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ORIGINAL ARTICLE

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Structure-activity study with bioreductive benzoquinone alkylating agents: effects on DT-diaphorase-mediated DNA crosslink and strand break formation in relation to mechanisms of cytotoxicity

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Abstract *Purpose*: Structure-activity studies were carried out with the model bioreductive alkylating agent benzoquinone mustard (BM) and its structural analogs. The specific objectives were: (1) to investigate the effects of functional group substitutions to the benzoquinone ring on DNA crosslink and strand break formation subsequent to reduction of the analogs by DT-diaphorase (DTD) in vitro, (2) to correlate DNA crosslink and strand break formation by the analogs with anaerobic reduction of the BM analogs by DTD and their redox cycling in vitro, and (3) to correlate DNA crosslink and strand break formation by the BM analogs with their cytotoxic effects in cancer cells. *Methods*: DNA interstrand crosslink and single-strand break formation were assessed using agarose gel assays. To determine DNA interstrand crosslinks or single-strand breaks, linearized or supercoiled plasmid DNA, respectively, were incubated with purified human DTD and increasing concentrations of each BM analog. Subsequently, DNA was electrophoresed on an agarose gel and DNA crosslink and strand break formation were quantified using densitometry. The rates of reduction of the BM analogs by purified human DTD were measured in vitro under hypoxic conditions, and the redox cycling potential was determined under aerobic conditions using HPLC analysis. The cytotoxic activities of these agents in human tumor cell lines were measured by the MTT assay, with and without the DTD inhibitor, dicoumarol. *Results*: BM analogs with electron-donating groups (MeBM, MBM, m-MeBM), electron-withdrawing groups (CBM, FBM), sterically bulky groups (PBM, m-PBM, m-TBM) and positional isomers (MeBM, m-MeBM, PBM, m-PBM) were synthesized. After reduction by DTD, the BM analogs produced a concentration-dependent increase in DNA crosslink and DNA strand break formation. The E_{10} (extent of DNA crosslink formation produced by 10 μM BM analog) for DNA crosslink formation displayed the rank order MeBM≈MBM > m-MeBM≈PBM≈BM > CBM > FBM > m-PBM≈m-TBM. For DNA strand break formation, the E_{10} values (extent of DNA strand break formation produced by 10 μM BM analog) displayed the rank order MeBM > MBM > m-MeBM > PBM > BM≈CBM > FBM > m-PBM≈m-TBM. Importantly, the cytotoxic activity of the BM analogs in SK-Mel-28 human melanoma cells correlated positively with the E_{10} values for DTD-mediated DNA crosslink formation ($r_s = 0.87$, $P < 0.05$) and DNA strand break formation ($r_s = 0.95$, $P < 0.05$). Similar correlations were observed in NCI-H661 human lung carcinoma cells. Furthermore, the D_{10} values (concentration of BM analog that decreased the surviving cell fraction to 0.1) for cytotoxic activity of the BM analogs correlated with the maximum levels of DNA crosslinks formed with each BM analog, with r_s values of −0.85 ($P < 0.05$) for the NCI-H661 cell line, and −0.81 ($P < 0.05$) for the SK-MEL-28 cell line. The half-time of reduction ($t_{1/2}$) of the BM analogs by DTD did not correlate with DNA crosslink formation, DNA strand break formation, or cytotoxic potency of the analogs. *Conclusions*: Functional groups on the benzoquinone ring affect the ability of BM to produce DNA crosslinks and strand breaks following reduction by DTD. Electron-donating groups increased DNA damage, whereas electron-withdrawing groups and sterically bulky groups at the C6 position had no effect or decreased the ability of the compounds to produce DNA damage compared to BM. Moreover, both DNA

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crosslink and strand break formation appear to have an important impact on the cytotoxicity of the BM analogs. These results may have significance for optimal use of BM-based antitumor agents and for rationalization of the development of novel therapeutic compounds that require bioactivation by DTD.

Keywords Bioreductive alkylating agents · DT-diaphorase · Structure-activity studies · DNA crosslinks · DNA strand breaks

Abbreviations *AZQ* 2,5-Diaziridinyl-3,6-bis(carboethoxyamino)-1,4-benzoquinone · *BM* 2-[Di(chloroethyl)amino]-1,4-benzoquinone · *CBM* 5-Chloro-2-[di(chloroethyl)amino]-1,4-benzoquinone · *DCPIP* 2,6-Di-chlorophenolindophenol · *DIC* Dicoumarol · *DMF* Dimethylformamide · *DTD* NAD(P)H:quinone oxidoreductase · *DZQ* 3,6-Diaziridinyl-1,4-benzoquinone · *EO9* 3-Hydroxymethyl-5-aziridinyl-1-methyl-2-(H-indole-4,7-dione)-prop-2-en-1-ol · *FBM* 5-Fluoro-2-[di(chloroethyl)amino]-1,4-benzoquinone · *MBM* 5-Methoxy-2-[di(chloroethyl)amino]-1,4-benzoquinone · *MeBM* 5-Methyl-2-[di(chloroethyl)amino]-1,4-benzoquinone · *m-MeBM* 6-Methyl-2-[di(chloroethyl)amino]-1,4-benzoquinone · *MeDZQ* 2,5-Diaziridinyl-3,6-dimethyl-1,4-benzoquinone · *PBM* 5-Phenyl-2-[di(chloroethyl)amino]-1,4-benzoquinone · *m-PBM* 6-Phenyl-2-[di(chloroethyl)amino]-1,4-benzoquinone · *m-TBM* 6-*t*-Butyl-2-[di(chloroethyl)amino]-1,4-benzoquinone · *RH1* 2,5-Diaziridinyl-3-hydroxymethyl-6-methyl-1,4-benzoquinone

Introduction

Bioreductive alkylating agents have two main structural elements, a bioreductive element and a cytotoxic element. For some agents, such as tirapazamine [27], the bioreductive and cytotoxic elements are the same moiety, whereas for other agents, including mitomycin C [20, 35], the bioreductive element regulates a separate cytotoxic element. In the case of mitomycin C, and aziridinylquinones such as AZQ, for example, the bioreductive element is a quinone moiety, which can undergo both one- and two-electron reduction, and the cytotoxic element is an alkylating group that is activated when the quinone is reduced [3, 5, 10, 12, 13, 26]. One-electron reduction of the quinone moiety is mediated by enzymes such as NADPH:cytochrome P-450 reductase [1, 30], and results in the formation of semiquinone reactive intermediates which can react with oxygen to produce superoxide and other reactive oxygen species. These reactive intermediates in turn can undergo nucleophilic additions with DNA, and produce DNA strand breaks [13, 34]. Two-electron reduction, converts the parent quinone moiety to its hydroquinone form, which may react with oxygen to produce oxidative damage, and crosslink DNA through the activated cytotoxic element [2, 36].

The obligatory two-electron reductase DT-diaphorase (NAD(P)H: quinone oxidoreductase, EC1.6.99.2) (DTD) is elevated in many tumors such as non-small-cell lung carcinoma [22]. Bioactivation of bioreductive agents by DTD to species with increased cytotoxic activity is considered central to the enzyme-directed approach to bioreductive drug development. With this approach, the aim is to design cytotoxic agents with increased tumor selectivity through activation by enzymes such as DTD, which are overexpressed in tumor cells compared to normal cells [22, 38].

DTD is pivotal in the reductive activation of mitomycin C [32], aziridinylbenzoquinones such as RH1 [37], AZQ and MeDZQ [19, 31] and the indoloquinone, EO9 [2, 3, 29], to cytotoxic species capable of alkylating and crosslinking DNA. DNA interstrand crosslink formation is considered one of the most cytotoxic lesions formed by bioreductive alkylating agents. In the case of mitomycin C, DTD-mediated reduction leads to the activation of two electrophilic sites located at the C(1) and C(10) positions of the molecule. Crosslink formation involves first the formation of a covalent bond between one of these activated sites on mitomycin C and the NH_2 at the 2 position of guanine. Subsequently, crosslink formation is completed through a second alkylation reaction of the other activated site on mitomycin C to the NH_2 at the 2 position of an adjacent guanine [35].

