Cytocidal Action of the Quinone Group and Its Relationship to Antitumor Activity

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ABSTRACT

Many clinically useful antitumor agents have a quinone group in their structure. A series of model compounds has been studied to determine if the quinone group is involved in the cytotoxic activity of these agents and to investigate the mechanism of this action. Hydrolyzed benzoquinone mustard, which contains a quinone group, produced significant cell kill of L5178Y lymphoblasts, whereas hydrolyzed aniline mustard, which has a similar chemical structure but contains no active functional groups, showed little activity against this cell line. Benzoquinone mustard, which possesses both a quinone moiety and an active alkylating group, was approximately 30,000 times more active against L5178Y cells than was hydrolyzed benzoquinone mustard. It was approximately 600 times more active than aniline mustard, a compound which has the same alkylating group but no quinone function, and 200 times more active than an equimolar combination of aniline mustard and hydrolyzed benzoquinone mustard. The cytotoxic activity of hydrolyzed benzoquinone mustard was inhibited by either superoxide dismutase or catalase, while catalase but not superoxide dismutase inhibited the activity of benzoquinone mustard. Neither enzyme had any effect on the cytotoxic action of aniline mustard. These studies suggest that the presence of a quinone group in the chemical structure of a compound can result in significant cell kill by a mechanism that appears to involve free radicals and active oxygen species. In addition, if the compound contains an alkylating group which can bind to DNA, as well as a quinone moiety, the cytotoxic activity of the agent appears to be enhanced and is greater than that resulting from an additive effect of the two groups.

INTRODUCTION

Many clinically useful antitumor agents, including Adriamycin, daunorubicin, actinomycin D, mitomycin C, and teniposide, contain a quinone or quinone-like moiety in their structure. These agents are capable of interacting directly with DNA by intercalation (10) or by alkylation and cross-linking (1, 18) and have been shown to induce damage to DNA (12, 21, 26, 31, 32). The quinone group is able to undergo oxidation-reduction reactions leading to the formation of free radicals and active oxygen species such as semiquinones, superoxide radicals, hydrogen peroxide, and hydroxyl radicals (3, 18, 20, 21, 25). Bachur has suggested that "site-specific free radicals" may be formed as a result of binding of the agents to DNA and that these radicals may be responsible for the DNA damage (4). Recently, it was demonstrated that free radicals can be generated within the cell nucleus itself (2).

The exact role of the quinone-induced DNA damage is still unclear. It has been proposed that cardiotoxicity (23) and other toxic side effects (28) of the anthracyclines are due to free radical formation. The daunorubicin analogue 5-imino daunorubicin, which is less cardiotoxic than other anthracyclines (29, 34), has been shown to have a decreased ability to generate free radicals and to damage DNA (19). In addition, free radical scavengers such as α-tocopherol (15, 27, 30) and chelating agents which retard the generation of hydroxyl radical (16) were found to protect the myocardium from anthracycline-induced toxicity. In contrast, evidence has been obtained that microsomes from murine tumor cells have a higher activity than do microsomes from normal mouse spleens in the activation of quinone antitumor agents (4). Furthermore, some tumor cells have a lower oxidation-reduction potential than do normal cells (7, 8), as well as suppressed levels of the protective enzymes superoxide dismutase and catalase (17, 24). These factors may account for the therapeutic effectiveness of quinone agents.

The quinone-containing antitumor agents all have a complex chemical structure with many potentially active functional groups. Thus, it has not been possible to demonstrate that the quinone moiety is directly involved in the cytotoxic action of these agents or to evaluate its role in their therapeutic effectiveness. In this study, the cytotoxic activity of a series of model compounds has been investigated in order to determine whether: (a) the quinone moiety can produce cell kill; (b) the formation of free radicals is involved in this cell kill; and (c) the ability to interact with DNA can enhance the activity of the quinone group.

