Activity of Quinone Alkylating Agents in Quinone-resistant Cells

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ABSTRACT

The role of the quinone group in the antitumor activity of quinone alkylating agents, such as mitomycin C and 2,5-diaziridinyl-3,5-bis(carboethoxymino)-1,4-benzoquinone, is still uncertain. The quinone group may contribute to antitumor activity by inducing DNA strand breaks through the formation of free radicals and/or by influencing the alkylating activity of the quinone alkylators. The cytotoxic activity and DNA-DNA cross-linking by the model quinone alkylating agents, benzoquinone mustard and benzoquinone dimustard, were compared in L5178Y murine lymphoblasts sensitive and resistant to the model quinone antitumor agent, hydroxylated benzoquinone mustard. The resistant cell lines, L5178Y/HBM2 and L5178Y/HBM10, have increased concentrations of glutathione and elevated catalase, glutathione S-transferase, and DT-diaphorase activity. L5178Y/HBM2 and L5178Y/HBM10 cells had 7.4- and 8.5-fold less sensitive to benzoquinone mustard and 1.7- and 4.3-fold less sensitive to benzoquinone dimustard, respectively, compared with sensitive cells, but showed no resistance to the non-quinone alkylating agent, aniline mustard. The formation of DNA double-strand breaks by benzoquinone mustard was reduced by 2- and 8-fold in L5178Y/HBM2 and L5178Y/HBM10 cells, respectively, while double-strand break formation by benzoquinone dimustard was reduced only in the L5178Y/HBM10 cells. The number of DNA-DNA cross-links produced by benzoquinone mustard was 3- and 6-fold lower, and the number produced by benzoquinone dimustard was 35% and 2-fold lower in L5178Y/HBM2 and L5178Y/HBM10 cells, respectively, compared with L5178Y parental cells. In contrast, cross-linking by aniline mustard was unchanged in sensitive and resistant cells. Dicoumarol, an inhibitor of DT-diaphorase, increased the cytotoxic activity of both benzoquinone mustard and benzoquinone dimustard in L5178Y/HBM10 cells. This study provides evidence that elevated DT-diaphorase activity in the resistant cells contributes to resistance to benzoquinone mustard and benzoquinone dimustard, possibly by decreasing the formation of the semiquinone intermediates of these agents. The altered reduction of the quinone groups in the resistant cells may be responsible for the decreased DNA-DNA cross-linking and lowered induction of DNA strand breaks by the quinone alkylating agents. These findings demonstrate that the quinone group may modulate the activity of quinone alkylating agents. The study also suggests that the semiquinone intermediates of benzoquinone mustard and benzoquinone dimustard may be the active alkylating species of these two agents.

INTRODUCTION

Antitumor agents such as MMC, AZQ, and triazinequinox contain both a quinone ring and alkylating groups in their structure. It has been suggested that the quinone group may play a role in the cytotoxic activity of these agents. Reduction-oxidation reactions of the quinone group may lead to the formation of oxygen free radicals which can induce DNA damage (1), and this has been observed with MMC (2, 3) and AZQ (4). Additionally, the quinone group may influence the alkylating activity of these agents. Studies have shown that the alkylating activity of MMC is dependent on reduction of the quinone group (2, 5-9). Similarly, AZQ was shown to produce DNA cross-links only after reduction (4, 10).

We have previously studied the activity of model quinone alkylating agents in L5178Y lymphoblasts. BM and BDM showed significantly greater potency in L5178Y cells compared with the nonquinone alkylating agent, AM (11, 12). The quinone agents induced both DNA strand breaks and DNA-DNA cross-links in L5178Y cells, and both their cytotoxic activity and strand break activity could be partially inhibited by the protective enzyme, catalase (11-13). Studies with these model compounds in L5178Y cells resistant to alkylating agents also indicated a significant role for DNA-DNA cross-linking in the cytotoxic activity of BM and BDM (14).