Depending on the ambient oxygen tension, the structure of the drug and its redox potential, and the characteristics of reduction of the quinone, both DNA strand break and crosslink formation may be important mechanisms of cytotoxicity for a given agent, and may produce synergistic effects in terms of the overall cytotoxic potency of the drug. For example, benzoquinone mustard (BM), which has a quinone group that can edox cycle and a nitrogen mustard cytotoxic element that can crosslink DNA, was compared with its aniline mustard derivative which does not have a quinone and is unable to redox cycle, or with the hydrolyzed BM derivative which has no crosslinking ability. BM was approximately 600-fold more active than its aniline mustard derivative, and 30,000-fold more active than the hydrolyzed BM derivative, against L5178Y mouse lymphoblasts [4].

Although the cytotoxic effects of benzoquinone-based bioreductive agents are thought to be due to DNA crosslink and strand break formation, there are limited data demonstrating a direct association between the cytotoxic activity of benzoquinone bioreductive agents and DTD-mediated reduction kinetics or the extent and type of DNA damage. Several reports have suggested qualitative associations between cytotoxicity of the diaziridinylbenzoquinones in different cell lines and DNA crosslink formation [10, 15, 34]. Furthermore, in experiments using a series of aziridinylbenzoquinones,

the ease of reduction by DTD has been shown to closely follow both their in vitro cytotoxicity and their ability to crosslink DNA [15]. To this end, it would be more informative to test structure-activity relationships for DNA crosslink or strand break formation using a broad range of concentrations. This would ensure that the correlations were based on drug concentrations within the linear range of the dose-response relationships, thus avoiding conclusions based on measurements in the saturation or floor portions of the dose-response curves.

At present it is unclear how varying functional group substitutions on the benzoquinone moiety of bioreductive alkylating agents may impact their cytotoxic activity and ability to form DNA crosslinks or strand breaks. Previously, we have provided evidence that electron-donating groups substituted at the C5 position of our model BM produce compounds with increased cytotoxic potency compared to BM, while sterically bulky groups at the C6 position decrease both the in vitro rate of reduction by DTD and the cytotoxic activity of the analogs compared to BM [11]. Furthermore, alkyl group substitutions to the quinone moiety of aziridinylquinones affect their crosslinking efficiency subsequent to chemical reduction [15], while functional group substitutions to cyclopent[b]indoles have led to the identification of several structure-activity relationships and the design of new bioreductive alkylating agents with increased DNA alkylating ability subsequent to reduction by DTD [33].

Currently it is unknown whether differences in kinetic parameters of reduction by DTD or alternatively, differences in effects at the DNA drug target are most important in determining the cytotoxic potency. This information would be important to increase our understanding of the mechanisms of cytotoxic activity of these agents, and for the development of future generations of quinone-based antitumor agents with increased efficacy.

The aim of the present study was to investigate structure-activity relationships between a series of BM analogs with a wide range of functional group substitutions and different mechanisms of cytotoxicity. Specifically, based on our previously established structure-activity relationships, we expanded the range of functional group substitutions to BM, to determine their effects on DTD-mediated reduction kinetics and cytotoxic potency in human tumor cell lines. We also investigated the effects of these functional group substitutions on DTD-mediated DNA crosslink or strand break formation by employing a concentration-effect study design, which would ensure that comparisons were made within the linear range of the concentration effect profiles, in order to characterize true structure-activity relationships. Furthermore, we determined whether differences produced by functional group substitutions on DTD-mediated reduction kinetics or DNA crosslink and strand break formation impacted on the cytotoxic activity of the BM analogs in human tumor cell lines.

Materials and methods

Materials

All media and fetal bovine serum were obtained from Gibco BRL (Grand Island, N.Y.). Dichloromethane, methanol, hexanes, ethanol, ethyl acetate, silica gel (1000 mesh), glacial acetic acid, anhydrous ether, hydrogen peroxide solution and potassium fluoride were from Mallinkrodt (Paris, Ky.) and Baker (Phillipsburg, N.J.). Purified recombinant human wildtype DTD was obtained from Dr. Sushu Pan (University of Pittsburgh Cancer Institute, Pittsburgh, Pa.). The DTD from Dr. Sushu Pan was used for the DNA crosslink and strand break experiments, and its activity was determined to be 33 μmol/min per mg protein, as measured by the dicoumarol-sensitive reduction of DCPIP [6]. DTD-mediated reduction experiments were carried out using the purified recombinant human wildtype DTD stock that was previously used to determine the half-times of reduction of a subset of the BM analogs in our previous study [11]. This stock of DTD was obtained from Dr. D. Ross (University of Colorado Health Sciences Center, Denver, Colo.), and its activity was determined to be 880 μmol/min per mg protein as measured by the dicoumarol-sensitive reduction of DCPIP [6]. NADH, FAD, dicoumarol, di(chloroethyl)amine hydrochloride, Tris-HCl, vanillin, chromic acid, methylbenzoquinone, fluorobenzoquinone, *t*-butyl benzoquinone, Tris (hydroxymethyl)amino-methane, Na_2EDTA and SYBR Green I nucleic acid gel stain were from Sigma-Aldrich (St. Louis, Mo.). Novagen pellet paint coprecipitant and sodium acetate were from VWR International (Mississauga, ON, Canada). Klenow fragment of DNA polymerase I and agarose were obtained from Invitrogen Canada (Burlington, ON, Canada). [α-^{32}P]dATP (6000 Ci/mmol), EcoRI and Nick columns (to separate unincorporated ^{32}P-labeled nucleotides) were purchased from Amersham Biosciences (Baie d'Urfe, QC, Canada), while cupric acetate, *N*,*N*,-dimethyl formamide and dimethyl sulfoxide were from Fisher Scientific (Fair Lawn, N.J.).

Preparation of BM analogs

The syntheses of BM and its analogs MBM, CBM, MeBM, PBM and m-PBM have been reported in full previously [11]. m-MeBM was synthesized using a combination of previously described methods [8, 21]. Di(chloroethyl)amine hydrochloride, potassium fluoride and cupric acetate were added to a solution of methylbenzoquinone [7] in 95% ethanol. The reaction mixture was stirred in the dark at room temperature for 72 h, at which time no methylbenzoquinone remained as determined by thin-layer chromatography. The mixture was filtered and the precipitate washed with ethyl acetate (30 ml). The combined organic phases were concentrated and the residual oil taken up in ethyl acetate (150 ml). This solution was washed with 0.15 *M* HCl (100 ml) and then brine. Drying over anhydrous sodium sulfate, concentration and crystallization from hot methanol afforded the 6-methylbenzoquinone mustard as red crystals (2.40 g, 22% yield), mp 90–91°C, NMR (CDCl3) 6.47 (1H, dq), 5.63 (1H, d), 3.80 (4H, m), 3.71 (4H, m), 2.03 (3H, d). The structure was confirmed by X-ray crystallographic analysis.

FBM was prepared starting from fluorobenzoquinone synthesized as follows. Fluorohydroquinone (1.00 g, 7.6 mmol) was added to a solution of ceric ammonium nitrate (8.67 g, 15.3 mmol) in distilled water (50 ml). After stirring for 1 h at room temperature, the mixture was extracted with ethyl ether (3×30 ml). The extracts were dried over sodium sulfate and concentrated affording yellow crystals of fluorobenzoquinone (760 mg), mp 77°C. The crystals rapidly darkened in the presence of light but were of satisfactory purity to be used directly in the preparation of the corresponding mustard. Di(chloroethyl)amine hydrochloride and cupric acetate were added at room temperature to a stirred solution of fluorobenzoquinone in 95% ethanol. Potassium fluoride was added, and the mixture was stirred in the dark at room temperature

for 72 h, at which time no starting fluorobenzoquinone remained as determined by thin-layer chromatography. The mixture was filtered, and the precipitate washed with ethyl acetate (3×100 ml). The combined organic phases were washed with 0.1 *M* HCl (100 ml) and then brine. Drying over anhydrous sodium sulfate and concentration afforded a red oil which was crystallized from 95% ethanol affording red crystals of 5-fluorobenzoquinone mustard (170 mg, 11% yield), mp 142–144°C. The structure was confirmed by NMR spectroscopy.

m-TBM was synthesized using previously described methods [8, 21], and starting from *t*-butylbenzoquinone as described for m-MeBM. The reaction mixture was stirred in the dark at room temperature for 72 h, at which time no *t*-butylbenzoquinone remained as determined by thin-layer chromatography. The mixture was filtered and the precipitate washed with ethyl acetate (4×20 ml). The combined organic phases were washed with 0.1 *M* HCl (100 ml) and then brine. Drying over anhydrous sodium sulfate and concentration afforded a red oil. This oil was purified by flash chromatography on silica gel using ethyl acetate-hexanes (1:2) affording pure 6-*t*-butylbenzoquinone mustard as a red oil (400 mg, 16% yield). The structure was confirmed by NMR spectroscopy.

