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ORIGINAL ARTICLE

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Structure-activity study of the interaction of bioreductive benzoquinone alkylating agents with DNA topoisomerase II

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Abstract *Purpose*: Quantitative structure-activity studies were performed on a series of benzoquinone mustard (BM) bifunctional alkylating agents to determine whether DNA topoisomerase II (topo II) inhibition was responsible for cell growth inhibition. *Methods*: Topo II inhibition was evaluated by decatenation and agarose gel electrophoresis assays. *Results*: The BM compounds were shown to potently inhibit the decatenation activity of topo II. Though BM compounds promoted the formation of protein-DNA complexes in isolated nuclei and cells, this effect was undiminished when levels of topo II varied. The BM compounds had little activity in a topo II-mediated DNA cleavage assay, suggesting that they do not function as topo II poisons. Rather, BM-induced protein-DNA complex formation was likely due to the bifunctional alkylating reactivity of these compounds. Finally, the growth inhibitory properties of these compounds did not correlate with their ability to inhibit topo II, indicating that these compounds did not exert their cellular activity through inhibition of topo II. Some BM compounds reacted very quickly with glutathione and cysteine, likely initially through an electrophilic Michael addition. In the absence of cysteine, the growth inhibitory effects of BM were increased tenfold, indicating the modulatory effect of cysteine sulfhydryl adducts. EPR studies showed that a semiquinone-free radical was produced by some BM compounds. *Conclusions*: BM compounds likely exert their action through DNA cross-linking and/or by inducing oxidative stress. Although topo II is not a direct target of these agents, this enzyme may play a role in processing the consequences of direct DNA adduction and/or oxidative DNA damage.

Keywords Bioreductive alkylating agents · Topoisomerase II · Cytochrome P450-reductase · EPR · DNA · Quinone

Abbreviations BM: Benzoquinone mustard · BSA: Bovine serum albumin · CC: Closed circular DNA · CHO: Chinese hamster ovary cell line · DMSO: Dimethyl sulfoxide · DTT: Dithiothreitol · DZR: Dexrazoxane-resistant cell line derived from the parent CHO cell line · NQO1: (DT-diaphorase), NAD(P)H: quinone oxidoreductase, EC 1.6.99.2 · EDTA: Ethylenediaminetetraacetic acid · EPR: Electron paramagnetic resonance · Na_2EDTA: Disodium EDTA · GSH: Glutathione · Hepes: N-[2-hydroxyethyl]piperazine-N′-[2-ethanesulfonic acid] · IC_{50}: 50% inhibitory concentration · kDNA: Kinetoplast DNA · LIN: Linear pBR322 DNA · LUMO: Energy of the lowest unoccupied molecular orbital · MTT: 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide · NC: Nicked circular DNA · ROS: Reactive oxygen species · RLX: Relaxed pBR322 DNA · SC: Supercoiled pBR322 DNA · SDS: Sodium dodecyl sulfate · TAE: Tris base (4 mM)/glacial acetic acid (0.11% (v/v))/Na_2EDTA (2 mM) buffer Tris, tris(hydroxymethyl)aminomethane · UV: Ultraviolet

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Introduction

Bioreductive anticancer drugs undergo reductive activation and/or electrophilic Michael addition in vivo to produce cytotoxic species [8, 30, 32]. The bioreductive alkylating agents contain, in addition to a functional group that can be reduced and undergo redox cycling, an alkylating functionality that is activated upon reduction. One-electron reduction of quinones by reductases can yield a semiquinone-free radical that can reduce oxygen to form superoxide, which in turn dismutates to hydrogen peroxide and can produce the extremely damaging hydroxyl radical in the presence of iron [8, 30, 32]. Likewise two-electron reduction by NQO1 can produce the hydroquinone which can reoxidize to produce ROS [8, 13, 14, 32]. Bioreductive alkylating agents have good antitumor activity, particularly towards hypoxic solid tumors [43]. We have previously reported on the NQO1 reduction and activation of a series of benzoquinone bifunctional bioreductive agents under hypoxic and oxic conditions [14], and more recently we showed that these benzoquinone alkylating agents were activated by NQO1 to produce DNA cross links and strand breaks [13].

Topoisomerase II alters DNA topology by catalyzing the passing of an intact DNA double helix through a transient double-stranded break made in a second helix and is critical for relieving torsional stress that occurs during replication and transcription and for daughter strand separation during mitosis [12, 26]. Several widely used anticancer agents, including doxorubicin and other anthracyclines, amsacrine, etoposide and mitoxantrone, also target topoisomerase II and are thought to be cytotoxic because they are topoisomerase II poisons [4, 12, 15, 26]. A number of studies have shown that a variety of quinones interact with and inhibit the activity of topoisomerase II [17, 18, 24, 25, 41]. The electrophilic reaction of the quinone with critical sulfhydryl groups on topoisomerase II are thought, in part, to be responsible for the inhibition of topoisomerase II [18, 24, 41]. The potency of quinone-induced topoisomerase IIα-mediated DNA damage has been shown to parallel the rate of reaction with glutathione [41]. What is less clear is whether quinones in general, or only certain quinones, have the ability to stabilize a covalent topoisomerase II-DNA intermediate (the cleavable complex) and act as topoisomerase II poisons [7, 10, 12, 15, 26, 27]. We have also recently shown using a proteomics approach that cisplatin may be inhibiting topoisomerase II by reacting with critical free sulfhydryl groups [23]. Earlier studies indicated that the toxic benzene metabolite benzoquinone does not stimulate production of cleaved linear DNA [24], while a recent study indicated that this quinone is a topoisomerase II poison [27]. The orthoquinone etoposide metabolite [17, 18] and some substituted quinones [41] and β-lapachone [16, 31] do stimulate topoisomerase II-mediated cleavage reactions in an isolated enzyme system. Given this literature we have carried out a structure-activity study of the interaction of topoisomerase II with a series of benzoquinone alkylating analogs in order to determine if these compounds exert their growth inhibitory effects by targeting topoisomerase II.

