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STUDIES ON THE MECHANISM OF ACTION OF QUINONE ANTITUMOR AGENTS*

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Abstract—The presence of a quinone group in the structure of a compound has been shown to produce cell kill and DNA strand breaks by a mechanism involving free radicals and active oxygen species. The ability of the compound to bind to DNA appeared to increase the DNA damage induced and the cytotoxic activity. A new series of model compounds has been used to investigate further the role of the quinone group in the mechanism of action of quinone antitumor agents. Bis(dimethylamino)benzoquinone, which contains a quinone group, produced significant cell kill of L5178Y lymphoblasts and induced concentration-dependent single-strand and double-strand breaks in the DNA of these cells. Benzoquinone dimustard, which possesses a quinone moiety and active alkylating groups, was approximately 2500 times more cycotoxic to L5178Y cells than was bis(dimethylamino)benzoquinone and was approximately 200-fold more active in inducing DNA double-strand breaks than was the quinone agent. Benzoquinone dimustard induced no apparent DNA single-strand breaks, but produced significant DNA cross-linking, a process which interferes with the assay for single-strand breaks. The cell kill produced by both quinone agents was inhibited by catalase, but not by superoxide dismutase. The cytotoxic activity of bis(dimethylamino)benzoquinone and two other quinone model compounds, hydrolyzed benzoquinone mustard and benzoquinone mustard, appeared to correlate with the induction of DNA strand breaks, while there appeared to be no correlation between cell kill and DNA double-strand breaks induced by benzoquinone dimustard. However, the cytotoxicity of benzoquinone dimustard appeared to be related to the cross-linking activity of this agent. These studies have provided additional evidence that the presence of a quinone group in the structure of a compound can result in significant cell kill by a mechanism that appears to involve active oxygen species. Quinone containing agents can induce DNA strand breaks, and this effect is enhanced when the agent is able to bind to DNA. The induction of DNA strand breaks appeared to correlate with cytotoxic activity for bis(dimethylamino)benzoquinone, hydrolyzed benzoquinone mustard and benzoquinone mustard, but not for benzoquinone dimustard, suggesting that the contribution of quinone-induced strand breaks to the overall cytotoxicity of an agent may vary considerably.

Recent studies have shown that the presence of a quinone group in the structure of a compound can result in significant tumor cell kill [1] and the induction of DNA single- and double-strand breaks [2]. The mechanisms of both the cell kill and the DNA damage appeared to involve free radicals and active oxygen species, and both activities were enhanced when the quinone agent had the ability to bind to DNA by alkylation. Furthermore, it was suggested that a correlation might exist between the cytotoxic activity and the induction of DNA damage [2].

These studies are of interest because many clinically useful antitumor agents, such as adriamycin, daunorubicin, and mitomycin C, contain a quinone moiety. The quinone group, which is able to generate free radicals and active oxygen species through oxidation–reduction reactions, has been implicated in the antitumor activity [3] and host toxicity [4–6] of these agents and has been shown to induce damage to DNA [7, 8].

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Because the quinone antitumor agents have a complex chemical structure, it has been difficult to prove a direct involvement by the quinone group in the activity of these agents. The present study has made use of a series of model compounds to investigate further the role of the quinone group in the mechanism of action of quinone antitumor agents.

MATERIALS AND METHODS

Bis(dimethylamino)benzoquinone, [2,5-bis(dimethylamino)-1,4-benzoquinone], was prepared as described previously [9] and was recrystallized from methanol (m.p. = 169.5–171°). Benzoquinone dimustard, [2,5-bis(di(2′-chloroethyl)amino)-1,4-benzoquinone], was prepared by a known procedure [10] and was recrystallized from ethanol (m.p. = 131–133°). Benzoquinone mustard, [di(2′-chloroethyl)amino-1,4-benzoquinone], was prepared as described previously [11] and was recrystallized twice from ethanol (m.p. = 112–113°). Superoxide dismutase from bovine blood (sp. act. 2,700 units/mg protein) and catalase from bovine liver (sp. act. 17,600 units/mg protein) were obtained from the Sigma Chemical Co., St. Louis, MO. [2-^{14}C]Thy-

midine (sp. act. 50 mCi/mmole) and [*methyl*-^{3}H] thymidine (sp. act. 50–80 Ci/mmole) were obtained from the New England Nuclear Corp., Boston, MA. Proteinase K was from E. Merck, Darmstadt, West Germany, and tetrapropylammonium hydroxide was from the Eastman Kodak Co., Rochester, NY.

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 [12]. Cytotoxicity studies were performed on suspension cultures of L5178Y cells as described previously [1, 13, 14]. Drugs in dimethyl sulfoxide were added to cell suspensions in a 1:20 dilution for bis(dimethylamino)benzoquinone and in a 1:100 dilution for benzoquinone dimustard. Cloning efficiency ranged from 36 to 72%. A linear regression analysis of each concentration-survival curve was obtained and the concentration required to reduce the surviving cell fraction to 0.1 (D_{10}) 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 treated for 1 hr with drug. The cell kill was determined by the clonogenic assay.