Measurement of DNA crosslink formation

DNA interstrand crosslink formation, subsequent to bioreductive activation of BM and its analogs, was assessed based on a previously developed assay [16]. In brief, pBR322 plasmid DNA was isolated from *E. coli* (DH5 alpha) by large-scale plasmid preparations. The plasmid DNA was linearized by digestion with EcoRI and 3′-end-labeled with [α-^{32}P]dATP (5 μl, 6000 Ci/mmol) and Klenow fragment of DNA polymerase I. Unincorporated dATP was removed by column filtration and DNA was eluted in TE buffer (10 m*M* Tris, pH 7.5, 1 m*M* Na_2EDTA) at a final concentration of 1 ng/μl. Reactions were performed in 1.5-ml microfuge tubes in a final volume of 500 μl reaction buffer (25 m*M* Tris-HCl, pH 7.4). Freshly prepared NADH and FAD were added to the reaction buffer to give final concentrations of 400 μ*M* and 0.1 μ*M*, respectively. The reaction mixture was purged with nitrogen at 37°C for 30 min to create anaerobic reaction conditions [11]. DTD (prepared fresh for each experiment from −80°C frozen stock and activated with 0.01% Tween 20) at a final concentration of 6.3 ng/ml, followed by 30 ng end-labeled DNA were added to the reaction buffer. Reactions were initiated by the addition of freshly prepared BM analog in DMF (1 n*M* to 75 μ*M*, final DMF concentration of 0.2%), followed by incubation at 37°C for 15 min while anaerobic reaction conditions were maintained.

Subsequently the reactions were terminated by the addition of 2 μl Novagen pellet Paint coprecipitant and 5 μl sodium acetate (3 *M*, pH 5.2) to a 50 μl aliquot of the reaction mixture. This was followed by the addition of 100 μl 100% ethanol, and incubation at room temperature for 3 min. The reaction mixture was then centrifuged at 14,000 *g* for 10 min and the supernatant was removed. The DNA pellets were washed with 70% ethanol, centrifuged for 5 min at 14,000 *g*, followed by removal of the supernatant. The microfuge tubes were inverted and the DNA pellets were dried for 30 min. The DNA pellets were dissolved in 20 μl strand separation loading dye (35% DMSO, 1 m*M* Na_2EDTA, pH 7, 10 m*M* Tris, pH 7, 0.05% bromophenol blue, 0.05% xylene cyanol).

DNA strand separation consisted of incubation of the DNA in a 70°C water bath for 3 min, followed by immediate cooling in an ice-water bath for 30 min. The DNA was subsequently loaded onto a 1.6% agarose gel containing 0.4 μg/ml ethidium bromide, followed by electrophoresis (125 V for 30 min) in TAE buffer, pH 7 (40 m*M* Tris base, 40 m*M* acetic acid, 1 m*M* EDTA). The gel was dried at 45°C for 6 h, and the double-stranded and single-stranded conformations of DNA were visualized and quantified by densitometry using the STORM 860 image-scanning system (Molecular Dynamics, Sunnyvale, Calif.). The quantity of the crosslinked double-stranded DNA as a percent of the total amount of DNA loaded per well on the agarose gel was determined for each BM analog, and data are expressed as the means ± SEM of four to seven experiments. Visual inspection of the DNA crosslink versus BM analog concentration curves indicated a hyperbolic function. Hence data were fitted to a hyperbola equation through nonlinear regression analysis using the Statistical Package for Social Sciences (SPSS, Chicago, Ill.) version 11.0 to determine the maximum amount of DNA crosslinks formed by each BM analog.

Measurement of DNA strand break formation

DNA single-strand break formation, subsequent to bioreductive activation of BM and its analogs, was assessed based on the DNA supercoiled relaxation method which measures the conversion of the supercoiled form of plasmid to its open relaxed circular conformation [36]. Briefly, pBR322 plasmid DNA was isolated from *E. coli* (DH5 alpha) by large-scale plasmid preparations. This was followed by purification of the supercoiled conformation of the plasmid DNA by gel electrophoresis. Reactions were performed in 1.5-ml microfuge tubes in a final volume of 200 μl reaction buffer (10 m*M* Tris, pH 7.5, 1 m*M* Na_2EDTA). Freshly prepared NADH and FAD were added to the reaction buffer to give final concentrations of 1 m*M* and 0.25 μ*M*, respectively. A 1.5-inch stainless steel needle through which air was blown was inserted into the reaction tube to a position just above the reaction buffer to create aerobic reaction conditions for 30 min at 37°C. DTD (prepared fresh from −80°C frozen stock and activated with 0.01% Tween 20) at a final concentration of 6.3 ng/ml, followed by 150 ng DNA were added to the reaction buffer. Reactions were initiated by the addition of freshly prepared BM analog in DMF (1 n*M* to 100 μ*M*, final DMF concentration of 0.2%), followed by incubation at 37°C for 15 min under aerobic conditions. Control reactions incubated at 37°C for 15 min under aerobic conditions consisted of: (1) buffer, DTD, NADH, FAD, with no BM analog, and (2) buffer, NADH, FAD, BM analog, with no DTD. Reactions were terminated by the addition of 2 μl of Novagen pellet Paint coprecipitant and 20 μl sodium acetate (3 *M*, pH 5.2). This was followed by the addition of 400 μl 100% ethanol, and incubation at room temperature for 3 min. The reaction buffer was then centrifuged at 14,000 *g* for 10 min and the supernatant was removed.

The DNA pellets were washed with 70% ethanol, centrifuged for 5 min at 14,000 *g*, followed by removal of the supernatant. The microfuge tubes were inverted and the DNA pellets were dried for 30 min. The DNA pellets were dissolved in 15 μl loading dye, comprising 30% sucrose in 10 m*M* Tris-HCl, pH 8, and 1 m*M* EDTA and containing 0.1% bromophenol blue, and the total volume of dissolved DNA was loaded into a 2.5% agarose gel. Following electrophoresis (30 V for 40 h) in TAE buffer (40 m*M* Tris base, 40 m*M* acetic acid, 1 m*M* EDTA), the gel was stained with SYBR green I nucleic acid stain (0.01% in TAE buffer, pH 8) and dried at 45°C for 6 h.

The DNA was quantified by densitometry using the STORM 860 fluorescence scanning system (Molecular Dynamics). To account for the lower binding constant of SYBR green I nucleic acid stain to the supercoiled plasmid compared to the relaxed circular conformation, values for the supercoiled bands were multiplied by a factor of 1.6. This correction factor was determined densitometrically by measuring the fluorescent emissions of equal concentrations of linearized and supercoiled pBR322 plasmid DNA, subsequent to agarose gel electrophoresis and staining with SYBR green I stain [17]. The quantity of the relaxed circular conformation of DNA as a percent of the total amount of DNA loaded per well on the agarose gel for each of the BM analogs was determined, and data are expressed as means ± SEM of four to seven experiments.

Cells

NCI-H661 human non-small-cell lung carcinoma cells and SK-MEL-28 human malignant melanoma cells were obtained from the American Type Culture Collection (Rockville, Md.). The NCI-H661 cells were grown in RPMI-1640 plus 10% fetal bovine serum,

and the SK-MEL-28 human malignant melanoma cells were grown in DMEM/F12 1:1 plus 10% fetal bovine serum. The levels of DTD activity in the NCI-H661 and SK-MEL-28 cell lines were 112.7 ± 12.4 and 586.7 ± 19.6 nmol/min per mg protein, respectively [9].