Materials and methods

Materials

pBR322 plasmid DNA was obtained from MBI Fermentas (Burlington, Canada) or New England Biolabs (Beverly, MA) and kDNA from Topogen (Columbus, OH). The human recombinant NADPH cytochrome P450-reductase (EC 1.6.2.4) was from Gentest (Woburn, MA). The indolequinone EO9 was a gift from Dr. H. R. Hendricks, New Drug Development Office, European Organization for Research and Treatment of Cancer, Amsterdam. Unless indicated, other chemicals were from Sigma (St. Louis, Missouri). Except where indicated, the errors quoted are standard errors from non-linear least squares analysis (SigmaPlot, SPSS, Chicago, IL). The preparation and characterization of the other BM analogs (Fig. 1) has been previously described [14]. The bisBM analog was prepared as described [28] and its structure confirmed by ^{1}H-NMR.

Kinetics of the reaction of BM analogs with glutathione, cysteine, DTT or BSA

The reaction of these sulfhydryl compounds with the BM analogs was followed spectrophotometrically by repeated spectral scanning on a Cary 1 double beam

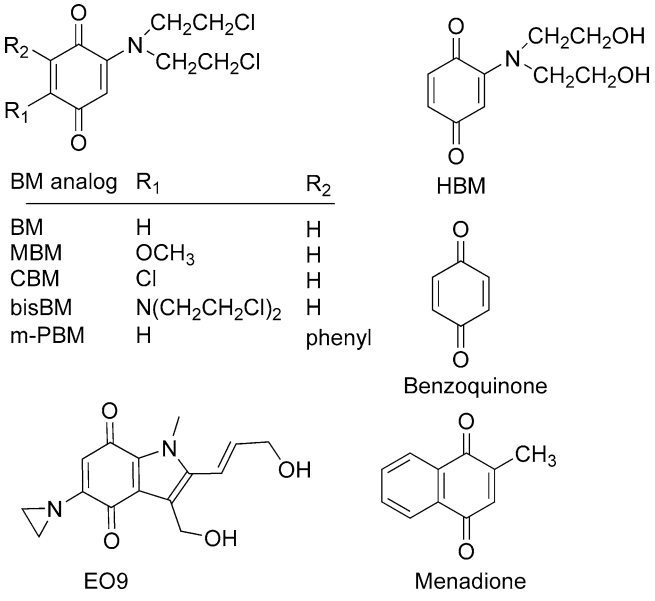


Fig. 1 Molecular structures of the benzoquinone mustard BM and its analogs, EO9, benzoquinone and menadione

spectrometer (Varian, Mississauga, Canada) with a thermostated cell compartment. The reactions were initiated by adding a small volume of stock sulfhydryl compound to 1 ml of freshly prepared 100 μM BM analog in a 1 cm silica cell.

Cell culture, growth inhibition assays, cell nuclei and nuclear extract preparation

Chinese hamster ovary (CHO) cells (type AA8; ATCC CRL-1859), obtained from the American Type Culture Collection (Rockville, MD), and DZR cells (a dexrazoxane-resistant CHO cell line with a point mutation in topoisomerase IIα) [22, 44] were grown in minimum essential medium (α-MEM; Invitrogen, Burlington, Canada) containing 20 mM Hepes (Sigma, St. Louis, MO), 100 units/ml penicillin G, 100 μg/ml streptomycin, 250 ng/ml amphotericin B, 10% fetal bovine serum (Invitrogen) in an atmosphere of 5% CO_2 and 95% air at 37°C (pH 7.4). For the measurement of cytotoxicity using the MTT assay, cells in exponential growth were harvested and seeded at either 2,000 cells/well (CHO) or 6,000 cells/well (DZR) in 96-well plates (200 μl/well) and were allowed to attach for 24 h. The drugs were dissolved in DMSO or dimethylformamide. When organic solvents were used for solubility, the final concentration did not exceed 0.5% (v/v). The cells were continuously incubated with the drugs for 72 h, or for the time indicated, and then assayed with MTT. The spectrophotometric 96-well plate cell growth inhibition MTT assay, which measures the ability of the cells to enzymatically reduce MTT after 72 h of treatment to various concentrations of drugs, has been described [21]. Four replicates were measured at each drug concentration and the IC_{50} values for growth inhibition were measured by fitting the absorbance-drug concentration data to a four-parameter logistic equation as described [21].

Human leukemia K562 cells, obtained from the American Type Culture Collection (Rockville, MD) and K/VP.5 cells (a 26-fold etoposide-resistant K562-derived cell line with decreased levels of topoisomerase IIα protein and mRNA) [11, 35] were maintained as suspension cultures in DMEM (Invitrogen, Grand Island, NY) containing 10% fetal calf serum and 2 mM L-glutamine. Nuclear extracts from CHO or K562 cells in exponential growth were prepared as described previously [20]. Nuclei from K562 and K/VP.5 cells were prepared as described previously [36].

Computational methodologies

The structures were first molecular-mechanics (MM2) energy minimized with the Chem3D module of ChemDraw 8 Ultra (CambridgeSoft, Cambridge, MA) on a PC-compatible computer. The optimized geometries were then fully minimized using the AM1 Hamiltonian of the semi-empirical molecular orbital program CS MOPAC Pro Chem3D (CambridgeSoft). The Wang-Ford calculated atomic charges and LUMO energies were computed from these minimized structures.