Uptake of superoxide dismutase by L5178Y cells was determined by incubating 50-ml aliquots of cells at 2.5×10^5/ml with superoxide dismutase (2400 units/ml) for various times in Fischer's medium containing 10% horse serum. Cells were washed twice with Dulbecco's phosphate-buffered saline (PBS), suspended in 1 ml of water, sonicated (4×30 sec), and spun at 1500 *g* for 10 min to remove cell membranes. The supernatant fraction (0.75 ml) was mixed with a 1 mM xanthine solution (0.5 ml), a 1 mM EDTA solution (0.5 ml), a 50 μM cytochrome *c* solution (2 ml) and a solution of PBS at pH 7.8 containing 33 μM potassium cyanide (1.25 ml). This solution (3.5 ml) was put into a u.v. cuvette, and the absorbance at 550 nm was measured. Xanthine oxidase solution (0.18 μg/ml in PBS, pH 7.8) was added (100 μl) and the absorbance at 550 nm was measured every 2 min for 30 min. The superoxide dismutase concentration was calculated from the slope of the absorbance versus time curve for the first 20 min using a calibration curve. The endogenous level of superoxide dismutase in L5178Y cells was determined as above except with no extracellular enzyme added during the incubation.

Uptake of catalase by L5178Y cells was determined by incubating 40-ml aliquots of cells at 2.5×10^5/ml with catalase (4400 units/ml) for various times in Fischer's medium containing 10% horse serum. Cells were washed twice with PBS, suspended in 2 ml of water, sonicated (4×30 sec), and spun at 1500 *g* for 10 min. The supernatant fraction (2 ml) was added to 8 ml of a 25 mM hydrogen peroxide solution and the decomposition of peroxide at 0° was determined as follows. At timed intervals, 1 ml of the reaction mixture was withdrawn and transferred to a 50-ml volumetric flask containing 5 ml of 10% sulfuric acid and the whole was made up to 50 ml with water. To 0.5 ml of this solution was added successively 0.5 ml of 1 M potassium iodide, 0.5 ml of 1 mM ammonium molybdate, 0.25 ml of 2% starch solution and 8.25 ml of water. The absorbance at 580 nm of the resulting blue solution was determined. The catalase concentration was calculated from the slope of a plot of the log(absorbance) versus time for 60 min using a calibration curve. The endogenous level of catalase in L5178Y cells was determined as above except with no extracellular enzyme added during the incubation.

Elution assays for measuring DNA double-strand breaks, DNA single-strand breaks and DNA–DNA cross-linking were carried out as described previously [15–18]. L5178Y cells labeled with [^{14}C]thymidine were treated with drugs as described above for the clonogenic assay. For the DNA single-strand break assay, proteinase K was not used so that only non-protein associated single-strand breaks were measured. The level of DNA single- and double-strand breaks was calculated from the elution profiles and was expressed as rad equivalent (dose of radiation inducing an equivalent number of breaks) as determined from calibration curves. The level of cross-linking was calculated as described by Kohn *et al.* [17]. The concentration–response curves for DNA strand breaks and DNA cross-linking were obtained by linear regression analysis. For inhibition studies, cell suspensions were preincubated for 30 min with or without catalase as described above. DNA strand breaks and DNA–DNA cross-linking were determined using the elution assays.

RESULTS

Model compounds. The model compounds used in this study are shown in Fig. 1. They both consist of a single ring structure making it unlikely that they would bind to DNA by intercalation. Bis(dimethylamino)benzoquinone contains a quinone group as well as two secondary amino groups which would be expected to show little activity. The amino groups may, however, be important in modifying the redox potential of the quinone moiety. This compound served as a model for the activity of the quinone. Benzoquinone dimustard contains both a quinone function and two active nitrogen mustard alkylating groups and thus should be able to bind directly to DNA. Although these compounds appear to have little clinical utility, they are useful models for evaluating the activities of the quinone and alkylating groups.

Cytotoxicity of the model compounds against L5178Y lymphoblast in vitro. The cytotoxic activities of the model compounds against L5178Y lymphoblasts *in vitro* were determined by a soft agar cloning assay [13] and are shown as concentration–survival curves in Fig. 2. Treatment of cells at 37° for 1 hr

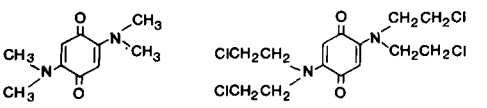

Fig. 1. Model compounds used in this study. Bis-(dimethylamino)benzoquinone contains a quinone group. Benzoquinone dimustard contains both a quinone moiety and active alkylating groups.

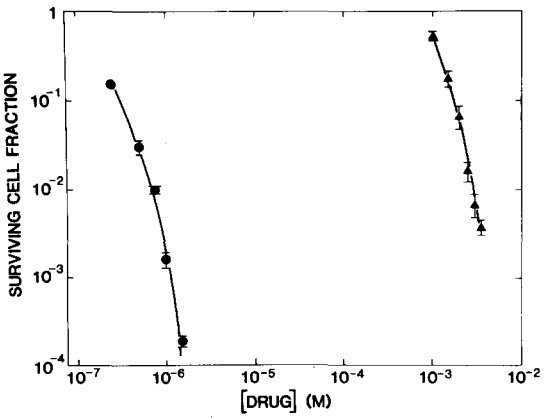

Fig. 2. Concentration–survival curves for L5178Y lymphoblasts treated with model compounds. Cells were incubated at 37° for 1 hr with benzoquinone dimustard (●) or bis-(dimethylamino)benzoquinone (▲) at the concentrations shown. The surviving cell fraction was determined by a clonogenic assay as described in the text and previously [13, 14] and is plotted against the concentration of drug used. Each point represents the mean of 5–10 quadruplicate determinations. Bars = S.E. On occasion, the confidence intervals were too small to be shown.

with bis(dimethylamino)benzoquinone resulted in significant cell kill, with the surviving cell fraction being reduced to approximately 3×10^{-3} at a drug concentration of 3.5 mM. The D_{10} (concentration of drug required to reduce the surviving cell fraction to 0.1) for the quinone agent was 1.12 ± 0.06 mM. The alkylating quinone, benzoquinone dimustard, was considerably more toxic reducing the surviving cell fraction of L5178Y cells treated under the same conditions to approximately 10^{-4} at a drug concentration of 1.5 μM. Benzoquinone dimustard had a D_{10} of 0.427 ± 0.023 μM and was approximately 2500 times more active against the lymphoma cells than was the quinone agent bis(dimethylamino)-benzoquinone.