Reduction of BM analogs by purified DTD

Reductions were carried out using methods identical to those described previously [11]. Briefly, reduction reactions occurred in 1 ml reaction buffer (25 m*M* Tris-HCl, pH 7.4) containing NADH (100 μ*M*) and FAD (0.5 μ*M*). These reaction mixtures were then purged with nitrogen gas or air for 3 h to attain anaerobic or aerobic reaction conditions, respectively. DTD (prepared fresh for each experiment from −80°C frozen stock of DTD and activated with 0.01% Tween 20) at a final concentration of 0.2 μg/ml, was added to the reaction mixture, and the reactions were initiated by addition of the BM analog in DMF (prepared fresh before the start of the reduction reactions, final DMF concentration of 1%) at a final concentration of 50 μ*M*. Nitrogen or air was bubbled into the reaction for the entire time course to maintain anaerobic or aerobic reaction conditions, respectively. At various times, dicoumarol (10 μ*M*) was added to terminate the reaction, and the reaction solution was immediately frozen at −80°C for HPLC analysis as described previously [11, 12].

Cytotoxicity studies

Cell lines were incubated with or without 100 μ*M* dicoumarol for 20 min at 37°C and then with BM analog for 1 h. The concentrations of each BM analog used in the experiments in the presence or absence of dicoumarol were estimated to be the concentrations required to decrease the surviving cell fractions to approximately 50% of baseline values, and were determined from the surviving cell fraction versus BM analog concentrations graphs. Cytotoxicity was determined by the MTT assay [18], and is presented as the surviving cell fraction (optical density at 540 nm of drug-treated cells as a fraction of the optical density of control cells) as we have previously described [9]. The cytotoxic activities of the BM analogs were compared with that of the parent compound BM by *t*-tests which assessed differences between the slopes of the linear regression lines of the optical density versus drug concentration curves. The D_{10} value (concentration of each BM analog that reduced the surviving cell fraction to 0.1) was calculated from the inverse of the slope of the linear regression lines of the optical density versus drug concentration curves in the absence of dicoumarol. The effects of dicoumarol on the cytotoxic activity of each BM analog in the NCI-H661 and SK-MEL-28 cell line were assessed by two-tailed *t*-tests, comparing the mean surviving cell fraction of cells treated without, or with, dicoumarol. Data are expressed as means ± SEM of 4 to 16 experiments.

Statistical analysis for DNA strand break and DNA crosslink assays

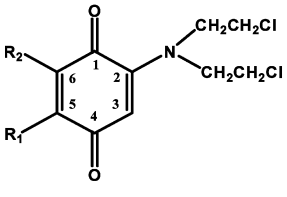
The data analysis was conducted using the Statistical Package for Social Sciences (SPSS, Chicago, Ill.) version 11.0. One-way analyses of variance (ANOVA) were performed to test the contribution of the functional group substitutions to variability in E_{10} values (extent of DNA damage at a 10 μ*M* concentration of BM analog) for DNA crosslink or DNA strand break formation in vitro. E_{10} values were used for this analysis because the extent of DNA crosslink or strand break formation at a concentration of 10 μ*M* was within the linear portion of both the DNA crosslink versus BM analog concentration and the DNA strand break versus BM analog concentration curves, respectively. The independent variable in the ANOVA was the BM analog (BM, MeBM, MBM, CBM, FBM, PBM, m-MeBM, m-PBM or m-TBM), and the dependent variable was either E_{10} for DNA crosslink formation or E_{10} for DNA strand break formation.

The normal distribution of the dependent variable (BM analog) and homogeneity of variance were tested using the Kolmogorov-Smirnov and the Levene tests, respectively. When a deviation from normal distribution or lack of homogeneity of variances for the dependent variable was found, the dependent variable was transformed (square root) and analyzed again for normal distribution and homogeneity of variances prior to ANOVA. Subsequent to ANOVA, paired multiple comparisons were performed using the Least Significant Difference (LSD) test to identify individual differences between BM and its analogs with different functional group substitutions. To assess the mechanism of variability in cytotoxicity among the BM analogs, Spearman rank-order non-parametric correlation analyses were performed between the D_{10} values for cytotoxicity and the E_{10} values for DNA crosslink or strand break formation. P values < 0.05 were accepted as statistically significant in all analyses.

Results

Description of the BM analogs

The synthesis of BM, MBM, CBM, MeBM, PBM and m-PBM have been previously reported [11]. Here we report the synthesis of new BM analogs FBM, m-MeBM and m-TBM to complete the series of BM analogs investigated (Fig. 1). MeBM, MBM, CBM, FBM and PBM all have functional group substitutions at the C5 position of the benzoquinone. Specifically, MeBM has a weak electron-donating methyl group, MBM has an electron-donating methoxy group, CBM has a weak electron-withdrawing chloro group, FBM has a weak electron-withdrawing fluoro group and PBM has a sterically bulky phenyl group substituted on the benzoquinone. In contrast, m-MeBM, m-PBM and m-TBM have functional group substitutions at the C6 position of the benzoquinone. m-MeBM has a weak electron-donating methyl group, m-PBM has a sterically bulky phenyl group, and m-TBM has a very large sterically bulky tertiary-butyl group attached to the benzoquinone moiety. The analogs were studied to identify the effect of functional groups on the mechanisms of cytotoxicity of the analogs subsequent to activation by DTD, on the



BM analog	R_1	R_2
BM	H	H
MeBM	CH_3	H
MBM	OCH_3	H
m-MeBM	H	CH_3
CBM	Cl	H
FBM	F	H
PBM	Phenyl	H
m-PBM	H	Phenyl
m-TBM	H	$C(CH_3)_3$

Fig. 1 Molecular structures for BM and its structural analogs

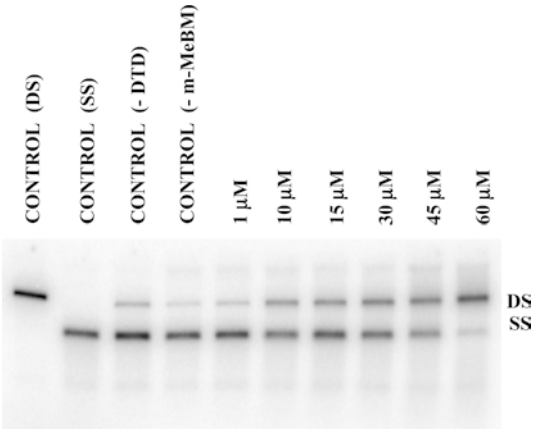


Fig. 2 Autoradiogram of a 1.6% neutral agarose gel showing concentration-dependent DNA interstrand crosslink formation in linearized pBR322 DNA by m-MeBM. DNA was incubated for 15 min in 25 m*M* Tris-HCl, pH 7.4, with increasing concentrations of m-MeBM with 6.3 ng/ml DTD under anaerobic conditions. DNA markers were: *control (DS)* linearized pBR322 plasmid not subjected to strand separation at 70°C, *control (SS)* linearized pBR322 plasmid subjected to strand separation at 70°C. Control reaction mixtures were: *control (*−DTD) incubated in the absence of DTD in the presence of m-MeBM (75 μM), *control (*−m-MeBM) incubated in the presence of DTD without m-MeBM. *DS* double-stranded DNA, *SS* single-stranded DNA

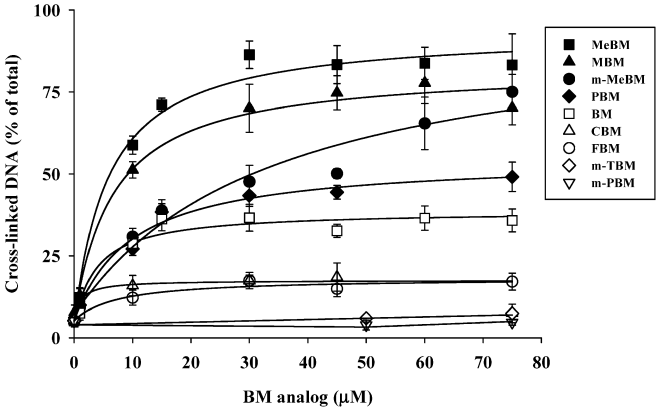


Fig. 3 Concentration-dependence of DTD-mediated interstrand DNA crosslink formation by the BM analogs in linearized pBR322 DNA. Linearized pBR322 plasmid was incubated for 15 min with increasing concentrations of each of the BM analogs, and purified recombinant human DTD (6.3 ng/ml) in 25 m*M* Tris-HCl buffer under anaerobic conditions. Baseline reaction mixtures did not contain BM analog. The DNA was denatured at 70°C and run on a 1.6% neutral agarose gel and the percentage of crosslinked DNA was determined by densitometric analysis. The data are presented as means ± SEM of four to seven experiments