Protein–DNA complexes

Protein–DNA complex formation in intact cells and in isolated nuclei was measured as previously described [37]. Mid-log growth K562 or K/VP.5 cells were labeled for 24 h with 0.5 μCi/ml [methyl−^{3}H]thymidine (0.5 Ci/mmol) and 0.1 μCi/ml [^{14}C] leucine (318 mCi/mmol) in DMEM containing 7.5% (v/v) fetal calf serum. Cells were then pelleted and resuspended in fresh DMEM/7.5% calf serum and incubated for 1 h at 37°C. Cells were pelleted and resuspended in buffer (pH 7.4) containing 115 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 5 mM NaH_2PO_4, 25 mM HEPES and 10 mM glucose at 37°C at a final cell density of 1.0×10^6 cells/ml for experimentation. For isolation of nuclei, cells were washed in ice-cold buffer A (1 mM KH_2PO_4 5 mM $MgCl_2$ 150 mM NaCl, 1 mM EGTA, pH 6.4). Cells were then resuspended in 1 ml of buffer A, lysed by the addition of 9 ml of buffer B (0.3%(w/v) Triton X-100 in buffer A), and incubated on ice for 30 min. After lysis, 40 ml of buffer A was added, and nuclei were pelleted by centrifugation at 150*g* for 10 min. The density of nuclei was adjusted to 1×10^6/ml in 37°C (pH 7.4) buffer A containing 1 mM ATP for experimentation. Cells or nuclei were then incubated with the BM compounds, etoposide, or 0.4% DMSO (control). Reactions were stopped by adding 1 ml of cell or nuclei suspension to 10 ml of ice-cold PBS. Cells or nuclei were then pelleted, lysed, cellular DNA sheared, and protein-DNA complexes precipitated with SDS and KCl as described in [37]. Protein–DNA complexes were quantified by scintillation counting, and ^{3}H-DNA was normalized to cell number using the co-precipitated ^{14}C-labeled protein as an internal control.

Topoisomerase IIα preparation

A high copy yeast expression vector for production and purification of human topoisomerase IIα in yeast was constructed. A 2μ plasmid pEG(KT) bearing the *GAL* promoter, the URA3 marker for selection, and the *leu2-d* allele was utilized for plasmid amplification of a high copy yeast expression vector. Beginning with a centromeric plasmid pYX113 containing full-length human topoisomerase IIα cDNA, a SacI restriction site 5′ to the ATG start site of topoisomerase IIα was engineered to facilitate excision of full-length topoisomerase IIα using a single restriction enzyme since a SacI site is present 1 Kb downstream of the topoisomerase IIα coding sequence. SacI digestion of the engineered pYX113 plasmid-liberated topoisomerase IIα which was ligated into the SacI site of pEG(KT) downstream of the *GAL* promoter to yield the pSY3 topoisomerase IIα

expression plasmid. Transformation of pSY3 into the protease-deficient topoisomerase I-negative yeast strain JelΔ Top1 (*Mat* a, *trp1, leu2, ura-52, pBR322 DNA-1122, pep4–3, his3::PGAL10-GAL4, TOP1::LEU2*) that was made auxotrophic for leucine by inserting the *his3* gene in the LEU2 locus was followed by selection under URA− conditions. Yeast were grown in leucine-free media to promote plasmid amplification followed by addition of galactose for induction. Full-length human topoisomerase IIα was extracted and purified as described previously [38]. Final DTT concentration used in purification and storage buffers was 500 μM. For DNA cleavage assays described below, enzyme was diluted into final experimental mixes so that the final DTT concentration was 7.6 μM.

kDNA decatenation assays and pBR322 DNA relaxation and cleavage assays

A modified and improved spectrofluorometric decatenation assay was used to determine the inhibition of topoisomerase II by the BM analogs [5, 19]. kDNA consists of highly catenated networks of circular DNA. Topoisomerase II decatenates kDNA in an ATP-dependent reaction to yield individual minicircles of DNA. The 20 μl reaction mixture contained 0.5 mM DTT, 0.5 mM ATP, 50 mM Tris–HCl (pH 8.0), 120 mM KCl, 10 mM $MgCl_2$, 30 μg/ml BSA, 40 ng kDNA and 5 ng of CHO nuclear extract, the amount that gave approximately 80% decatenation. The assay incubation was carried out at 37°C for 20 min and was terminated by the addition of 12 μl of 250 mM Na_2EDTA. Samples were centrifuged at 8,000*g* at 25°C for 15 min and 20 μl of the supernatant was added to 180 μl of 600-fold diluted PicoGreen dye (Molecular Probes, Eugene, OR) in a 96-well plate. The fluorescence, which was proportional to the amount of kDNA, was measured in a BMG Fluostar Galaxy (Durham, NC) fluorescence plate reader using an excitation wavelength of 485 nm and an emission wavelength of 520 nm.

Topoisomerase II-cleaved DNA complexes produced by anticancer drugs may be trapped by rapidly denaturing the complexed enzyme with SDS [9]. The cleavage of double-stranded closed circular pBR322 DNA to form linear DNA was followed by separating the SDS-treated reaction products using ethidium bromide gel electrophoresis as described in [9]. The 20 μl cleavage assay reaction mixture contained K562 nuclear extract or topoisomerase IIα protein, pBR322 plasmid DNA, 0.5 mM ATP and drug (1 μl in DMSO) in assay buffer (10 mM Tris, 50 mM KCl, 50 mM NaCl, 0.1 mM EDTA, 5 mM $MgCl_2$, 2.5% (v/v) glycerol, pH 8.0). The order of addition was BM analog, a buffer/ATP/DNA mixture and then topoisomerase II (1 μl). The reaction mixture was incubated at 37°C for 10 min and quenched with 1% (v/v) SDS/25 mM Na_2EDTA. The reaction mixture was treated with 0.25 mg/ml proteinase K (Sigma) at 55°C for 30 min to digest the protein. The linear pBR322 DNA cleaved by topoisomerase II was separated by electrophoresis (2 h at 8 V/cm) on a TAE ethidium bromide (0.5 μg/ml) agarose gel (1.2%, wt/v). The DNA in the gel was imaged by its fluorescence on a Alpha Innotech (San Leandro, CA) Fluorochem 8900 imaging system equipped with a 365 nm UV illuminator and a CCD camera or using a Stratagene Eagle Eye II (La Jolla, CA).