Effects of superoxide dismustase and catalase on the cytotoxic activity of the model compounds. The effects of the cell protective enzymes, superoxide dismutase and catalase, on the cytotoxic activity of the model compounds were studied using procedures described previously [1], and the results are shown in Fig. 3. Incubation of cells for 30 min with superoxide dismutase (2400 units/ml) prior to treatment with the quinone agents, bis(dimethylamino)benzoquinone and benzoquinone dimustard, did not appear to inhibit the cytotoxic activity of these agents. However, pretreatment for 30 min with catalase (4400 units/ml) significantly inhibited the cell kill produced by both agents. The combination of superoxide dismutase and catalase proved no more effective than catalase alone.

Uptake of superoxide dismutase and catalase by L5178Y lymphoblasts. Uptake of the enzymes superoxide dismutase and catalase by L5178Y cells at 37° is shown in Fig. 4. The intracellular concentration of superoxide dismutase increased from an endogenous level of 3.26×10^{-7} units/cell to a maximum concentration of 5.08×10^{-7} units/cell after 2 hr of incubation. Similarly, the intracellular catalase concentration increased from an endogenous level of 1.01×10^{-6} units/cell to a maximum concentration of 1.42×10^{-6} units/cell.

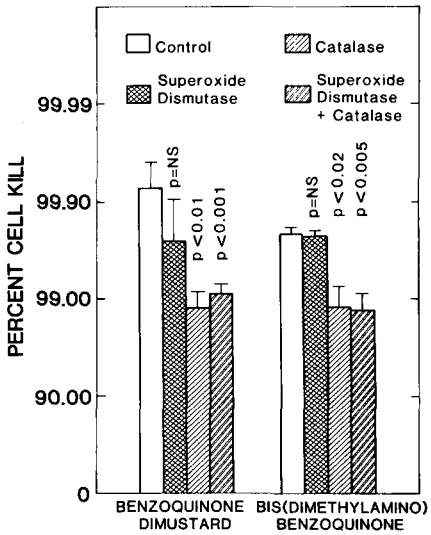

Fig. 3. Effects of superoxide dismutase and catalase on the cytotoxicity of the model compounds. L5178Y cells were preincubated at 37° for 30 min with or without superoxide dismutase (2400 units/ml) and/or catalase (4400 units/ml). Model compounds were added and cells were incubated at 37° for 1 hr with 2.5 μM benzoquinone dimustard or 3.5 mM bis(dimethylamino)benzoquinone. The percent cell kill was determined by the clonogenic assay. Data represent the mean of 4–8 quadruplicate determinations. Bars = S.E. The results were statistically evaluated by a two-tailed *t*-test comparing the significance of the difference of the means. NS = not significant.

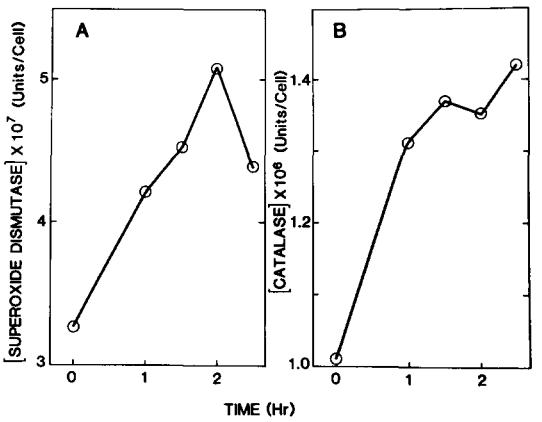

Fig. 4. Uptake of superoxide dismutase and catalase by L5178Y lymphoblasts. (A) L5178Y cells were incubated at 37° with 2400 units/ml of superoxide dismutase for the times shown. The intracellular concentration of superoxide dismutase at 0 time represents the endogenous level of enzyme. For determination of the endogenous level of intracellular superoxide dismutase no extracellular enzyme was added. The intracellular concentration of superoxide dismutase was determined as described in the text. (B) L5178Y were incubated at 37° with 4400 units/ml of catalase for the times shown. The intracellular concentration of catalase at 0 time represents the endogenous level of enzyme. For determination of the endogenous level of intracellular catalase no extracellular enzyme was added. The intracellular concentration of catalase was determined as described in the text.