Table 1 Maximum levels of DNA interstrand crosslink formation in linearized pBR322 DNA following reduction of the BM analogs by DTD. Linearized pBR322 plasmid was incubated for 15 min with increasing concentrations of each of the analogs, and purified recombinant human DTD (6.3 ng/ml) in 25 mM Tris-HCl buffer under anaerobic conditions. Baseline reaction mixtures did not contain BM analog. The DNA was denatured at 70°C and run on a 1.6% neutral agarose gel and the percentage of crosslinked DNA was determined by densitometric analysis. Data were fitted to a hyperbola equation through nonlinear regression analysis to determine the maximum amount of DNA crosslinks formed by each BM analog

BM analog	Maximum level of crosslinked DNA (% of total)
BM	32.1
MeBM	88.9
MBM	77.2
m-MeBM	89.3
CBM	11.5
FBM	10.9
PBM	49.6
m-PBM	0
m-TBM	2.2

Table 2 DTD-mediated interstrand DNA crosslink formation by the BM analogs in linearized pBR322 DNA. Linearized pBR322 plasmid was incubated for 15 min with increasing concentrations of each of the analogs, and purified recombinant human DTD (6.3 ng/ml) in 25 m*M* Tris-HCl buffer under anaerobic conditions. Baseline reaction mixtures did not contain BM analog. The DNA was denatured at 70°C and run on a 1.6% neutral agarose gel and the percentage of crosslinked DNA was determined by densitometric analysis. Data are presented as the level of crosslinked DNA formed by each of the BM analogs at a 10 μ*M* concentration (E_{10} value) in the presence of DTD. The E_{10} value is equal to the percent of the total amount of DNA loaded per well on the agarose gel that is in the crosslinked conformation and is corrected for baseline values of DNA crosslink formation. The E_{10} values of the BM analogs were compared to that of BM by one-way ANOVA, followed by post-hoc LSD tests. Data are presented as means ± SEM of four to seven experiments (*NS* not significant)

BM analog	BM analog concentration (μ*M*)	DNA crosslink formation (% of total)	*P* value
BM	10	23.5 ± 1.8	
MeBM	10	53.6 ± 2.8	< 0.05
MBM	10	46.0 ± 2.5	< 0.05
m-MeBM	10	25.6 ± 2.6	NS
CBM	10	14.1 ± 4.0	< 0.05
FBM	10	7.0 ± 2.3	< 0.05
PBM	10	21.9 ± 2.0	NS
m-PBM	10	1.5 ± 0.7	< 0.05
m-TBM	10	1.4 ± 0.7	< 0.05

reduction kinetics of the benzoquinone group by DTD and on the cytotoxic activity of the analogs.

DNA crosslink formation by the BM analogs

All BM analogs except m-PBM and m-TBM produced a concentration-dependent increase in DNA interstrand crosslink formation in linearized pBR322 plasmid DNA, as measured by an agarose gel assay (Fig. 2), following reduction by DTD under anaerobic conditions (Fig. 3). Functional group substitutions produced differences in the maximum levels of DNA crosslinks that ranged from 0% to 89.3% (Table 1). E_{10} values displayed the rank order MeBM≈MBM > m-MeBM≈PBM≈BM > CBM > FBM > m-PBM≈m-TBM (Table 2). DTD-mediated reduction of MeBM and MBM led to the formation of DNA-damaging species with an increased ability to

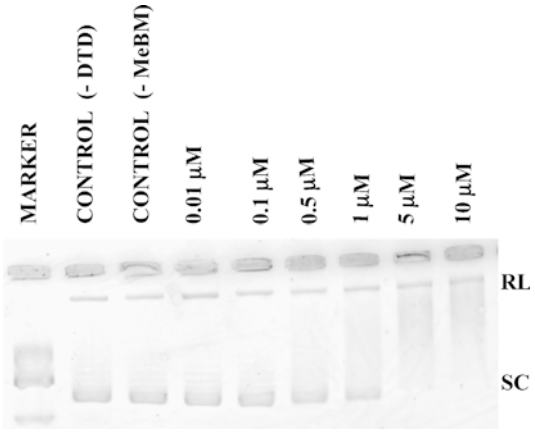


Fig. 4 Autoradiogram of a 2.5% neutral agarose gel showing concentration-dependent DNA single-strand break formation in supercoiled pBR322 DNA by MeBM. DNA was incubated for 15 min in 10 m*M* TAE buffer, pH 7.5, with increasing concentrations of MeBM and 6.3 ng/ml DTD under aerobic conditions. Control reaction mixtures were: *control* (*−DTD*) incubated in the absence of DTD in the presence of MeBM (10 μ*M*), *control* (*−MeBM)* incubated in the presence of DTD without MeBM. Molecular weight markers were λDNA digested with *Hin*d III (*RL* relaxed circular DNA, *SC* supercoiled DNA)

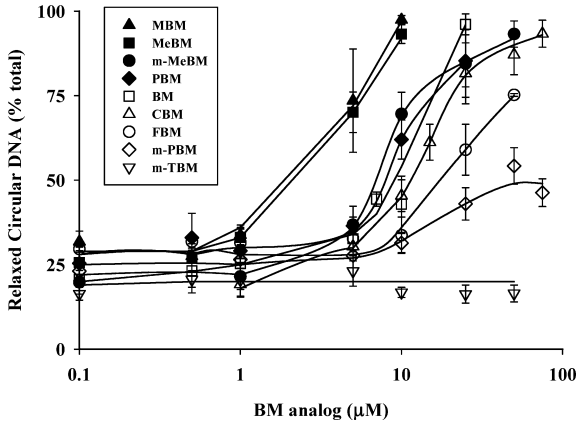


Fig. 5 Concentration-dependence of DTD-mediated single-strand break formation by the BM analogs in supercoiled pBR322 DNA. PBR322 plasmid was incubated for 15 min with increasing concentrations of each of the BM analogs, and purified recombinant human DTD (6.3 ng/ml) in 10 m*M* TEA buffer (pH 7.5) under aerobic conditions. Baseline reaction mixtures did not contain BM analog. The DNA was run on a 2.5% neutral agarose gel and the percentage of relaxed circular DNA was determined by densitometric analysis. The data are presented as means ± SEM of four to seven experiments

produce DNA crosslinks compared to BM, with E_{10} values for MeBM and MBM of 53.6 ± 2.8% and 46.0 ± 2.5%, respectively, compared to an E_{10} value of 23.5 ± 1.8% for BM ($P < 0.05$, Table 2). Bioactivation of m-MeBM and PBM by DTD resulted in DNA crosslink formation that was similar to that produced by BM, while CBM and FBM produced fewer DNA crosslinks than BM ($P < 0.05$, Fig. 3). In contrast, incubation of m-PBM and m-TBM with DTD produced no DNA crosslinks. None of the BM analogs produced DNA crosslinks in the absence of DTD.

DNA strand break formation by the BM analogs

All BM analogs, except m-TBM, produced concentration-dependent increases in DNA single-strand break formation in supercoiled PBR322 plasmid DNA, as measured by an agarose gel assay (Fig. 4), following reduction by DTD under aerobic conditions (Fig. 5). E_{10} values for the BM analogs displayed the rank order MeBM > MBM > m-MeBM > PBM > BM≈CBM > FBM > m-PBM≈m-TBM. MeBM, MBM, m-MeBM and PBM, with E_{10} values of 72.6 ± 4.3%, 62.8 ± 1.3%, 48.7 ± 4.1% and 39.6 ± 8.7% (mean ± SEM), respectively, produced DNA single-strand breaks more readily than the parent compound BM (24.4 ± 2.7%; $P < 0.05$, Table 3). The E_{10} values for DNA strand break formation were similar for BM and CBM (Table 3); FBM and m-PBM produced fewer DNA strand breaks than BM ($P < 0.05$, Table 3), while m-TBM did not produce any DNA strand breaks. None of the BM analogs produced DNA strand breaks in the absence of DTD.