We have isolated and characterized L5178Y cell lines resistant to the nonalkylating quinone model compound, HBM (15). Resistance in these cell lines appeared to be multifactorial. L5178Y/HBM2 cells, which were 2.5-fold resistant to HBM, showed reduced drug uptake, had slightly elevated levels of superoxide dismutase and catalase activity, a 2-fold increase in intracellular glutathione, and a 3-fold increase in GST and DT-diaphorase activity compared with L5178Y sensitive cells. L5178Y/HBM10 cells, which were 6-fold resistant to HBM compared with sensitive cells, showed reduced drug uptake and similar increases in superoxide dismutase activity and glutathione levels. However, the L5178Y/HBM10 cells also had a 3-fold increase in catalase activity, an 11-fold increase in GST activity, and a 24-fold increase in DT-diaphorase activity. HBM produced significantly reduced levels of DNA strand breaks in the resistant cells.

In the present study we have examined the activity of the model quinone alkylating agents, BM and BDM, in the quinone-resistant cell lines in order to evaluate the role of the quinone group in the cytotoxic activity of these agents.

MATERIALS AND METHODS

Materials. Benzoquinone mustard [di(2'-chloroethyl)amino-1,4-benzoquinone], benzoquinone dimustard [2,5-bis(di(2'-chloroethyl)amino)-1,4-benzoquinone], aniline mustard [N,N-di(2'-chloroethyl)aniline], and hydroxylated benzoquinone mustard [di(2'-hydroxyethyl)amino-1,4-benzoquinone] were prepared as described previously (11, 13). Fischer's medium and horse serum were obtained from Grand Island Biological Co., Grand Island, NY. [3H]Thymidine (specific activity, 50 mCi/mmol) and [3H]histidine (specific activity, 50 to 80 Ci/mmol) were obtained from Du Pont-New England Nuclear, Boston, MA. Proteinase K was from E. Merck, Darmstadt, Federal Republic of Germany; tetrapropylammonium hydroxide was from Eastman Kodak Co., Rochester, NY; polycarbonate filters (0.8 f and 2.0 T) were from Nucleopore Corp., Pleasanton, CA; and dicoumarol was from Sigma Chemical Co., St. Louis, MO.

Cell Lines. The L5178Y, L5178Y/HBM2, and L5178Y/HBM10 cell lines used in this study have been described previously (15). L5178Y cells were grown in Fischer's medium containing 12% horse serum, L5178Y/HBM2 cells were grown in Fischer's medium containing 12% horse serum and 0.2 mm HBM, and L5178Y/HBM10 cells were grown in Fischer's medium containing 12% horse serum and 1.0 mm HBM.

Cytotoxicity Assays. L5178Y parental or resistant cells were incubated with [3H]thymidine (10^6 cells/ml) in the presence of various concentrations of the alkylating agents (15). Cell survival was determined by counting the surviving plated cells.

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3 The abbreviations used are: MMC, mitomycin C; AZQ, 2,5-diaziridinyl-3,6-bis(carboethoxymino)-1,4-benzoquinone; BM, benzoquinone mustard; BDM, benzoquinone dimustard; AM, aniline mustard; HBM, hydroxylated benzoquinone mustard; GST, glutathione S-transferase; Dm, concentration of drug reducing the surviving cell fraction to 0.1.

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bated with different concentrations of the model antitumor agents for 1 h at 37°C in medium containing horse serum. Cytotoxic activity in the parental and resistant cell lines was determined by a soft agar cloning assay, as described previously (11, 16). Cloning efficiencies ranged from 35 to 66% for L5178Y parental cells, from 51 to 90% for L5178Y/HBM2 cells, and from 37 to 80% for L5178Y/HBM10 cells. A linear regression analysis of each concentration-survival curve was obtained, and the $D_{10}$ was derived from the negative reciprocal of the regression slope as previously described (17). The degree of resistance of the L5178Y/HBM2 and L5178Y/HBM10 cells was determined from the ratio of the $D_{10}$ value in the resistant cells to the $D_{10}$ in the parental cells. The cytotoxicity of each agent in the sensitive and resistant cell lines was compared statistically by a $t$ test comparing the significance of the differences of the slopes of the concentration-survival curves.