EPR experiments on BM and its analogs

A freshly prepared 15 μl aliquot of the drug in buffer was injected into a 8 cm length of gas-permeable Teflon tubing which was then folded at both ends and inserted into a quartz EPR tube open at both ends, and placed in the EPR cavity as described in [6]. The EPR spectra were recorded with a Bruker (Milton, Canada) EMX EPR spectrometer. Pre-purified grade thermostated (37°C) argon (400 l/h) was flowed continuously over the sample while the spectra were recorded. Recording of the first-derivative EPR spectra was started approximately 3 min after the sample was prepared. A total of 10 spectra (42 s/scan) were recorded and their signals averaged. For recording of the spectra the instrument settings were: microwave power 20 mW, modulation frequency 100 kHz, microwave frequency 9.24 GHz, modulation amplitude 2.0 G, time constant 0.02 s, gain 20,000, 1024 data points/scan, magnetic field centered at 3,315 G, and a 50 G scan range.

Results

The BM compounds inhibit cell growth and inhibit the catalytic activity of topoisomerase II

The structures of the BM compounds and others utilized are shown in Fig. 1. In the experiments shown in Fig. 2a, BM potently inhibited the cell growth of both the CHO and DZR cell lines. The DZR cell line contains one-half the level of topoisomerase IIα compared to parental CHO cells and it contains a point mutation in the enzyme that imparts high fold resistance to bisdioxopiperazine catalytic inhibitors of topoisomerase II. As shown in Fig. 2b, BM also potently inhibited the decatenation activity of CHO nuclear extract topoisomerase II. The IC_{50} values for cell growth inhibition of CHO and DZR cells, and inhibition of topoisomerase II catalytic activity of the BM analogs and benzoquinone and menadione are given in Table 1. As shown by this data the DZR cells were not cross-resistant to the growth inhibitory properties of the BM compounds. Because this cell line contains one-half the level of topoisomerase II, as compared with the parental CHO cells [22], this result suggests that the BM compounds do not act as topoisomerase II poisons. We previously showed that the DZR cell line was

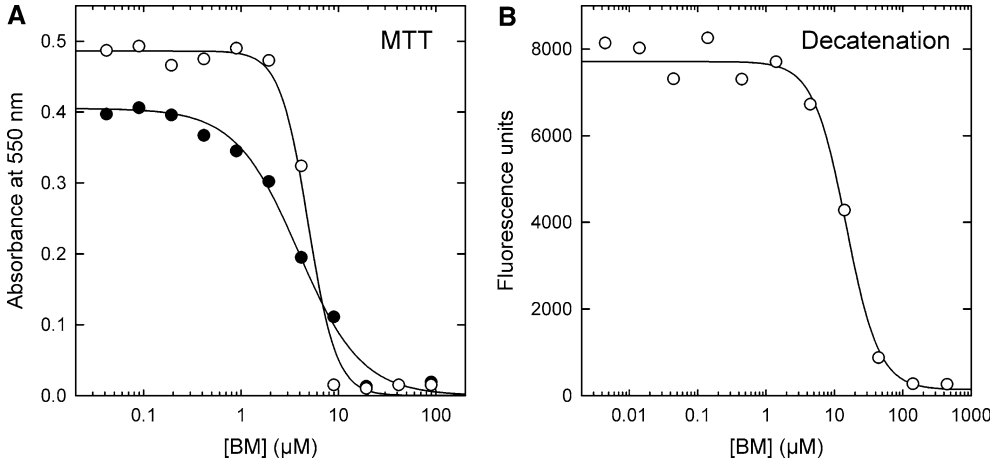


Fig. 2 BM inhibits CHO and DZR cell growth and the catalytic decatenation activity of topoisomerase II. **a** Inhibition of growth of CHO (○) and DZR (●) cells by BM. Cells were treated with BM for 72 h prior to the assessment of growth inhibition by an MTT assay. The *curved solid lines* are non-linear least squares fits to a 4-parameter logistic equation and yield IC_{50}'s of 4.9 ± 0.3 and 3.9 ± 0.5 μM, respectively for CHO and DZR cells. **b** Effect of BM on the catalytic decatenation activity of CHO topoisomerase II nuclear extract determined by the fluorescence decatenation assay as described in the Methods section. The *curved solid line* is a non-linear least squares fit to a 4-parameter logistic equation and yields an IC_{50} of 15 ± 2 μM

8.5-fold cross-resistant to the growth inhibitory effects of the topoisomerase II poison etoposide [22]. HBM, which has non-alkylating hydroxyethyl groups different from the other BM analogs, was the weakest inhibitor of topoisomerase II and the least active in inhibiting cell growth. This result suggested that the chloroethyl alkylating groups of the BM analogs were critical for their activity. The unsubstituted benzoquinone and menadione (which lacks alkylating side chains) were about 10-fold less potent in inhibiting topoisomerase II compared to BM, the most potent BM compound studied, though they were slightly more potent in inhibiting cell growth (Table 1). The cell growth inhibitory properties of the BM analogs in CHO and DZR cells parallel those seen for NCI-H661 human non-small-cell lung carcinoma and SK-MEL-28 human malignant melanoma cells that we previously determined [13, 14].