Table 3 DTD-mediated single strand break formation by the BM analogs in supercoiled pBR322 DNA. PBR322 plasmid was incubated for 15 min with increasing concentrations of each of the analogs, and purified recombinant human DTD (6.3 ng/ml) in 10 m*M* TEA buffer (pH 7.5) under aerobic conditions. Baseline reaction mixtures did not contain BM analog. The DNA was run on a 2.5% neutral agarose gel and the percentage of relaxed circular DNA was determined by densitometric analysis. Data are presented as the amount of relaxed circular DNA formed by each of the BM analogs at a 10 μ*M* concentration (E_{10} value). The E_{10} value is equal to the percent of the total amount of DNA loaded per well on the agarose gel that is in the relaxed circular conformation and is corrected for baseline values of DNA strand break formation. The E_{10} values of the BM analogs were compared to that of BM by one-way ANOVA, followed by post-hoc LSD tests. Data are presented as means ± SEM of four to seven experiments (*NS* not significant)

BM analog	BM analog concentration (μ*M*)	DNA strand break formation (% of total)	*P* value
BM	10	24.4 ± 2.7	
MeBM	10	72.6 ± 4.3	< 0.05
MBM	10	62.8 ± 1.3	< 0.05
m-MeBM	10	48.7 ± 4.1	< 0.05
CBM	10	29.0 ± 4.0	NS
FBM	10	7.5 ± 5.0	< 0.05
PBM	10	39.6 ± 8.7	< 0.05
m-PBM	10	5.7 ± 2.0	< 0.05
m-TBM	10	2.2 ± 2.2	< 0.05

Reduction of BM analogs by purified DTD

The reduction kinetics of two novel BM analogs, FBM and m-TBM were determined in this study. Data on half-times of reduction of the other BM analogs investigated in the present study have been reported previously [11]. The in vitro reduction of the BM analogs (50 μM) by purified human recombinant DTD in the

Table 4 Cytotoxic activity of the BM analogs in NCI-H661 human non-small-cell lung cancer cells and SK-MEL-28 human melanoma cells. Cells were incubated with BM analog for 1 h. Cytotoxicity was determined by the MTT assay and is presented as the D_{10} value (concentration of each BM analog which reduced the surviving cell fraction to 0.1). D_{10} was calculated from the inverse slope of the linear regression lines of the optical density versus BM analog concentration curves. Slopes of the linear regression lines were compared by two-tailed *t*-tests comparing the differences in the slopes between BM and its analogs. Data are presented as the means ± SEM of 4 to 16 experiments (*NS* not significant)

BM analog	NCI-H661 cell line			SK-MEL-28 cell line		
	D_{10} (μ*M*)	*P* value	Reference	D_{10} (μ*M*)	*P* value	Reference
BM	4.87 ± 0.14		11	7.57 ± 0.76		11
MeBM	0.56 ± 0.18	< 0.05	11	0.76 ± 0.02	< 0.001	11
MBM	0.40 ± 0.02	< 0.001	11	1.39 ± 0.04	< 0.05	11
m-MeBM	0.45 ± 0.02	< 0.001		2.31 ± 0.13	< 0.05	
CBM	1.67 ± 0.15	< 0.05	11	4.71 ± 0.45	NS	11
FBM	5.30 ± 0.41	NS		17.70 ± 2.92	< 0.05	
PBM	2.16 ± 2.65	< 0.05	11	3.11 ± 0.27	< 0.05	11
m-PBM	5.05 ± 1.37	NS	11	7.32 ± 0.37	NS	11
m-TBM	40.7 ± 3.97	< 0.001		106 ± 9.42	< 0.001	

Table 5 Cytotoxic activity of the BM analogs in NCI-H661 human non-small-cell lung cancer cells and SK-MEL-28 human melanoma cells in the absence and presence of dicoumarol (DIC). Cells were incubated without or with 100 μ*M* DIC for 20 min and then with BM analog for 1 h. The concentration of each BM analog used was estimated to be the concentration required to decrease the surviving cell fraction to approximately 0.5. Cytotoxicity was determined by the MTT assay and is presented as the surviving cell fraction (optical density in BM analog-treated cells as a fraction of the optical density in control cells). The results are presented as the means ± SEM of 4 to 16 determinations. The effect of DIC on the cytotoxic activity of each BM analog in each cell line was compared by two-tailed *t*-tests comparing the significance of the differences of the mean surviving cell fraction of cells treated without or with DIC (*NS* not significant)

BM analog	NCI-H661 cell line				SK-MEL-28 cell line				Reference
	Drug dose (μ*M*)	Surviving cell fraction		*P* value	Drug dose (μ*M*)	Surviving cell fraction		*P* value	
		−DIC	+DIC			−DIC	+DIC		
BM	2	0.37 ± 0.08	0.06 ± 0.01	< 0.05	2	0.74 ± 0.05	0.20 ± 0.09	< 0.001	11
MeBM	0.01	0.75 ± 0.07	0.58 ± 0.09	NS	0.2	0.56 ± 0.07	0.28 ± 0.08	< 0.05	11
MBM	2	0.09 ± 0.01	0.20 ± 0.02	< 0.05	2	0.10 ± 0.03	0.28 ± 0.06	< 0.05	11
m-MeBM	0.25	0.69 ± 0.04	0.37 ± 0.07	< 0.05	2	0.54 ± 0.04	0.06 ± 0.01	< 0.001	
CBM	2	0.24 ± 0.06	0.30 ± 0.06	NS	2	0.54 ± 0.06	0.38 ± 0.10	NS	11
FBM	1	0.58 ± 0.08	0.41 ± 0.09	NS	5	0.44 ± 0.02	0.29 ± 0.06	< 0.05	
PBM	0.25	0.56 ± 0.09	0.55 ± 0.11	NS	1	0.62 ± 0.10	0.63 ± 0.10	NS	11
m-PBM	2	0.48 ± 0.06	0.31 ± 0.05	NS	2	0.77 ± 0.06	0.70 ± 0.10	NS	11
m-TBM	30	0.51 ± 0.06	0.60 ± 0.03	NS	30	0.70 ± 0.05	0.68 ± 0.04	NS	

presence of cofactors FAD and NADH was carried out under anaerobic or aerobic conditions to study redox cycling. The extent of reduction was measured using HPLC to follow the loss of enzyme cofactor, NADH. Under hypoxic conditions, approximately half (one equivalent) of the NADH present in the reaction solution was consumed with FBM, and NADH decreased from 1.0 to 0.6 ± 0.1 nmol (mean ± SEM) over the 90-min incubation period. The half-time of reduction for FBM was determined to be 4 min. In contrast, a very small amount of NADH was consumed under hypoxic conditions in the case of m-TBM, indicating that this compound was reduced slowly by DTD, and to a significantly lesser extent, compared to the other BM analogs studied. When FBM was reduced by purified DTD under aerobic conditions, greater than one equivalent of NADH was consumed over the 90-min incubation time, with NADH values decreased from 1.0 to 0.1 ± 0.1 nmol (mean ± SEM), indicating redox cycling. In contrast, slow redox cycling was observed with m-TBM, compared to the other BM analogs studied.

Cytotoxic activity of the BM analogs and the relationship to DNA crosslink and strand break formation

The cytotoxic activities of three new BM analogs (m-MeBM, FBM and m-TBM) were determined by the MTT assay in NCI-H661 human non-small-cell lung cancer cells and SK-MEL-28 human melanoma cells, which have moderate and high levels of DTD, respectively. The cytotoxic activities of the other BM analogs, determined using identical methods and cell lines to those used in the present study, have been reported previously [11] (Table 4). m-MeBM had a