MATERIALS AND METHODS

Hydrolyzed benzoquinone mustard [di(2'-hydroxyethyl)amino-1,4-benzoquinone] was prepared as described previously (6) and purified by recrystallization from ethanol (m.p. 159–161°). Hydrolyzed aniline mustard [N-(phenyldiethanolamine) (11) was obtained from Aldrich Chemical Co., Inc., Milwaukee, Wis., and was purified by recrystallization from ether (m.p. 56–58°). Benzoquinone mustard [di(2’-chloroethyl)amino-1,4-benzoquinone] was prepared as described previously (22) and was recrystallized from methanol (m.p. 112–113°). Aniline mustard [N,N-di(2’-chloroethyl)aniline] was prepared by a known procedure (11) and recrystallized from methanol (m.p. 41.5–43°). Superoxide dismutase (specific activity, 2700 units/mg protein), catalase (specific activity, 11,000 units/mg protein), xanthine oxidase (specific activity, 4 units/mg protein), and cytochrome c were obtained from Sigma Chemical Co., St. Louis, Mo.

The L5178Y lymphoma used in this study was obtained from Dr. G. J. Goldenberg, University of Manitoba, and arose as a spontaneous neoplasm in a DBA/2 mouse (13). Cytotoxicity studies were performed on suspension cultures of L5178Y cells incubated in vitro at a cell concentration of approximately 2 X 10^6 cells/ml in Fischer’s medium with 10% horse serum. Drug, which was added to the suspension in dimethyl sulfoxide in a 1:100 dilution, and cells were incubated at 37° for 1 hr. The cells were washed twice with cold medium, counted with an electronic particle counter (Coulter Counter Model ZB, Coulter Electronics, Inc., Hialeah, Fla.), and diluted to a concentration ranging from 100 to 100,000 cells/tube with Fischer’s medium containing 15% horse serum and agar (4.4 mg/ml) in culture tubes in quadruplicate (9, 14). After incubation at 37° in a humidified atmosphere containing 6% CO_2 in air for 9 to 11 days, colonies were counted, and the surviving cell fraction was calculated. Cloning efficiency ranged from 35 to 65%. A linear regression analysis of each dose-survival curve was obtained.

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and the dose of drug required to reduce the surviving cell fraction to 0.1 was derived from the negative reciprocal of the regression slope as previously described (14).

For inhibition studies, cell suspensions were preincubated at 37° for 30 min with or without superoxide dismutase (2400 units/ml) and/or catalase (4400 units/ml) and then were treated with drug for 1 hr. The surviving cell fraction was determined by the clonogenic assay described above. The surviving cell fraction for control experiments ranged from $7 \times 10^{-3}$ to $5 \times 10^{-4}$.

To determine if the model compounds could inactivate superoxide dismutase, the enzyme at 2400 units/ml was incubated in Dulbecco's phosphate-buffered saline for 1 hr with or without 60 nm benzoquinone mustard, 30 μM aniline mustard, or 2 mM hydrolyzed benzoquinone mustard. The enzyme solution (0.5 ml) was added to a solution of xanthine (143 μM), EDTA (143 μM), and cytochrome c (29 μM) and diluted to 6 ml with Dulbecco's phosphate-buffered saline. The reaction solution (3.5 ml) was transferred to a UV cuvet, and the absorbance at 550 nm was measured using a Beckman Model DU-8 spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.). Absorbance at 550 nm was measured every min for 30 min after addition of 10 μl of xanthine oxidase (0.18 μg/ml) (5).

RESULTS

Model Compounds. The model compounds used in this study are shown in Chart 1. They all contain a bifunctional nitrogen mustard group either in an active form or in the inactive hydrolyzed form. Benzoquinone mustard contains both a quinone moiety and an active mustard group and might be expected to interact with DNA by alkylation as well as through the action of the quinone group. Aniline mustard has a nitrogen mustard group but no quinone and served as a control for the cytotoxic activity of the alkylating group. Hydrolyzed benzoquinone mustard contains only a quinone group and was used to study the cytotoxic activity of the quinone group, while hydrolyzed aniline mustard, which appears to have no active groups and would be expected to exhibit little cytotoxic activity, served as a control for any activity due to the ring structure or the hydrolyzed mustard group. Aniline mustard has been shown to be an effective antitumor agent in a number of animal tumor systems (33), presumably as a result of alkylation and cross-linking of cellular DNA, while the antitumor activity of the other model compounds has not been examined previously. Although these compounds appear to have little value as clinical agents, they have been chosen for this study because they contain few other features which might interfere with the evaluation of the cytotoxic activity of the quinone moiety.