For inhibition studies with dicoumarol, L5178Y or L5178Y/HBM10 cells were pretreated with or without 100 $\mu M$ dicoumarol for 15 min. L5178Y cells were then treated with either 30 $nM$ BM or 3 $\mu M$ BDM, while L5178Y/HBM10 cells were treated with either 0.5 $\mu M$ BM or 10 $\mu M$ BDM at 37°C for 1 h. Cytotoxicity was determined by clonogenic assay. The results were analyzed statistically by a two-tailed $t$ test comparing the significance of the difference of the mean surviving cell fraction in the absence or presence of dicoumarol. The concentration of dicoumarol used in this study was not toxic to the cells.

Determination of DNA Double Strand Breaks. Cells labeled with $[^{3}C]$/thymidine were treated with various concentrations of HBM, BM, or BDM for 1 h at 37°C in medium containing horse serum. DNA double-strand breaks were measured using an elution assay as described previously (13, 18, 19). The level of DNA strand breaks was calculated from the elution profiles and was expressed as rad equivalents (dose of radiation inducing an equivalent number of breaks) as determined from calibration curves. A linear regression analysis of each concentration-activity curve was obtained, and the strand-breaking activity of each agent in the sensitive and resistant cell lines was compared statistically by a $t$ test comparing the significance of the difference of the slopes of the concentration-activity curves.

Determination of DNA-DNA Cross-Linking. Cells labeled with $[^{3}C]$/thymidine were treated with various concentrations of BM, BDM, or AM for 1 h at 37°C in medium containing horse serum. DNA-DNA cross-links were measured using an elution assay as described previously (13, 20). The level of cross-linking was calculated as described by Kohn et al. (20). A linear regression analysis of each concentration-activity curve was obtained, and the cross-linking activity of each agent in the sensitive and resistant cell lines was compared statistically by a $t$ test comparing the significance of the difference of the slopes of the concentration-activity curves.

RESULTS

Cytotoxicity of Model Compounds in L5178Y Sensitive and Resistant Cells. The cytotoxic activities of the model antitumor agents, HBM, BM, BDM, and AM, in parental L5178Y cells and in the resistant cells, L5178Y/HBM2 and L5178Y/HBM10, were determined by a soft agar cloning assay after treatment of cells with drug for 1 h at 37°C (Fig. 1). A comparison of the $D_{10}$ values (Table 1) shows that the L5178Y/HBM2 and L5178Y/HBM10 cells were 2.5- and 6.2-fold resistant to HBM, 7.4- and 8.5-fold resistant to BM, and 1.7- and 4.3-fold resistant to BDM, respectively, compared with parental L5178Y cells. In contrast, the resistant cells showed no cross-resistance to the nonquinone model agent, AM.

Effect of Dicoumarol on the Cytotoxicity of Model Compounds in L5178Y Sensitive and Resistant Cells. L5178Y/HBM10 cells were treated with 0.5 $\mu M$ BM or 10 $\mu M$ BDM in the presence or absence of 100 $\mu M$ dicoumarol. The presence of dicoumarol significantly increased the cytotoxicity of both BM and BDM in L5178Y/HBM10 cells (Fig. 2), but the level of resistance to these agents was only partially reversed. L5178Y cells were treated with 30 $nM$ BM or 3 $\mu M$ BDM in the presence or absence of 100 $\mu M$ dicoumarol. Although there was a slight increase in the cytotoxicity of both BM and BDM in the presence of dicoumarol, this effect was not statistically significant. The concentration of BM and BDM used in these studies produced similar levels of cell kill in L5178Y and L5178Y/HBM10 cells in the absence of dicoumarol.