Reaction of BM analogs with glutathione, cysteine, DTT or BSA

It is well known that quinones can react with sulfhydryl compounds at rates that vary depending on the quinone [1, 33, 41, 42]. Thus, we decided to investigate the reactivity of the BM analogs with cysteine and glutathione, which are both present in cells, and with BSA which has a single free cysteine group, and with DTT, which was present in our topoisomerase II nuclear extract and in our purified topoisomerase IIα stock solutions. At pH 8, BM reacted very quickly (within the mixing time) with glutathione (Fig. 3a), cysteine (Fig. 3b) and DTT (Fig. 3c), but reacted slowly with BSA (Fig. 3d). BSA contains a single reactive free cysteine sulfhydryl group that is more sterically hindered than the low-molecular weight sulfhydryl compounds and thus would be expected to react more slowly than the low-molecular weight sulfhydryl compounds. The reactions of BM with glutathione, cysteine and DTT were all characterized by a fast ($t_{1/2} < 1$ min) initial drop in absorbance at 495 nm, which was then followed by much slower increases in absorbance in both the UV and

Table 1 Inhibition of topoisomerase II and CHO and DZR cell growth inhibitory effects of a series of quinones[a]

Quinone	Topoisomerase II IC_{50} (μM)	CHO IC_{50} (μM)	DZR IC_{50} (μM)	LUMO (eV)	Vinylic carbon partial charge
BM	15 ± 2	4.9 ± 0.3	3.9 ± 0.5	−1.7646	−0.187
MBM	89 ± 2	0.14 ± 0.07	0.12 ± 0.02	−1.7332	−0.381
CBM	18 ± 2	4.7 ± 0.2	5.2 ± 0.3	−1.9789	−0.090
HBM	280 ± 70	> 600	> 600	−1.5839	−0.193
BisBM	240 ± 60	2.3 ± 0.2	1.9 ± 0.1	−1.5618	−0.347
m-PBM	340 ± 150	2.2 ± 0.1	3.2 ± 0.3	−1.6299	−0.157
Benzoquinone	97 ± 30	2.6 ± 0.8	1.4 ± 0.2	−1.7296	−0.184
Menadione	105 ± 30	4.5 ± 0.4	2.5 ± 0.2	−1.5001	−0.264

[a]The IC_{50} for topoisomerase II inhibition was measured in a decatenation assay and the IC_{50} for CHO and DZR growth inhibition was measured in an MTT assay after 72 h of continuous exposure to the drug. The Wang-Ford calculated partial charge on the most electropositive vinylic carbon atom was obtained using MOPAC as were the LUMO values

