degree of cytotoxicity.

In air, DIC had no effect on the rate of uptake of [3H]POR into EMT6 cells (Fig. 1). DIC, however, increased the extent of drug efflux at DIC concentrations of 100 µM (Fig. 1) and 300 µM (data not shown). In hypoxia, DIC at 100 µM may have increased slightly the uptake of [3H]POR into EMT6 cells (Fig. 2), but the uptake process was not increased further with higher concentrations of DIC (data not shown). DIC increased both the rate and the extent of POR efflux in hypoxia.
The cytotoxicities of the mitomycin antibiotics are thought to be associated with the formation of DNA-DNA cross-links (15–17). In hypoxic cells, MC-induced DNA cross-links (Fig. 3) and MC-induced cytotoxicity (9) both increased progressively with increasing DIC concentrations over the range of 100 to 1000 μM. In air, the formation of MC-induced cross-links decreased to a value indistinguishable from zero as the concentration of DIC was raised from 30 to 100 μM (Fig. 3), while the cytotoxicity of MC decreased as the concentration of DIC was raised from 100 to 300 μM (9). Single-strand breaks were not detectable when cells were treated with MC plus 100 μM DIC (data not shown). At these drug concentrations, no DNA cross-links were detected (Fig. 3), but MC was still cytotoxic (9). DIC alone was not cytotoxic and did not form cross-links or single-strand breaks under the conditions used in these experiments.

DIC is an inhibitor of quinone reductase, an enzyme which metabolizes quinones by a two-electron transfer mechanism (7). To gain additional information on the role of quinone reductase in influencing the toxicity of MC to neoplastic cells, the effect of DIC on the cytotoxicity of MC was evaluated in L1210 cells, which contain no measurable quinone reductase activity (19, 22). As shown in Table 2, DIC significantly increased the toxicity of MC to L1210 cells in hypoxia. This result was similar to that obtained with EMT6 cells. In air, however, DIC increased slightly the toxicity of MC to L1210 cells (Table 2); this finding contrasted with the protective effect of DIC in aerobic EMT6 cells treated with MC (Table 1) (9).

Table 2 Effect of DIC on the cytotoxicity of MC to aerobic and hypoxic L1210 cells

<table>
<thead>
<tr>
<th>DIC alone</th>
<th>MC alone</th>
<th>MC + DIC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Air</td>
<td>Hypoxia</td>
<td></td>
</tr>
<tr>
<td>Surviving fraction</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.01 (0.07–1.26)</td>
<td>0.76 (0.73–0.79)</td>
<td>0.040 (0.28–0.058)</td>
</tr>
</tbody>
</table>

DISCUSSION

During investigations using the prototypical bioreductive alkylating agent MC, we discovered that DIC, an inhibitor of quinone reductase (7), increased the toxicity of MC to hypoxic cells while decreasing the toxicity of this agent to aerobic cells (8). Studies in animals, examining the clinical potential of this phenomenon, revealed that regimens combining X-rays with DIC and MC produced supraadditive cytotoxicities to the cells of solid EMT6 mammary tumors and produced a small increase in the therapeutic ratio over that obtained with MC and X-rays without DIC (8). To elucidate the biochemical basis for this phenomenon, we have been evaluating the role of quinone reductase in the intracellular metabolism of MC.

For a number of years, it has been presumed that quinone reductase protects cells from the injury caused by the reduction of quinones to corresponding semiquinone radicals by flavoproteins such as NADPH-cytochrome c reductase (10–12). This assumption is based largely on observations of increased toxicities when cells were exposed to quinones simultaneously with DIC (10–12, 22–24). In contrast, Talcott et al. (25) reported that DIC decreased the toxicity of 3-bromomethylmenadione in Salmonella typhimurium, while Akman et al. (22) showed that DIC increased the toxicity of menadione in aerobic L1210 cells, which have no measurable quinone reductase activity (19, 22).