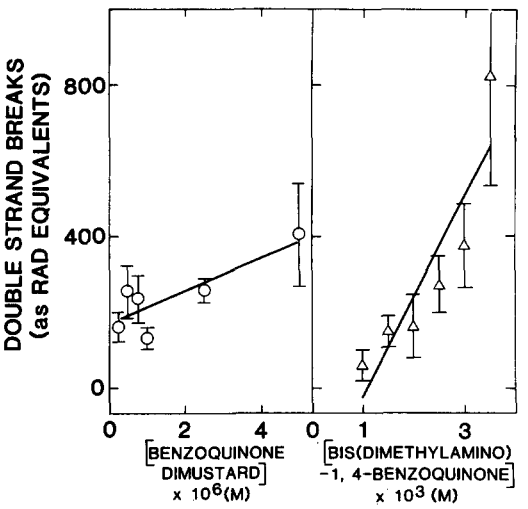

Fig. 5. Concentration–response plot of DNA double-strand breaks induced by the model compounds. L5178Y cells were incubated at 37° for 1 hr with benzoquinone dimustard (○) or bis(dimethylamino)benzoquinone (△) at the concentrations shown. DNA double-strand breaks induced by each compound were determined by the elution assay described in the text and previously [15, 16]. The number of breaks, expressed as rad equivalents, is plotted against the concentration of drug used. Each point represents the mean of 4–6 determinations. Bars = S.E. The lines were determined by linear regression analysis.

Induction of DNA double-strand breaks in L5178Y lymphoblasts in vitro. DNA double-strand breaks induced by the model compounds in L5178Y cells were measured using a neutral elution assay [15, 16]. Cells were treated at 37° for 1 hr with the concentrations of drug shown (Fig. 5). Bis(dimethylamino)benzoquinone induced approximately 800 rad equivalents of DNA double-strand breaks at a concentration of 3.5 mM. The DNA damage induced showed significant concentration-dependence with a correlation coefficient of 0.895 ($P < 0.02$). Treatment of cells with benzoquinone dimustard also resulted in a concentration-dependent production of double-strand breaks with a correlation coefficient of 0.847 ($P < 0.04$) and approximately 400 rad equivalents of breaks at a drug concentration of 5 μM. Benzoquinone dimustard was approximately 200-fold more active in inducing DNA double-strand breaks than was bis(dimethylamino)-benzoquinone as determined from the ratio of the slopes of the concentration–response curves.

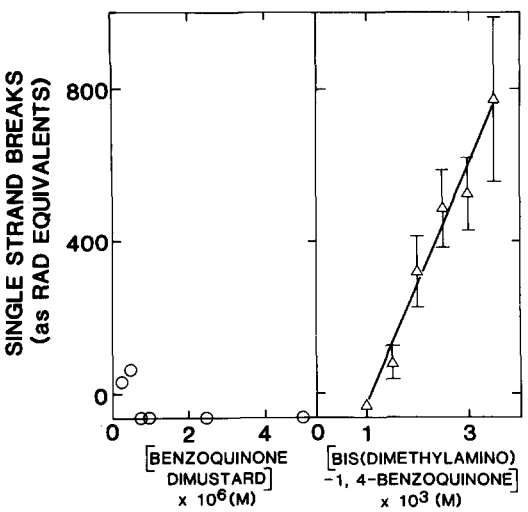

Fig. 6. Concentration–response plot of DNA single-strand breaks induced by the model compounds. L5178Y cells were incubated at 37° for 1 hr with benzoquinone dimustard (○) or bis(dimethylamino)benzoquinone (△) at the concentrations shown. DNA single-strand breaks induced by each compound were determined by alkaline elution assay as described in the text and previously [17, 18]. The number of breaks, expressed as rad equivalents, is plotted against the concentration of drug used. Each point represents the mean of 2–5 determinations. Bars = S.E. On occasion, the confidence intervals were too small to be illustrated. The line was determined by linear regression analysis.

Induction of DNA single-strand breaks in L5178Y cells in vitro. DNA single-strand breaks induced by the model compounds in L5178Y lymphoblasts were measured using an alkaline elution assay [17, 18]. Incubation of cells at 37° for 1 hr with bis-(dimethylamino)benzoquinone resulted in the induction of approximately 800 rad equivalents of single-strand breaks at a drug concentration of 3.5 mM (Fig. 6). The level of DNA damage increased with increasing drug concentration, and the concentration–response curve had a correlation coefficient of 0.988 ($P < 0.001$). In contrast, treatment of cells with benzoquinone dimustard at drug concentrations up to 5 μM resulted in few, if any, apparent single-strand breaks.

The ratio of single-strand breaks (excluding those arising from double-strand breaks) to double-strand breaks induced by bis(dimethylamino)benzoquinone was determined as described previously [19, 20]. The ratio at a drug concentration of 3.5 mM was calculated as being between 7 and 35, values which are only slightly below those reported in the literature for X-irradiation [20–23].

DNA–DNA cross-linking in L5178Y cells in vitro. DNA cross-linking can interfere with the alkaline elution assay for DNA single-strand breaks but does not interfere with the elution assay for DNA double-strand breaks. DNA–DNA cross-linking induced by the model compounds was measured using a second alkaline elution assay [17, 18]. L5178Y cells treated at 37° for 1 hr with the alkylating quinone, benzoquinone dimustard, showed significant levels of cross-linking with approximately 100 rad equivalents of cross-links being produced at a drug concentration of 1.5 μM. This level of cross-linking was sufficient to mask at least 500 rad equivalents of single-strand breaks. In contrast, bis(dimethylamino)benzoquinone which does not contain an alkylating group produced little or no DNA-DNA cross-linking.