![Chart 1](attachment:chart1.png)

Chart 1. Model compounds used in this study. Benzoquinone mustard contains both a quinone group and an active mustard moiety. Aniline mustard contains an active mustard group. Hydrolyzed benzoquinone mustard contains only an active quinone moiety, while hydrolyzed aniline mustard has no active functional groups.

Cytotoxic Activity against L5178Y Lymphoblasts in Vitro. The cytotoxicity of the model compounds against L5178Y lymphoblasts in vitro was evaluated by soft agar cloning (9), and the dose-survival curves are shown in Chart 2. Hydrolyzed benzoquinone mustard produced substantial cell kill, reducing the surviving cell fraction to approximately $10^{-3}$ at a drug concentration of 1 μM. Treatment of cells with aniline mustard resulted in a similar level of cell kill at a drug concentration of 30 μM, while benzoquinone mustard was highly cytotoxic, reducing the surviving cell fraction to $10^{-5}$ at a concentration of approximately 50 μM. In contrast, hydrolyzed aniline mustard showed little activity at concentrations of up to 50 μM.

The relative cytotoxic activity of the 4 model compounds is shown in Table 1. Benzoquinone mustard was greater than 30,000 times more effective in killing L5178Y lymphoblasts than was hydrolyzed benzoquinone mustard and greater than 600 times more effective than aniline mustard.

In order to determine if the enhanced cytotoxicity of benzoquinone mustard was due to a synergistic effect arising from the action of both a quinone group and an alkylating moiety, L5178Y cells were treated with a combination of equal con-

![Chart 2](attachment:chart2.png)

Chart 2. Dose-survival curves for L5178Y lymphoblasts treated with model compounds. Cells were incubated at 37° for 1 hr with benzoquinone mustard (C), aniline mustard (D), hydrolyzed benzoquinone mustard (O), or hydrolyzed aniline mustard (O) at the concentrations shown. The surviving cell fraction was determined by a clonogenic assay as described in the text and previously (9, 14) and is plotted against the concentration of drug used. Each point represents the mean of 5 to 14 quadruplicate determinations. Bars, S.E. On occasion, the confidence intervals are too small to be shown.

<table>
<thead>
<tr>
<th>Model compound</th>
<th>$D_{0.1}$ (μM)</th>
<th>Relative cytotoxic activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydrolyzed benzoquinone mustard</td>
<td>$5.69 \pm 0.79 \times 10^{-4}$</td>
<td>1</td>
</tr>
<tr>
<td>Hydrolyzed aniline mustard</td>
<td>$6.08 \pm 1.96 \times 10^{-4}$</td>
<td>0.01</td>
</tr>
<tr>
<td>Benzoquinone mustard</td>
<td>$1.65 \pm 0.15 \times 10^{-4}$</td>
<td>30,216</td>
</tr>
<tr>
<td>Aniline mustard</td>
<td>$1.20 \pm 0.08 \times 10^{-4}$</td>
<td>47</td>
</tr>
<tr>
<td>Aniline mustard + hydrolyzed benzoquinone mustard</td>
<td>$3.26 \pm 1.69 \times 10^{-4}$</td>
<td>171</td>
</tr>
</tbody>
</table>

* $D_{0.1}$ dose of drug required to reduce the surviving cell fraction to 0.1.
* Mean ± S.E.
centrations of aniline mustard and hydrolyzed benzoquinone mustard (Table 1) and approximated that expected for essentially no cell kill at the same drug concentrations as those used for benzoquinone mustard. The dose of drug required to reduce the surviving cell fraction to 0.1 for the drug combination was approximately 200-fold greater than that of benzoquinone mustard (Table 1) and approximated that expected for an additive effect of the 2 model compounds.