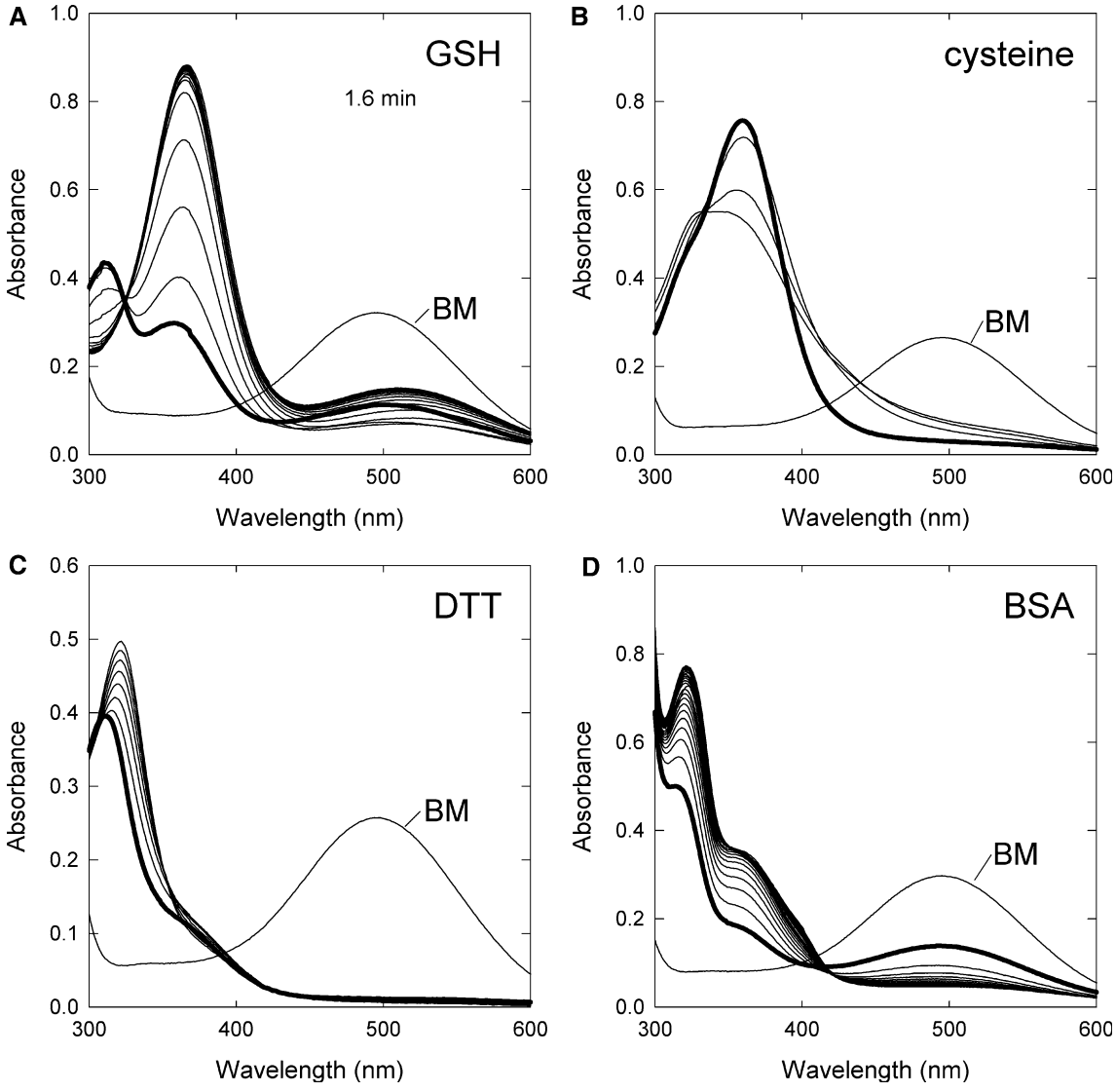


Fig. 3 BM reacts with glutathione, cysteine, DTT and BSA. In each case the spectrum-labeled BM was recorded prior to addition of reactant and the spectrum with the thick line was recorded at the time indicated after addition of reactant. **a** Spectral changes occurring upon the addition of 100 μM BM to 100 μM glutathione (pH 8.0, 25°C). The spectrum-labeled BM was recorded before the addition of glutathione, the second 0.8 min (*thick line*) after the addition, and the subsequent spectra every 1.6 min. The absorbance of BM at the peak maximum of 495 nm rapidly decreased with time and then slowly increased. **b** Spectral changes occurring upon the addition of 100 μM BM to 100 μM cysteine (pH 8.0, 37°C). The spectrum-labeled BM was recorded before the addition of cysteine and the second (*thick line*) and subsequent spectra at 3 min intervals. The absorbance of BM at the peak maximum of 495 nm rapidly decreased with time and then slowly increased. **c** Spectral changes occurring upon the addition of 100 μM BM to 200 μM DTT (pH 8.0, 37°C). The first spectrum was recorded before the addition of DTT, the second (*thick line*) and subsequent spectra at 3 min intervals. The absorbance of BM at the peak maximum of 495 nm rapidly decreased with time and then slowly increased. **d** Spectral changes occurring upon the addition of 100 μM BM to 100 μM BSA (pH 8.0, 37°C). The first spectrum was recorded before the addition of BSA, the second (*thick line*) and subsequent spectra at 3 min intervals. The absorbance of BM at the peak maximum of 495 nm continuously decreased with time

visible regions. The lack of an isosbestic point in the spectrum of the reaction of BM with glutathione, cysteine, DTT and BSA indicates that there were more than two species present in solution. The slow second reaction of BM with glutathione (but not with cysteine or DTT) did display an isosbestic point at 325 nm, suggesting that the second slow reaction produced only a single product. It is, however, not possible to rule out further reactions with GSH that do not yield changes in the isosbestic point. The initial adduct of the reaction of GSH with benzoquinone, which has been characterized as a 1,4-reductive Michael addition, has been shown to be unstable even in the absence of oxygen with an intramolecular cyclization occurring by involving the α-amino group producing ultimately two characterized products and other multiple polymerization products [1]. Thus, it was probable that glutathione, cysteine and DTT likewise reacted with BM to undergo similar complex secondary reactions after an initial fast Michael addition reaction. DTT could also potentially cyclize

with BM as it is a dithiol. The reaction of BM with GSH was also followed at pH 4 and 5 in 10 mM acetate buffer and it was observed that the reaction slowed sufficiently ($t_{1/2} \sim 3.5$ min and ~ 1 min, respectively) and thus the decrease in absorbance at 495 nm with time could be followed (results not shown). This result is in accord with the studies of the pH dependence of GSH with a napthoquinone [42], and is likely due to the glutathione thiolate anion being the reactive species. Of the other BM analogs studied, CBM reacted quickly with DTT and GSH ($t_{1/2} < 1$ min, spectral data not shown). m-PBM and HBM, also reacted quickly with DTT ($t_{1/2} \sim 1$ min) but more slowly with GSH ($t_{1/2} > 10$ and 30 min, respectively). No reaction of MBM was detected with DTT or GSH. Thus, as has been reported for other quinones [33, 42], the BM quinones, depending upon their substitution pattern, also varied considerably in their reactivity with various sulfhydryl compounds.

The BM compounds induce the formation of protein-DNA complexes

Anticancer drugs that stabilize a covalent topoisomerase II-DNA intermediate (the cleavable complex) act as topoisomerase II poisons [12, 26]. One method by which cancer cells become resistant to topoisomerase II poisons is by lowering their level or activity of topoisomerase II [12, 36]. With less topoisomerase II, cells produce fewer DNA strand breaks in the presence of topoisomerase II poisons and are less lethal. These cell lines provide a convenient way to test whether a drug that inhibits topoisomerase II acts as a topoisomerase II poison. Conversely, a lack of change in the sensitivity of a putative topoisomerase II poison to a cell line with a lowered topoisomerase II level can be taken to indicate that poisoning of topoisomerase II is not a significant mechanism for this particular agent. We previously showed that the K/VP.5 cell line with acquired resistance to etoposide contained one-fifth the topoisomerase IIα content of the parental K562 cells [11, 35]. The K/VP.5 cell line provided the means to determine if the BM compounds were topoisomerase II poisons as well as catalytic inhibitors of topoisomerase II. We investigated the ability of these compounds to produce topoisomerase II protein–DNA complexes both in K562 and K/VP.5 nuclei and whole cells as previously described in [22, 37]. As shown in Fig. 4a and b, bisBM and especially BM and MBM were all effective in producing increases in protein–DNA complexes. BM was even more effective than etoposide in producing protein–DNA complexes. The etoposide-resistant K/VP.5 cell line [11, 35] displayed approximately the same pattern of protein–DNA complex formation as the parental K562 cell line in response to the BM compounds, while the level of etoposide-induced protein DNA–complexes was reduced in K/VP.5 compared to K562 cells and nuclei as expected. These results suggest that the protein–DNA complexes produced by the BM compounds were not topoisomerase II protein-DNA complexes. The fact that the non-alkylating HBM analog produced only a low-fold increase in protein–DNA complexes in both nuclei and cells suggests that the increases in BM-, MBM-, and bisBM-induced protein–DNA complexes were due to the bifunctional alkylating properties of these alkylat-