Inhibition of DNA damage by catalase. The effect of catalase on the induction of DNA strand breaks was studied using procedures described previously [2]. Incubation of cells for 30 min with catalase (4400 units/ml) prior to treatment with benzoquinone dimustard or bis(dimethylamino)benzoquinone significantly inhibited the induction of double-strand breaks by the quinone agents (Fig. 7). Strand breaks were inhibited to approximately 40% of control levels with benzoquinone dimustard and to approximately 30% of control with bis(di-

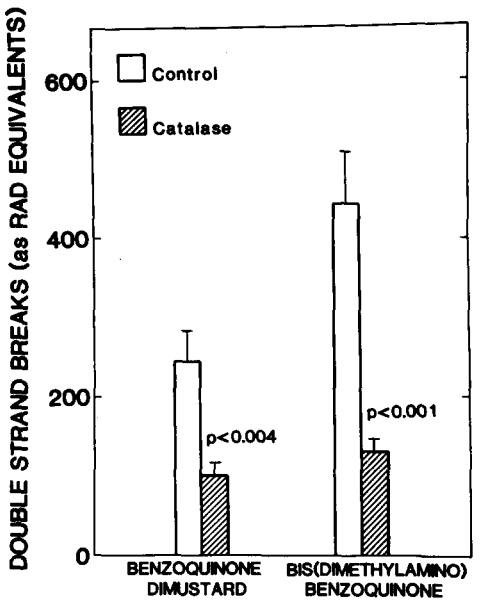

Fig. 7. Effect of catalase on the induction of DNA double-strand breaks by the model compounds. L5178Y cells labeled with [^{14}C]thymidine were preincubated at 37° for 30 min with or without catalase (4400 units/ml). Benzoquinone dimustard or bis(dimethylamino)benzoquinone was added to give final drug concentrations of 5 μM or 3.5 mM, respectively, and the cells were incubated at 37° for 1 hr. The number of DNA double-strand breaks induced was determined by elution assays and is presented as rad equivalents. Bars = S.E. The results were statistically evaluated by a two-tailed *t*-test comparing the mean number of DNA strand breaks induced in the presence or absence of catalase.

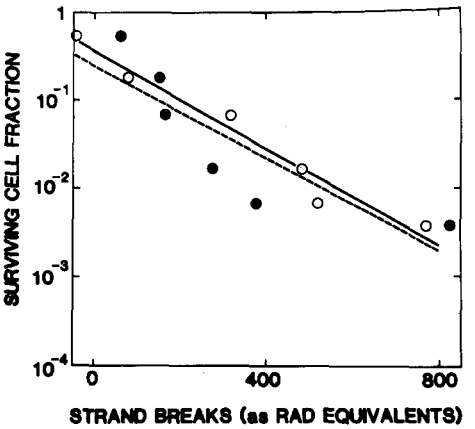

Fig. 8. Correlation of induction of DNA strand breaks with cytotoxic activity of bis(dimethylamino)benzoquinone. L5178Y lymphoblasts were incubated at 37° for 1 hr with 1.0, 1.5, 2.0, 2.5, 3.0 or 3.5 mM bis(dimethylamino)benzoquinone. DNA double-strand and DNA single-strand breaks induced in the cells were determined by elution assays as described in the text and Figs. 5 and 6. Cell cytotoxicity was determined by a clonogenic assay as described in the text and Fig. 2. Cell cytotoxicity, expressed as surviving cell fraction, produced by each concentration of drug is plotted against DNA double-strand breaks (●--●) or DNA single-strand breaks (○——○), expressed as rad equivalents, induced by the same drug concentration. The lines were determined by linear regression analysis. The linear regression equation for DNA double-strand breaks was $\log Y = -0.6275 - 0.0026X$ with a correlation coefficient of 0.851 ($P < 0.04$) and that for DNA single-strand breaks was $\log Y = -0.4545 - 0.0027X$ with a correlation coefficient of 0.981 ($P < 0.001$).

methylamino)benzoquinone. Catalase also inhibited the induction of single-strand breaks by the non-alkylating quinone but had no effect on the production of DNA-DNA cross-links by the alkylating quinone.

Correlation of cytotoxic activity with induction of DNA damage. To examine possible relationships between cytotoxic activity and the induction of DNA damage, the ability of the model compounds to produce cell kill was compared with their ability to induce DNA damage. The model compounds described in this publication, as well as others described previously, were included in this comparison [1, 2]. Table 1 shows the relative cytotoxicities of quinone-containing model compounds and their relative abilities to induce double- and single-strand

Table 1. Correlation of cytotoxic activity of model compounds with induction of DNA strand breaks*

Model compounds	D_{10} (M)	Relative cytotoxic activity	Relative induction of single-strand breaks	Relative induction of double-strand breaks
Bis(dimethylamino)benzoquinone	1.12×10^{-3}	1	1	1
Hydrolyzed benzoquinone mustard	5.59×10^{-4}†	2†	1.7‡	2.3‡
Benzoquinone dimustard	4.27×10^{-7}	2,623		170
Benzoquinone mustard	1.85×10^{-8}†	60,541†		31,722‡

* L5178Y lymphoblasts were incubated at 37° for 1 hr with various concentrations of the model compounds. The surviving cell fractions were determined by a clonogenic assay as described in the text and previously [1], and the DNA single- and double-strand breaks induced were determined by elution assays as described in the text and previously [2]. The relative cytotoxic activity was obtained from the ratio of the D_{10} (concentration of drug required to reduce the surviving cell fraction to 0.1) of the model compond to that of bis(dimethylamino)benzoquinone. The relative induction of single-strand breaks and double-strand breaks was obtained from the ratio of the slope of the concentration–response plot of the model compound to that of bis(dimethylamino)benzoquinone.

† Data were obtained from Ref. 1.

‡ Data were obtained from Ref. 2.