Induction of DNA Double Strand Breaks by Quinone Model Compounds in L5178Y Sensitive and Resistant Cells. L5178Y sensitive and resistant cells were incubated with various concentrations of HBM, BM, or BDM at 37°C for 1 h. The number of DNA double strand breaks induced in the L5178Y, L5178Y/HBM2, and L5178Y/HBM10 cells was determined by elution assay (Fig. 3). The induction of double strand breaks by HBM was reduced by 2-fold in L5178Y/HBM2 cells and by 30-fold in L5178Y/HBM10 cells compared with the level found in the sensitive cells, and the latter effect was statistically significant ($P < 0.01$). With BM, the level of DNA double strand breaks was reduced by 2-fold and 8-fold in L5178Y/HBM2 and L5178Y/HBM10 cells, respectively, compared with parental cells, and again the latter result was statistically significant. However, DNA double strand break formation by BDM was not significantly different in L5178Y and L5178Y/HBM2 cells, but was significantly reduced by only 40% to minimum detectable levels in L5178Y/HBM10 cells ($P < 0.01$).

Production of DNA-DNA Cross-Links by Model Alkylating Agents in L5178Y Sensitive and Resistant Cells. L5178Y sensitive and resistant cells were treated with various concentrations of BM, BDM, or AM at 37°C for 1 h, and the number of DNA-DNA cross-links produced in the L5178Y, L5178Y/HBM2, and L5178Y/HBM10 cells was determined by elution assay (Fig. 4). The production of cross-links by BM was reduced by 3-fold and 6-fold in L5178Y/HBM2 and L5178Y/HBM10 cells, respectively, compared with sensitive cells, while cross-link formation by BDM was 35% and 2-fold lower in these cells, respectively, compared with L5178Y cells. These effects were statistically significant ($P < 0.01$). In contrast, the number of cross-links produced by AM in sensitive and resistant cells was not significantly different.

DISCUSSION

The quinone ring may play an important role in the cytotoxic activity of a number of antitumor agents. Reduction-oxidation reactions of the quinone group can lead to the formation of oxygen free radicals and active oxygen species (1) which may contribute to the antitumor activity (3, 21) and cardiotoxicity of the anthracyclines (22-25) and to the antitumor action of MMC (2, 3), AZQ (4), and streptonigrin (26). In addition, the quinone group may influence the alkylating activity of alkylating quinones, such as MMC (2, 5-9) and AZQ (4, 10), which are activated by reduction of the quinone. In previous studies with model compounds we have shown that the model quinone alkylating agents, BM and BDM, show increased cytotoxicity and cross-linking activity in L5178Y lymphoblasts compared with a nonquinone alkylating agent, AM, and increased cytotoxicity and DNA double strand break formation compared with HBM (12). Additional work in alkylator-resistant cells indicated an important role for cross-linking in the cytotoxic activity of BM and BDM and suggested that the enhanced DNA strand break formation by BM and BDM may be due to the ability of these agents to generate free radicals close to their target by binding to DNA (14).

We have isolated and characterized L5178Y cell lines resist-
HBM10 cells are 6-fold resistant to this nonalkylating quinone compared with parental cells. These cells may be resistant to HBM because of reduced induction of DNA strand breaks resulting from decreased formation and/or increased inactivation of free radicals. Decreased formation of free radicals may be due to reduced drug uptake or increased drug inactivation and extrusion resulting from elevated levels of glutathione and GST activity. The present study provides evidence that the quinone group plays an important role in the cytotoxic activity of quinone alkylating agents. The quinone-resistant cell lines showed cross-resistance to the quinone alkylating agents, BM and BDM, but not to the nonquinone alkylating agent, AM. Since the L5178Y/HBM2 and L5178Y/HBM10 cells did not demonstrate any resistance to AM despite having elevated concentrations of glutathione and GST activity, it would appear that this detoxification mechanism did not play a major role in resistance to alkylating agents in these cells.