As our first approach to examining the role of quinone reductase in MC cytotoxicity, we examined quinones (Adriamycin and menadione) with known interactions with quinone reductase and compared the results obtained with these quinones to those obtained with MC, POR, and BMY-25282. The toxicities of both of the MC analogues were modulated by DIC in the same manner as for MC, even though BMY-25282 is preferentially toxic to aerobic cells while POR and MC are both preferentially toxic to hypoxic cells (Table 1) (26–28). In contrast, DIC increased the toxicity of menadione to both aerobic and hypoxic EMT6 cells and had no effect on the toxicity of Adriamycin under either condition (Table 1).

In L1210 cells, which have NADPH-cytochrome c reductase activity but no measurable quinone reductase activity (19, 22), DIC increased the toxicity of MC both in air and in hypoxia (Table 2). The effects of DIC on L1210 cells in hypoxia were similar to those with EMT6 cells but in air were the opposite of those obtained with EMT6 cells. These results suggest that, if quinone reductase is involved in the metabolism of MC by EMT6 cells, it must activate MC to a cytotoxic species under aerobic conditions. This hypothesis is also supported by the fact that MC is similarly toxic to EMT6 and L1210 cells in hypoxia but is much less toxic to L1210 cells than to EMT6 cells in air (Tables 1 and 2).

To further assess the effects of DIC on the intracellular metabolism of MC, we examined interactions of MC with DIC in aerobic and hypoxic EMT6 cells and in aerobic and hypoxic LI210 cells, as well as in L1210 cells. The results obtained with these quinones to those obtained with MC, POR, and BMY-25282. The toxicities of both of the MC analogues were modulated by DIC in the same manner as for MC, even though BMY-25282 is preferentially toxic to aerobic cells while POR and MC are both preferentially toxic to hypoxic cells (Table 1) (26–28). In contrast, DIC increased the toxicity of menadione to both aerobic and hypoxic EMT6 cells and had no effect on the toxicity of Adriamycin under either condition (Table 1).
metabolism of MC, we examined the effects of DIC on drug uptake and on drug-induced DNA cross-links. DIC had little or no effect on the uptake of [3H]POR by aerobic or hypoxic EMT6 cells but increased [3H]POR efflux under both conditions of oxygenation (Figs. 1 and 2). Our previous studies indicated that the rate of uptake is a critical determinant of POR cytotoxicity when POR is used as a single agent (13). When examining the formation of MC-related DNA cross-links, we found that in hypoxia DIC modulated the formation of cross-links in the same manner as the cytotoxicity; i.e., DIC increased both the number of MC-induced DNA cross-links and the amount of MC-induced toxicity in hypoxic EMT6 cells (Table 1; Fig. 3). In air, the dose-response curve for the decrease in MC-induced cross-links by DIC differed from the dose-response curve for the decrease in MC-induced cytotoxicity by DIC, indicating that cross-links and cytotoxicity are not synonymous events in aerobic cells. These findings support the concept that DIC modulates the intracellular metabolism of MC after drug uptake but before the formation of the DNA cross-links, which are the putative cytotoxic lesions. Moreover, these results, like those of others (29), suggest that, at least in aerobic cells, there may be another cytotoxic lesion in addition to DNA cross-links. The additional lesion does not appear to be DNA single-strand breaks, as single-strand breaks were not observed, even when DIC essentially abolished DNA cross-linking. Cytotoxicity was observed under these conditions, suggesting the occurrence of cytotoxic lesions other than single-strand breaks or cross-links.

In summary, our data indicate that DT-diaphorase may be involved in the aerobic bioactivation of MC to a toxic species when this enzyme is present in the cell. However, DT-diaphorase does not appear to be the DIC-inhibited enzyme involved in protecting cells from the toxic products produced from MC in hypoxia. The bioactivation of MC to toxic products involves multiple mechanisms, which may differ with the oxygenation status of the cells. In addition, modulation of the cytotoxicity of MC by DIC appears to involve intracellular events which precede the formation of the lethal drug-induced DNA lesions.

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REFERENCES

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