Table 2. Correlation of cytotoxic activity of model compounds with production of DNA-DNA cross-linking*

Model compound	D_{10} (M)	Cytotoxic activity	Production of DNA-DNA cross-linking
Aniline mustard	1.20×10^{-5}†	1	1‡
Benzoquinone dimustard	4.27×10^{-7}	28	90
Benzoquinone mustard	1.85×10^{-8}†	649	4171‡

* L5178Y lymphoblasts were incubated at 37° for 1 hr with various concentrations of the model compounds. The surviving cell fractions were determined by a clonogenic assay as described in the text and previously [1], and DNA-DNA cross-linking was determined by an alkaline elution assay as described in the text and previously [2]. The relative cytotoxic activity was obtained from the ratio of the D_{10} of the model compound to that of aniline mustard. The relative production of DNA-DNA cross-linking was obtained from the ratio of the slope of the concentration–response plot of the model compound to that of aniline mustard.

† Data were obtained from Ref. 1.

‡ Data were obtained from Ref. 2.

breaks in the DNA of L5178Y cells. No single-strand breaks were observed with the alkylating quinones, benzoquinone dimustard and benzoquinone mustard. For bis(dimethylamino)benzoquinone, hydrolyzed benzoquinone mustard and benzoquinone mustard, a relationship appeared to exist between relative cytotoxicity and induction of strand breaks, whereas for benzoquinone mustard no such relationship was apparent. Table 2 shows the relative cytotoxicities of the nitrogen-mustard-containing compounds and their relative abilities to produce DNA-DNA cross-linking in L5178Y cells. Cross-linking and cytotoxic activity appeared to be related for aniline mustard and benzoquinone dimustard but not for benzoquinone mustard.

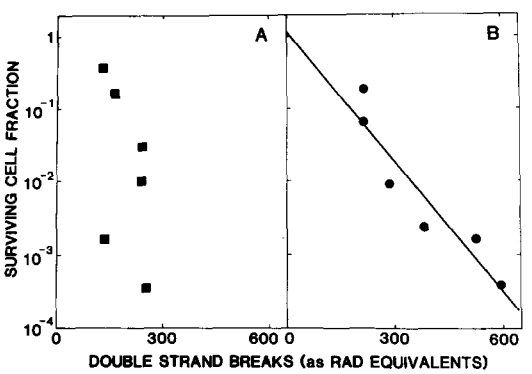

Fig. 9. Correlation of induction of DNA double-strand breaks with cytotoxic activity of benzoquinone dimustard and benzoquinone mustard. (A) L5178Y cells were incubated at 37° for 1 hr with 0.1, 0.25, 0.5, 0.75, 1.0 or 2.5 μM benzoquinone dimustard. (B) L5178Y cells were incubated at 37° for 1 hr with 10, 20, 30, 40, 50 or 60 nM benzoquinine mustard. DNA double-strand breaks induced in the cells were determined by the elution assay as described in the text and previously [2]. Cell cytotoxicity was determined by a soft agar cloning assay as described in the text and previously [1]. Cell cytotoxicity, expressed as surviving cell fraction, produced by each concentration of drug is plotted against DNA double-strand breaks, expressed as rad equivalents, induced by the same drug concentration. Correlations were determined by linear regression analysis. (A) Benzoquinone dimustard. The linear regression equation had a correlation coefficient of 0.424 (P = NS). (B) Benzoquinone mustard. The linear regression equation was $\log Y = 0.0609 - 0.0059X$ with a correlation coefficient of 0.937 ($P < 0.01$).

Possible direct linear correlations between cytotoxicity and DNA damage were examined. DNA damage (expressed as rad equivalents) resulting from treatment of L5178Y cells at 37° for 1 hr with various concentrations of bis(dimethylamino)benzoquinone was plotted against the cytotoxic activity (expressed as surviving cell fraction) produced by treatment of cells under the same conditions with the same concentrations of drug (Fig. 8). For both single-strand and double-strand breaks, a linear correlation appeared to exist between cell kill and DNA damage induced by this agent. For single-strand breaks, the correlation coefficient was 0.981 and this correlation was highly significant ($P < 0.001$), while for double-strand breaks the correlation coefficient of 0.851 was also statistically significant ($P < 0.04$).

A similar plot of cytotoxic activity versus double-strand breaks induced by benzoquinone dimustard showed no apparent linear correlation (Fig. 9A). In contrast, the alkylating quinone, benzoquinone mustard, showed a significant correlation between cytotoxic activity against L5178Y cells and the induction of double-strand breaks in these cells (Fig. 9B). The correlation coefficient was 0.937 and this was statistically significant ($P < 0.01$).

DISCUSSION

Considerable evidence has been presented to support the hypothesis that the quinone group is involved in the activity of such quinone-containing antitumor agents as adriamycin [3, 6, 8, 24, 25], daunorubicin [3, 4], streptonigrin [26] and others [27]. The generation of free radicals and active oxygen species during oxidation–reduction reactions of the quinone group may be involved in the mechanism of action of these agents [7, 25, 28–30]. The investigation of the role of the quinone group in the activity of these agents has been made difficult by their complex chemical structures. Recent studies

with model compounds have provided evidence that the quinone group can produce tumor cell kill and can induce DNA single- and double-strand breaks by mechanisms that appear to involve free radicals and active oxygen species [1, 2]. This report has presented experimental results that support an active role for the quinone group in the mechanism of action of antitumor agents. However, it has also been demonstrated that major differences can exist in the relative contribution of the quinone group to the overall activity of these agents.