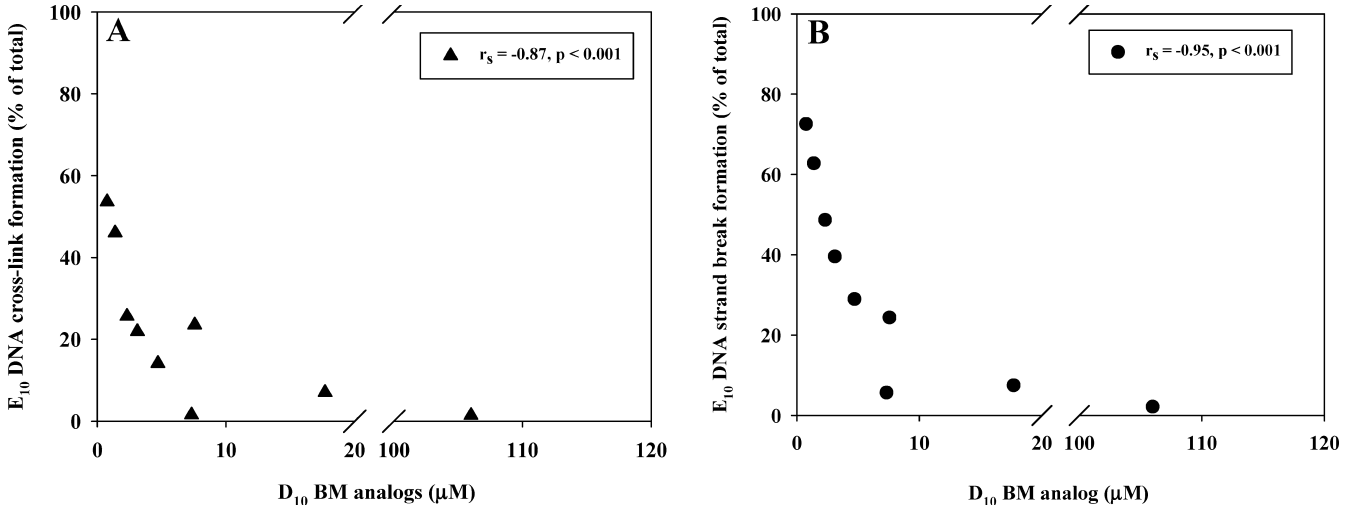


Fig. 6A, B Association between cytotoxic potency for each BM analog in the SK-MEL-28 cell line and DNA interstrand crosslink formation (**A**) or DNA single-strand break formation (**B**)

cytotoxic activity greater than that of BM in both the NCI-H661 ($P < 0.05$, Table 4) and SK-MEL-28 ($P < 0.001$, Table 4) cell lines. In contrast, FBM had a cytotoxic activity similar to that of BM in the NCI-H661 cell line, whereas its cytotoxicity was less than that of BM in the SK-MEL-28 cell line ($P < 0.05$, Table 4). The cytotoxic activity of m-TBM was less than that of BM in both cell lines ($P < 0.001$, Table 4). Pretreatment of the cell lines with dicoumarol (100 μ*M*) increased the cytotoxic potency of m-MeBM in both the NCI-H661 ($P < 0.05$, Table 5) and SK-MEL-28 ($P < 0.001$, Table 5) cell lines. Dicoumarol did not affect the cytotoxicity of FBM in the NCI-H661 cell line, but increased its cytotoxic activity in the SK-MEL-28 cell line ($P < 0.05$, Table 5). In contrast, the cytotoxic potency of m-TBM was not influenced by dicoumarol in either cell line (Table 5).

The E_{10} values of the BM analogs for DNA crosslink and DNA strand break formation correlated significantly with the D_{10} values for cytotoxic activity in both the NCI-H661 and the SK-MEL-28 cell lines. For the SK-MEL-28 cell line, the correlation coefficients (r_s) were −0.87 ($P < 0.001$, Fig. 6A) and −0.95 ($P < 0.001$, Fig. 6B) for DNA crosslink and strand break formation, respectively, while for the NCI-H661 cell line, the r_s values were –0.87 ($P < 0.05$) for crosslink formation and −0.92 ($P < 0.05$) for strand break formation. In addition, a significant correlation was observed between the maximum levels of DNA crosslinks formed and the D_{10} values for cytotoxicity, with r_s values of −0.85 ($P < 0.05$) for the NCI-H661 cell line and −0.81 ($P < 0.05$) for the SK-MEL-28 cell line. No significant correlations were found between the half-time of reduction of the BM analogs by DTD and DNA crosslink formation, DNA strand break formation or cytotoxic potency of the analogs ($P > 0.05$).

Discussion

The characterization of quantitative structure-activity relationships between a range of compounds and biological endpoints requires the use of a concentration-response study design which allows comparisons between compounds within the linear range of the concentration-effect profiles. Otherwise, the study conclusions may be subject to potential confounding due to, for instance, saturation of the target biological system at excessively high drug concentrations (ceiling effect) or alternatively due to a floor effect secondary to a concentration threshold which may need to be surpassed in order to produce the desired biological effect. With this in mind, we measured DNA crosslink and DNA strand break formation produced by a range of concentrations of BM analogs having a wide variety of functional groups, following reduction by DTD. We compared the effects of the functional groups on DTD-mediated DNA crosslink and strand break formation at a 10 μ*M* concentration of the BM analogs, which was within the linear range of the concentration-effect profiles. DTD-mediated DNA crosslink and strand break formation were significantly influenced by the characteristics and position of functional group substitutions on the benzoquinone moiety of BM.

Although the effects of functional group substitutions on quinone-based bioreductive alkylating agents have been previously reported in relation to effects on DNA sequence-specific alkylation [14, 19, 23], there are limited quantitative data on the effects of various functional groups on DNA crosslink formation. For instance, the level of DNA crosslinks produced following reduction of quinone methide aziridinylquinones decreased as the length of the methylene chain at the C5 position increased. However, it is not clear whether these relationships were determined at concentrations within the linear portion of the respective concentration-effect profiles [23].

The mechanism by which nitrogen mustards may form either DNA intra- or interstrand crosslinks involves aziridinium ion formation by each nitrogen mustard group which can subsequently react with the N7 position on guanine residues within opposite or the same strands of DNA [25]. In the present study, DTD-mediated reduction was required in order to observe the concentration-dependent increase in crosslinked DNA products with the BM analogs: reaction mixtures without the presence of DTD but in the presence of the highest concentration of each analog utilized in the concentration-effect studies produced only a small background level of crosslinked DNA products.

Our results indicate that the electron-donating methoxy and methyl groups, when substituted at the C5 position of the benzoquinone moiety, increased DTD-mediated crosslink formation compared to BM at the 10 μM concentration. The electron-donating effects of these functional groups may enhance activation of the nitrogen mustard groups, by facilitating leaving of the chloride groups, thus allowing aziridinium ion formation with greater ease. In fact, the electron-donating effects appear also to facilitate the intrinsic efficacy for crosslink formation, as the maximum levels of DNA crosslink formation by MeBM, MBM and m-MeBM were increased by more than twofold compared to that of BM. The E_{10} value for crosslink formation for m-MeBM was similar to that of BM, and therefore the activating effect observed appears more effective when the electron-donating groups are substituted in the para-position versus the meta-position to the cytotoxic element. In contrast, there was no difference observed between maximum levels of DNA crosslink formation by m-MeBM, MBM and MeBM, and therefore this parameter does not appear to be influenced by the position of the functional group substitution.

Electron-withdrawing groups substituted at the C5 position of the benzoquinone moiety decreased DTD-mediated crosslink formation compared to that by BM. Furthermore, there was a decrease in maximum levels of DNA crosslink formation by CBM and FBM, with values approximately one-third of that observed with BM. It is likely that these results are due to the inactivating effects of electron-withdrawing groups on the nitrogen mustard, an effect that would hinder leaving of the chloro groups, and thus, the formation of the aziridinium ion and crosslink formation.

The maximum levels of crosslinked DNA formed by analogs with sterically bulky groups at the C6 position of the benzoquinone were similar to the baseline values. Therefore, limited or no crosslinked DNA was formed following reduction of these analogs by DTD. This observation was not surprising for m-TBM, as this analog was reduced by DTD the slowest of all compounds studied, and therefore should not have been activated through this pathway to a significant extent. m-PBM was reduced more slowly than BM [11]. Although its rate of reduction was slow compared to that of BM, some DNA crosslinks would have been expected after the 15-min incubation of m-PBM with the DNA, as this compound did produce DNA strand breaks, which indicated redox cycling of the reduced product. Thus, the substitution of the phenyl group at the C6 position on the benzoquinone may produce steric interference, preventing crosslink formation. Substitution of the phenyl group at the C5 position allowed DNA crosslink formation with a concentration-effect profile similar to that of BM. Therefore, the steric hindrance which prevented DNA crosslink formation in m-PBM appeared to be limited to the C6 position of the benzoquinone.