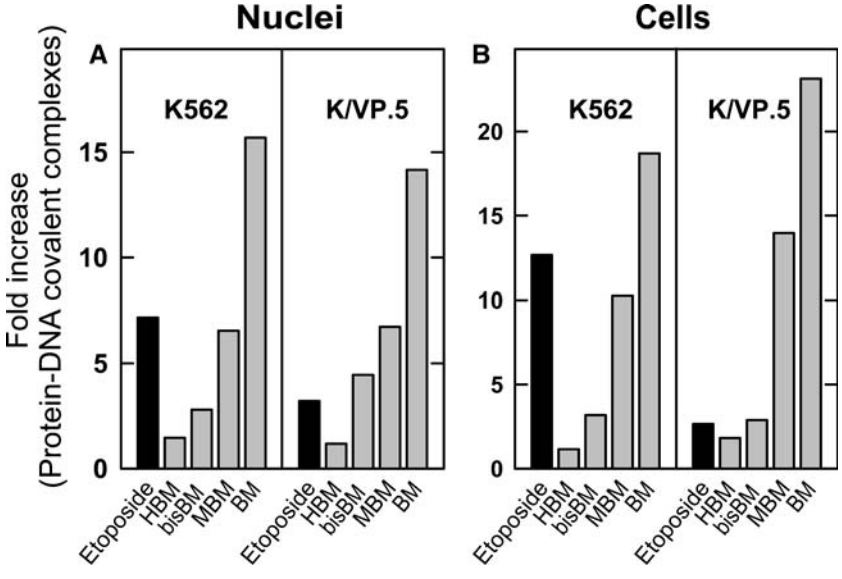


Fig. 4 Fold-increase in the formation of protein–DNA complexes in K562 and K/VP.5 nuclei and cells after treatment with various benzoquinone mustards (100 μM) and etoposide (50 μM). In both nuclei (**a**) and cells (**b**), bisBM, MBM, and BM greatly increased the amount of complex formation in both K562 and K/VP.5 nuclei and cells, while HBM only slightly increased the amount of protein–DNA complex formation. Etoposide also greatly increased the amount of complex formation in both K562 nuclei (**a**) and cells (**b**). However, the amount of etoposide-mediated complex formation was greatly reduced in K/VP.5 nuclei (**a**) and cells (**b**) that contain one-fifth the amount of topoisomerase IIα compared to K562 nuclei or cells

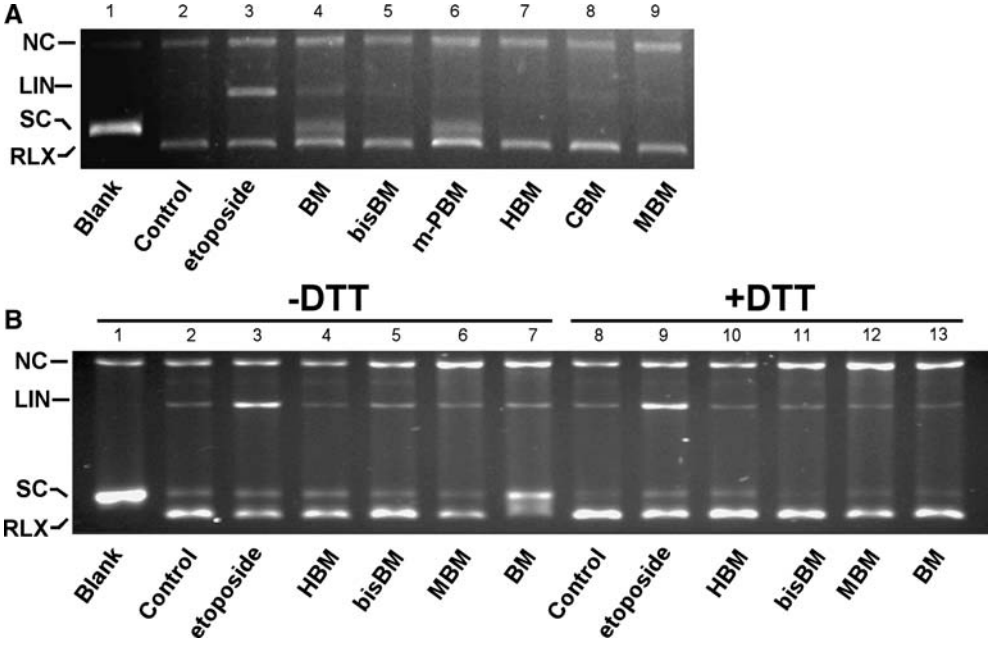
ing BM analogs. Incubation of nuclei for 30 min in the absence or presence of 10 mM GSH, prior to addition of 100 μM BM, resulted in a dramatic reduction in protein–DNA complexes from 13.9 ± 1.8-fold over control (in the absence of GSH) to 1.6 ± 0.6-fold increase in the presence of GSH (Mean ± SEM for five experiments; data not shown). These results are consistent with results from Figs. 3 and 4, indicating reaction of the BM analogs with GSH and cysteine and modulation of growth inhibitory activity subsequent to formation of sulfhydryl adducts.

Fig. 5 Effect of the benzoquinone mustards and etoposide on the human topoisomerase II nuclear extract and topoisomerase-IIα-mediated relaxation and cleavage of pBR322 DNA in the presence and absence of added 500 μM DTT. **a** The fluorescent image of the ethidium bromide-stained gel shows the effect on relaxation and cleavage of pBR322 DNA (100 ng) after treatment of topoisomerase II K562 cell nuclear extract (200 ng protein in 20 μl) with 200 μM of the drugs indicated. Etoposide treatment produced a significant amount of linear DNA, while only BM and the other quinones produced only a small amounts of linear DNA. All of the quinones increased the amount of nicked DNA. BM and m-PBM were the most effective in inhibiting the relaxing activity of topoisomerase II. The DTT concentration in the assay mixture was 12.5 μM. **b** The effect on relaxation and cleavage of pBR322 DNA (200 ng) after the treatment of topoisomerase IIα (250 ng protein in 20 μl) with 100 μM of the benzoquinone mustard compounds or 50 μM etoposide. The drugs were preincubated with pBR322 DNA in the assay buffer at room temperature in the absence or presence of added DTT (500 μM). Topoisomerase IIα (200 ng in 20 μl) was then added for a further 10 min at 37°C. When DTT was not added, the DTT concentration in the assay mixture was derived from the purified stock topoisomerase IIα and was present at a final concentration of 7.6 μM. In this assay the benzoquinone mustard compounds produced only small, but detectable, amounts of linear DNA above control levels either in the presence or absence of added DTT