The model compounds used in this study are similar to those used previously [1] in that they are single ring structures that are unlikely to bind to DNA by intercalation. In addition, they contain a limited number of active functional groups. Bis(dimethylamino)benzoquinone served as a model for a non-binding quinone agent, whereas benzoquinone dimustard, which contains both a quinone group and alkylating groups, was studied to determine if the ability to bind to DNA could enhance the activity of the quinone.

Cytotoxicity studies (Fig. 2) showed that the quinone agent bis(dimethylamino)benzoquinone produced significant tumor cell kill and that the presence of alkylating groups in benzoquinone dimustard appeared to enhance the cytotoxic activity. Although the alkylating quinone, benzoquinone dimustard, was approximately 2,500–fold more active than bis-(dimethylamino)benzoquinone, this difference in activity was considerably smaller than the 30,000-fold difference that was observed previously for other model quinones [1].

For both quinone compounds, the cytotoxicity was inhibited significantly by catalase, an enzyme that removes hydrogen peroxide from the cell, but was not affected by superoxide dismutase, which removes superoxide radicals (Fig. 3). This result provided evidence that the cytotoxic activity of these compounds, at least in part, involves active oxygen species such as hydrogen peroxide. The lack of inhibition observed with superoxide dismutase suggests that the mechanism of action of these agents may not involve superoxide radical. This finding is similar to results reported previously for other antitumor agents such as benzoquinone mustard [1], mitomycin C [7] and VP-16 [31]. A study of uptake of superoxide dismutase by L5178Y cells showed that under the conditions of the inhibition experiments the intracellular concentration of superoxide dismutase increased by approximately 40% compared to endogenous intracellular levels of this enzyme. Studies in other cells have shown a similar level of uptake for the enzyme and have demonstrated the ability of the enzyme to cross the cell membrane and become associated with cytosol, mitochrondria and nuclei [32, 33]. The apparent decrease in superoxide dismutase concentration after 2 hr probably results from degradation of the enzyme within the cell. In a similar experiment, the intracellular concentration of catalase increased by approximately 35% when L5178Y cells were incubated with catalase. The observed increased intracellular levels of both enzymes may result from binding of the enzymes to cell surface proteins. Although this explanation cannot be unequivocally excluded, previous observations with superoxide dismutase [33] and the removal of cell membranes prior to measuring the enzyme concentrations suggest that both superoxide dismutase and catalase are able to enter L5178Y cells.

It is possible that the inhibition of both the cytotoxicity and DNA strand breaks induced by the model compounds by catalase may be due to extracellular binding of the drugs to the protein. However, this explanation appears to be unlikely since preincubation of cells with catalase had no effect on DNA-DNA cross-linking produced by benzoquinone dimustard or on the cytotoxic activity of the alkylating agent aniline mustard [1]. The incomplete inhibition of cell kill by catalase may be due to the inability of the enzyme to achieve a high enough concentration within the cell or may result from the operation of additional mechanisms producing cell kill.

The concentration-dependent induction of DNA double- and single-strand breaks by bis(dimethylamino)benzoquinone and double-strand breaks by benzoquinone dimustard (Figs. 5 and 6) was similar to findings reported previously for other model compounds [2]. The inhibition of the DNA strand break activity of bis(dimethylamino)benzoquinone and benzoquinone dimustard by catalase is consistent with the involvement of hydrogen peroxide in the mechanism of DNA damage (Fig. 7). Furthermore, DNA damage was enhanced when the quinone agent had the ability to bind to DNA. However, benzoquinone dimustard was only 200-fold more active than bis(dimethylamino)benzoquinone, whereas benzoquinone mustard, an alkylating quinone, was approximately 15,000 times more active than hydrolyzed benzoquinone mustard, a non-alkylating quinone [2]. The apparent inability of benzoquinone dimustard to induce DNA single-strand breaks (Fig. 6) is probably due to its ability to cross-link DNA, a process that is known to interfere with the elution assay for single-strand breaks [17]. The level of cross-linking observed with this agent was high enough to mask at least 500 rad equivalents of single-strand breaks.

While it is possible that the observed differences in the effects of the model compounds may relate to differences in drug uptake, this explanation appears unlikely. All of the model agents are highly lipid soluble and would be expected to enter the cells readily by a passive diffusion mechanism. Experiments to test this hypothesis will be carried out.

The comparison of relative cytotoxicity and relative activity in inducing DNA strand breaks for the quinone model compounds (Table 1) showed an apparent relationship between DNA strand breaks and cell kill for the two quinone models, hydrolyzed benzoquinone mustard and bis(dimethylamino)-benzoquinone, and for the alkylating quinone, benzoquinone mustard. The statistically significant linear correlations between cell kill and DNA damage observed with these agents (Figs. 8 and 9B) are consistent with these results and provide evidence that such DNA damage may play an important role in the mechanism of action of these compounds. In contrast, no linear correlation was observed between DNA strand breaks and cell kill produced by ben-

zoquinone dimustard (Fig. 9A). This result, coupled with an apparent relationship between DNA-DNA cross-linking and cytotoxic activity for this quinone agent (Table 2), suggested that cross-linking may play a more important role in the cytocidal action of benzoquinone dimustard. The increased cytotoxic activity of benzoquinone dimustard compared to aniline mustard can be fully explained by the increased activity of the quinone compound in producing DNA-DNA cross-linking. However, the induction of DNA strand breaks by benzoquinone dimustard must contribute to the overall cytotoxic activity since both cell kill and the induction of double-strand breaks by this agent are inhibited by catalase, whereas the enzyme has no effect on DNA-DNA cross-link formation. These findings illustrate that, although DNA strand breaks may contribute to the cytotoxicity of an alkylating quinone, they need not be the primary mechanism involved. Further, these results demonstrate that major differences can exist in the relative contribution of quinone-induced DNA strand breaks to the overall activity of quinone antitumor agents.