The mechanism of induction of DNA strand breaks by DZQ is thought to be through reoxidation of the hydroquinone formed by reduction via DTD, and subsequent generation of reactive oxygen species [12, 24, 36]. Previously we have reported that electron-donating group substitutions on the benzoquinone moiety lead to compounds with increased potential to redox cycle subsequent to reduction by DTD, compared to BM and its analogs with electron-withdrawing or sterically bulky group substitutions [11]. In the present study, DTD-mediated DNA strand break formation was enhanced in compounds with electron-donating groups substituted on the benzoquinone moiety when compared to BM. The enhanced DNA strand break formation may have resulted from the electron-donating groups modifying the hydroquinone moiety to enhance its interaction with oxygen, leading to increased reactive intermediates. These intermediates include the semiquinone, which is formed due to one-electron oxidation, and reactive oxygen species. The electron-donating groups may presumably also affect the stability and the potential of the semiquinone to alkylate DNA. For instance, electron-donating groups, especially at the C5 position, could act to push the unpaired electron towards the cytotoxic groups at the C2 position, an effect which would lead to increased DNA alkylation. The observation that electron-donating groups led to compounds with an increased potential to produce DTD-mediated crosslinks and strand breaks contributes to the hypothesis that DNA strand break formation may be enhanced by the close proximity of the analog to DNA following crosslink formation.

The weak electron-withdrawing chloro group of CBM did not affect DNA strand break formation, which was similar to that of BM at the 10 μM concentration. In contrast, there was a decrease in DNA strand break formation observed with FBM, compared to BM at the 10 μM concentration. The reason for the difference in the effect of the two weak electron-withdrawing groups is unknown.

We observed an increase in DNA strand break formation with PBM, but analogs with sterically bulky groups at the C6 position were less efficient in producing DNA strand breaks than BM subsequent to reduction by DTD. The increase in DNA strand break formation observed with PBM was unexpected, and may have been

due to stabilization of the semiquinone by an electron-donating effect of the phenyl group when substituted in the para position to the mustard groups. However, this hypothesis needs further study. Substitutions of sterically bulky groups at the C6 position of the benzoquinone led to compounds with decreased DNA strand break formation. This was expected for m-TBM, as it is reduced very slowly by DTD. In the case of m-PBM, this compound did produce DNA strand breaks, but the level was decreased when compared to its positional isomer PBM. Several factors may be responsible for this observation. The absence of DNA crosslink formation by m-PBM may lead to less-efficient DNA strand break formation as a result of redox cycling and reactive oxygen species generation at positions further removed from the DNA. Furthermore, the rate of reduction of m-PBM was slower than that of PBM [11], which may lead to a decrease in the amount of reduced hydroquinone available to redox cycle and to produce DNA strand breaks.

Our previous study indicated that electron-donating or -withdrawing effects of functional groups are not important in affecting the rate of reduction of the BM analogs by DTD as both types of functional groups decreased the rate of reduction to a similar extent. In contrast, the steric effects of functional groups are most important in affecting DTD-mediated reduction kinetics [11]. To confirm these findings we synthesized FBM which has an electron-withdrawing fluorine group substitution, but no steric effects as the fluorine is sterically similar in size to the hydrogen at the C5 position of the benzoquinone moiety in BM. The half-time of reduction of FBM was the same as that of the parent structure BM, providing further evidence that electronic effects are not important in affecting the rate of reduction by DTD. Moreover, m-TBM, which has a very large sterically bulky group substituted at the benzoquinone was reduced very slowly by DTD. This result is consistent with our previous studies indicating that steric effects at the C6 position of the benzoquinone are important in decreasing the rate of reduction by DTD, presumably by steric interactions in the active site [11]. Similar results have been observed in studies with a series of indoloquinones, where large functional group substitutions at the 5 position (equivalent to the C6 position in our analogs), led to compounds that were poor substrates for DTD [28].

Previously we have reported that the cytotoxicity of CBM is equivalent to that of BM in the SK-MEL-28 cell line but is increased in the NCI-H661 cell line [11]. Here our results indicated that the cytotoxic activity of FBM was similar to or less than that of BM in the NCI-H661 and SK-MEL-28 cell lines, respectively. Furthermore, DNA crosslink formation was decreased for both CBM and FBM compared to that for BM, and DNA strand break formation was decreased for FBM but not altered for CBM compared to BM. The contrast in effects of CBM and FBM on mechanisms of DNA damage may explain the differing cytotoxic potencies of these compounds. The inefficient activation of m-TBM by DTD was supported by its decreased cytotoxic activity compared to BM in both cell lines. We also assessed the cytotoxicity of FBM and m-TBM in the presence and absence of dicoumarol to determine the overall contribution of DTD-mediated reduction to cytotoxic potency. The cytotoxic potency of FBM was similar in the presence or absence of dicoumarol in the NCI-H661 cell line, but was increased in the presence of dicoumarol in the SK-MEL-28 cell line, suggesting that in cell lines with high levels of DTD, this enzyme may act to inactivate FBM. The cytotoxic potency for m-TBM in the presence of dicoumarol was similar to that in the absence of dicoumarol, indicating that DTD does not contribute to its activation.

Previous results indicate that MeBM has a greater cytotoxic activity than BM, and that DTD is an inactivating enzyme for this compound [11]. Here, we synthesized m-MeBM to assess whether these observations were unique to substitution of the methyl group at the C5 position. In fact, m-MeBM did show greater cytotoxic potency than BM, and DTD-mediated reduction also appeared to be an inactivating pathway for this compound, indicating that the effects due to the methyl group substitution were not unique to its substitution on the C5 position on the benzoquinone. However, the inactivation effect of DTD observed with MeBM and m-MeBM could not be generalized to all electron-donating groups as DTD-mediated reduction acts as an activating pathway for MBM [11].

Although it has been suggested that the cytotoxic effects of quinone-based bioreductive agents are due to DNA crosslink and strand break formation, there are limited experimental data demonstrating a direct association between the cytotoxic activity of benzoquinone-based bioreductive agents and DTD-mediated reduction kinetics or the extent and type of DNA damage. The two-electron reduction of mitomycin C and a series of aziridinylbenzoquinones has been shown to be a bioactivation process, and leads to the formation of cytotoxic metabolites that produce DNA crosslinks and strand breaks. Such DNA damage is inhibited by dicoumarol, and the compounds are less toxic in the DTD-deficient BE cell line [12, 32]. In a series of indoloquinones, DNA strand break formation following reduction by human DTD has been found not to be a major mechanism of cytotoxicity, as cytotoxicity did not correlate with the extent of DNA strand break formation [28]. Although these reports provide some evidence on the mechanism of cytotoxicity associated with quinone analogs, correlations were often established at a single drug concentration, which may potentially lead to confounding effects.

A number of factors may affect the cytotoxic effects of specific classes of bioreductive alkylating agents include functional group characteristics, rate of reduction by bioactivating enzymes, oxygen radical generation, rate and extent of DNA alkylation and other types of DNA damage. It is not clear from current knowledge which factors are important in determining the overall

cytotoxicity. In this study, we found that both DTD-mediated crosslink formation and single-strand break formation positively correlated with the cytotoxic potency in two different cell lines for a broad range of BM analogs. This provides further quantitative evidence that DNA strand break and crosslink formation by the reduced products of BM analogs are important factors in the mechanism of cytotoxicity of these agents.

Electron-donating functional group substitutions at the C5 and C6 position of the quinone led to analogs with a greater cytotoxicity than BM, and a greater production of DNA crosslinks and strand breaks. Substitution of electron-withdrawing groups produced compounds that were less cytotoxic than compounds with electron-donating groups, while these groups produced either no effect or decreased the levels of DNA crosslink and strand break formation compared to BM.

PBM had a greater cytotoxic potency than BM but its concentration-DNA crosslink profile was similar to that of BM. In contrast, PBM did show greater DNA strand break formation than BM, which could explain the greater cytotoxicity than BM. Sterically bulky groups at the C6 position of the benzoquinone led to compounds with lower cytotoxic potency than BM, and decreased DNA crosslink and strand break formation.

Despite the correlation between cytotoxic effects and both DNA crosslink and DNA strand break formation, there was no correlation between DTD-mediated reduction and cytotoxicity or between reduction and DNA crosslink or strand break formation. This suggests that further downstream events following activation of analogs to cytotoxic metabolites are most important in influencing the cytotoxic character of the analogs. The structure-activity relationships identified in this study may be helpful in the design of new bioreductive agents with the right balance between efficacy in production of DNA damage and affinity to bioreductive activating enzymes such as DTD for improved antitumor activity.

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