Resistance mechanisms which can reduce the formation of free radicals or inactivate these reactive species may contribute to the resistance to BM and BDM. These include decreased drug uptake and elevated GST, catalase, and superoxide dismutase activity; and increased levels of glutathione. This suggestion was supported by the finding that the formation of DNA double strand breaks by BM and BDM was reduced in L5178Y/HBM10 cells. However, the induction of double strand breaks by these agents is not significantly reduced in L5178Y/HBM2 cells, and the reduced formation of double strand breaks did not correlate with the increased resistance of L5178Y/HBM2 cells to BM and BDM.
not correspond closely with the reduced sensitivity of the cells to the antitumor agents. Thus, other mechanisms must also contribute to resistance to BM and BDM in these cells.

There was a significant decrease in the production of DNA-DNA cross-links by BM and BDM in the L5178Y/HBM2 and L5178Y/HBM10 cells, but cross-linking by AM was unchanged in these cells compared with parental L5178Y cells. This finding suggests that the decreased alkylating activity of the quinone agents was not due to a direct effect on the nitrogen mustard alkylating group. It has been shown previously that reduction of the quinone group is required to activate the alkylating activity of bioreductive alkylating agents, such as MMC and AZQ (2, 8-10). In a similar manner, reduction of the quinone group may enhance the cross-linking activity of BM and BDM. Thus, mechanisms of resistance effecting reduction of the quinone group may be responsible for the reduced cross-linking observed in the L5178Y/HBM2 and L5178Y/HBM10 cells. However, cytochrome P-450 reductase activity, which is responsible for the one electron reduction of quinone groups (29), was unchanged in the resistant cells (15). In contrast, DT-diaphorase activity, which produces a two electron reduction of the quinone directly to the hydroquinone (28), was 3- and 24-fold elevated in L5178Y/HBM2 and L5178Y/HBM10 cells (15), respectively, compared with parental cells. The importance of DT-diaphorase in the mechanisms of resistance to the model quinone agents in these cells was further supported by the ability of dicoumarol, an inhibitor of DT-diaphorase activity, to increase the cytotoxic activity of BM and BDM in the resistant cells. The elevated DT-diaphorase activity in the resistant cells may result in increased reduction of BM and BDM directly to their hydroquinone forms without formation of semiquinone intermediates. These findings suggest that the semiquinone intermediates, obtained by one electron reduction of BM and BDM, may be responsible for the cytotoxic activity of these antitumor agents. If semquinones are the active alkylating species of BM and BDM and are responsible for the production of the DNA strand breaks by induction of oxygen free radicals, then elevated levels of DT-diaphorase activity in the resistant cell lines could decrease the formation of the semiquinones and could, at least in part, account for the reduced levels of cross-linking, double strand break formation, and cytotoxicity of the quinone alkylating agents in these cells.

These findings contrast with results obtained with other quinone alkylating agents. We have found that MMC produced...
increased cytotoxicity and DNA cross-linking in L5178Y/HBM10 cells compared with parental L5178Y cells. Both of these activities in L5178Y/HBM10 cells were decreased by the addition of dicoumarol (30). Similarly, Siegel et al. (31) have shown that AZQ was more cytotoxic to HT-29 human colon carcinoma cells compared with BE human colon carcinoma cells in vitro. HT-29 cells have approximately 140-fold greater alkalizing and cytotoxic activity of MMC and AZQ, resulting from two electron reduction of the quinone groups catalyzed by DT-diaphorase, may contribute to the alkylating and cytotoxic activity of MMC and AZQ.

In summary, we have shown that L5178Y lymphoblasts resistant to the model quinone antitumor agent, HBM, are cross-resistant to the quinone alkylating agents, BM and BDM, but not to the nonquinone alkylating agent, AM. Elevated levels of DT-diaphorase activity in the resistant cells contribute to resistance to BM and BDM, possibly by decreasing the formation of the semiquinone intermediates of these agents. The altered reduction of the quinone group may result in decreased DNA-DNA cross-linking and lowered induction of DNA strand breaks. Thus, the quinone group can modulate the activity of quinone alkylating agents. These studies also suggest that the semiquinone intermediates of BM and BDM may be the active alkylating species of these two agents.

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