Inhibition of Cytotoxicity by Superoxide Dismutase and Catalase. The effect of the cell protective enzymes superoxide dismutase and catalase on the cytotoxic activity of the model compounds was studied (Chart 4). Cells were preincubated with superoxide dismutase (2400 units/ml) and/or catalase (4400 units/ml) for 30 min and then for an additional hr in the presence of drug. Superoxide dismutase, catalase, or both enzymes significantly increased the surviving cell fraction of cells treated with hydrolyzed benzoquinone mustard (p < 0.02 or less). In contrast, neither enzyme had any effect on the cytotoxicity of aniline mustard. The cytotoxic activity of benzoquinone mustard was significantly inhibited by catalase (p < 0.001) but not by superoxide dismutase.

Possible Inactivation of Superoxide Dismutase by Model Compounds. In order to determine if the model compounds could inactivate superoxide dismutase, the enzyme (2400 units/ml) was incubated for 1 hr at 37°C with or without 60 nM benzoquinone mustard, 30 µM aniline mustard, or 2 mM hydrolyzed benzoquinone mustard. The activity of the treated and untreated enzyme in removing superoxide radical was compared by a spectrophotometric determination of reduced cytochrome c resulting from reaction with superoxide radical (5). No effect on the activity of the superoxide dismutase was observed with any of the model compounds.

DISCUSSION

Considerable evidence has been obtained that the quinone group is involved in the activity of many antitumor agents through the generation of free radicals and active oxygen species. For the anthracyclines, this activity has been linked to cardiotoxic side effects (16, 19, 23, 27, 30), while for other agents it may play a role in antitumor activity (4). However, because of the complex structure of the drugs involved, the cytotoxic action of the quinone group has not been directly demonstrated. Using model compounds, this study has shown that the quinone group can produce cell kill by a mechanism involving free radicals and active oxygen species.

The model compounds chosen for this study contained a limited number of potentially active functional groups so that the cytotoxic effects of each group could be evaluated. Hydrolyzed benzoquinone mustard was used to measure the cytotoxic activity of the quinone group. Hydrolyzed aniline mustard served as a control for any activity due to the basic ring structure or the hydrolyzed mustard group. Benzoquinone mustard contains both a quinone moiety and an active alkylating group which is capable of binding to DNA, resulting in the formation of “site-specific free radicals” (4). This compound was studied to determine if the ability to interact directly with DNA could enhance the cytotoxicity of the quinone group.
Aniline mustard, which contains only the active mustard function, was used as a control for the cytotoxic activity of the alkylating moiety.

Dose-survival studies of cytotoxic activity by the model compounds (Chart 2) demonstrated that hydrolyzed benzoquinone mustard produced significant tumor cell kill, whereas hydrolyzed aniline mustard showed little activity. This finding strongly suggested that the presence of the quinone group can result in significantly greater cell kill ($p < 0.001$) than did an additive effect of the 2 groups in different compounds. This suggested that the ability of a quinone compound to bind to DNA may enhance the cytotoxic activity of the quinone group.

Superoxide dismutase and catalase are enzymes which protect the cell from free radical-induced damage by removing superoxide radical and hydrogen peroxide. The finding that these enzymes significantly inhibited the cytotoxic activity of hydrolyzed benzoquinone mustard (Chart 4) indicated the involvement of active oxygen species in this activity. Since oxidation-reduction reactions of the quinone group are known to affect the formation of such active oxygen species, this result provides further evidence for a direct involvement of this group in the mechanism of cell kill. In contrast, the activity of aniline mustard, which is unable to generate free radicals, was not affected by the enzymes.

The cytotoxic activity of benzoquinone mustard was significantly inhibited by catalase but not by superoxide dismutase. A study to determine if the enzyme was inactivated by benzoquinone mustard, as has been found with streptonigrin, showed that the model compound had no effect on the activity of the enzyme. Other possible explanations for the lack of effect by superoxide dismutase are that the binding of the quinone agent to DNA makes the superoxide radical generated inaccessible to the enzyme or that a different mechanism of cell kill which does not involve superoxide radical is operative.

In summary, this study demonstrated that the presence of a quinone group in the chemical structure of a compound can result in significant cell kill by a mechanism that appears to involve free radicals and active oxygen species. Furthermore, the ability of a quinone-containing agent to bind to DNA appeared to enhance its cytotoxic activity.

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REFERENCES


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