In summary, this study has provided additional evidence that the presence of a quinone group in the structure of a compound can result in significant cell kill by a mechanism that appears to involve active oxygen species. Quinone containing agents can induce DNA strand breaks, and this effect is enhanced for agents that can bind to DNA. The observed correlation between the induction of strand breaks and cytotoxicity produced by some quinone agents provided further evidence for the involvement of quinone-induced DNA strand breaks in the activity of quinone compounds. However, considerable differences can exist in the relative importance of this mechanism to the overall activity of the agent.

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REFERENCES

1. A. Begleiter, *Cancer Res.* **43**, 481 (1983).
2. A. Begleiter and G. W. Blair, *Cancer Res.* **44**, 78 (1984).
3. N. R. Bachur, S. L. Gordon and M. V. Gee, *Cancer Res.* **38**, 1745 (1978).
4. J. W. Lown, H-H. Chen, J. A. Plambeck and E. M. Acton, *Biochem. Pharmac.* **28**, 2563 (1979).
5. J. F. Van Vleet, V. J. Ferrans and W. E. Weirich, *Am. J. Path.* **99**, 13 (1980).
6. J. H. Doroshow, G. Y. Locker, I. Ifrim and C. E. Myers, *J. clin. Invest.* **68**, 1053 (1981).
7. J. W. Lown, A. Begleiter, D. Johnson and A. R. Morgan, *Can. J. Biochem.* **54**, 110 (1976).
8. J. W. Lown, S-K. Sim, K. C. Majumdar and R-Y. Chang, *Biochem. biophys. Res. Commun.* **76**, 705 (1977).
9. R. N. Harger, *J. Am. chem. Soc.* **46**, 2540 (1924).
10. W. C. J. Ross, *Biochem. Pharmac.* **13**, 969 (1964).
11. A. N. Makarova and A. Y. Berlin, *Zh. obshch. Khim.* **37**, 637 (1967).
12. A. H. Greenberg, J. Manougian, M. Ray and G. J. Goldenberg, *Acta cytol.* **24**, 232 (1980).
13. M-Y. Chu and G. A. Fischer, *Biochem. Pharmac.* **17**, 753 (1968).
14. G. J. Goldenberg, R. M. Lyons, J. A. Lepp and C. L. Vanstone, *Cancer Res.* **31**, 1616 (1971).
15. M. O. Bradley and K. W. Kohn, *Nucleic Acids Res.* **7**, 793 (1979).
16. W. G. Woods, *Biochim. biophys. Acta* **655**, 342 (1981).
17. K. W. Kohn, R. A. G. Ewig, L. C. Erickson and L. A. Zwelling, in *DNA Repair, A Laboratory Manual of Research Techniques* (Eds. E. C. Friedberg and P. C. Hanawalt), p. 379. Marcel Dekker, New York (1981).
18. A. Begleiter, J. Grover, E. Froese and G. J. Goldenberg, *Biochem. Pharmac.* **32**, 293 (1983).
19. L. A. Zwelling, S. Michaels, L. C. Erickson, R. S. Ungerleider, M. Nichols and K. W. Kohn, *Biochemistry* **20**, 6553 (1981).
20. L. A. Zwelling, S. Michaels, D. Kerrigan, Y. Pommier and K. W. Kohn, *Biochem. Pharmac.* **31**, 3261 (1982).
21. J. T. Lett, I. Caldwell, C. J. Dean and P. Alexander, *Nature, Lond.* **214**, 790 (1967).
22. W. Veatch and S. Okada, *Biophys. J.* **9**, 330 (1969).
23. D. L. Dugle, C. J. Gillespie and J. D. Chapman, *Proc. natn. Acad. Sci. U.S.A.* **73**, 809 (1976).
24. E. H. Herman and V. J. Ferrans, *Fedn Proc.* **39**, 859 (1980).
25. C. E. Myers, W. P. McGuire, R. H. Liss, I. Ifrim, K. Grontizinger and R. C. Young, *Science* **197**, 165 (1977).
26. J. W. Lown and S-K. Sim, *Can. J. Biochem.* **54**, 446 (1976).
27. M. J. Egorin, B. M. Fox, J. F. Spiegal, P. L. Gutierrez, R. D. Friedman and N. R. Bachur, *Proc. Am. Ass. Cancer Res.* **23**, 177 (1982).
28. N. R. Bachur, S. L. Gordon and M. V. Gee, *Molec. Pharmac.* **13**, 901 (1977).
29. J. W. Lown, S-K. Sim and H-H. Chen, *Can. J. Biochem.* **56**, 1042 (1978).
30. R. I. Glazer, K. D. Hartman and C. L. Richardson, *Cancer Res.* **42**, 117 (1982).
31. A. J. Wozniak, K. R. Hande and W. E. Ross, *Proc. Am. Ass. Cancer Res.* **24**, 319 (1983).
32. K. Kelly, C. Barefoot, A. Sehon and A. Petkau, *Archs Biochem. Biophys.* **190**, 531 (1978).
33. J. R. Lepock, L. D. Arnold, A. Petkau and K. Kelly, *Biochim. biophys. Acta* **649**, 49 (1981).