The effects of the BM compounds on topoisomerase II-mediated double strand breaks

A cleavage assay [9], which tests for the formation of topoisomerase II-mediated drug-induced linear DNA, was also carried out to test whether the BM compounds were topoisomerase II poisons. As shown in lane 3 (Fig. 5a), using topoisomerase II nuclear extract, the topoisomerase II poison etoposide induced linear DNA formation from supercoiled pBR322 DNA as expected. BM did induce a very small amount of linear DNA (~ 7% of that found for etoposide, by densitometry), while the other BM analogs produced little or no detectable linear DNA. BM and m-PBM were effective in preventing the conversion of supercoiled pBR322 to relaxed closed circular DNA consistent with inhibition of topoisomerase II catalytic (relaxation) activity (Fig. 5a). BM and the other compounds (Fig. 5a) also induced formation of nicked circular DNA. Because the DTT concentration in the assay mixture was 12.5 μM (from the nuclear extract stock solutions) compared to 200 μM for the drug, only a small amount of the drug could have reacted with DTT in the assay mixture.

Similar experiments were also performed using purified human topoisomerase IIα in the presence and absence of a preincubation of the drugs and pBR322 DNA with 500 μM DTT (Fig. 5b). The results indicated that bisBM, MBM, and BM induced a small amount of DNA double strand cleavage (maximally for BM, ~20% of that found for etoposide) compared to the DMSO Control (absence of drug). The final DTT concentration in the assay mixture was 7.6 μM (derived from addition of purified topoisomerase IIα) compared to 100 μM for the drugs used. Only a small amount of each agent, therefore, would have reacted with DTT in the assay mixture. Hence, DTT reduction of the quinone mustards was not responsible for the low activity of these agents



as topoisomerase IIα poisons. Preincubation of drug with DTT enhanced the amount of nicked circular DNA that is observed with these compounds. Of the BM compounds tested, only BM inhibited relaxation activity (lane 7). DTT pretreatment largely antagonized the ability of BM to inhibit catalytic activity and to stimulate DNA cleavage (lane 13). The sulfhydryl adducts of the quinones are also well known to be reactive through the formation of the reactive hydroquinone [1, 8, 32], and it is likely that DTT activated these quinones to produce reactive intermediates and ROS that resulted in the formation of nicked circular DNA. Taken together, the results of these experiments suggest that the BM compounds were not effective topoisomerase II poisons and that the protein–DNA complexes induced in cells and nuclei (Fig. 4) were likely due to the bifunctional alkylating properties of these compounds.

The growth inhibitory effects of BM were increased in cysteine-free growth medium

The α-MEM growth medium used to obtain the growth inhibitory data of Table 1 contains 0.57 mM cysteine. The spectrophotometric results of Fig. 3b showed that cysteine reacted quickly with BM, which raised the possibility that cysteine in the growth medium could affect the growth inhibitory effects of BM. Thus, in order to test this possibility, experiments were conducted in which attached CHO cells were treated with BM for 1 h in PBS/1% glucose medium that contained neither cysteine nor 0.57 mM cysteine. After washing off extracellular drug, the cells were then allowed to grow for a further 72 h. As shown in Fig. 6, in the absence of cysteine in the medium, the IC_{50} for growth inhibition decreased tenfold, from 3.0 μM with added cysteine, to 0.29 μM in its absence. Thus, this result indicates that the growth inhibitory effects of BM was attenuated by the presence of cysteine in the medium. In addition, this result indicates that the adducts that BM formed with cysteine were also growth inhibitory. Cells typically contain several millimolar GSH and thus, cells treated with BM in the absence of cysteine in medium would still encounter intracellular GSH upon their uptake and would react to form BM-GSH adducts with altered activity.

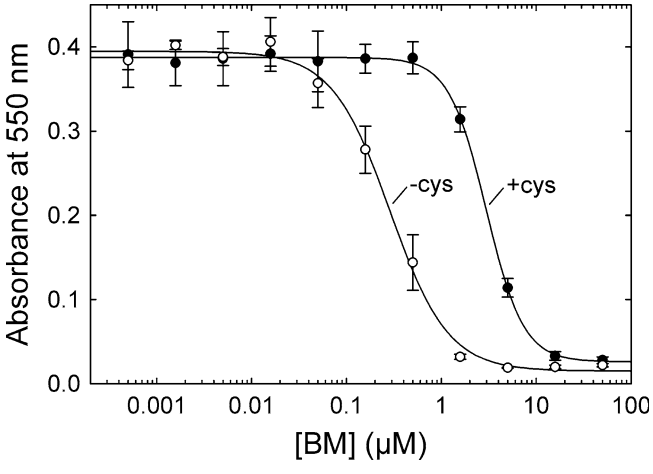


Fig. 6 Treating CHO cells in cysteine-free medium increased the growth inhibitory effect of BM. Attached CHO cells were washed with either PBS/0.1% (w/v) glucose (○) or with PBS/0.1% (w/v) glucose/0.57 mM cysteine (●). Cells were then treated with BM at the concentrations indicated for 1 h at 37°C in a CO_2 incubator. The medium was then removed and replaced with replete α-MEM medium that contained 0.57 mM cysteine. After 72 h the BM-treated cells were assessed for inhibition of growth by an MTT assay. The curved *solid lines* are non-linear least squares fits to a 4-parameter logistic equation and yield IC_{50}'s of 0.29 ± 0.02 and 3.0 ± 0.08 μM, respectively, for CHO cells treated with BM in the absence or presence of cysteine in the medium

EPR measurement of semiquinone free radical formation by BM and its analogs

Because various enzymatic reductases have the capability to effect a one-electron reduction of quinones to yield a semiquinone free radical, which can, in turn, through

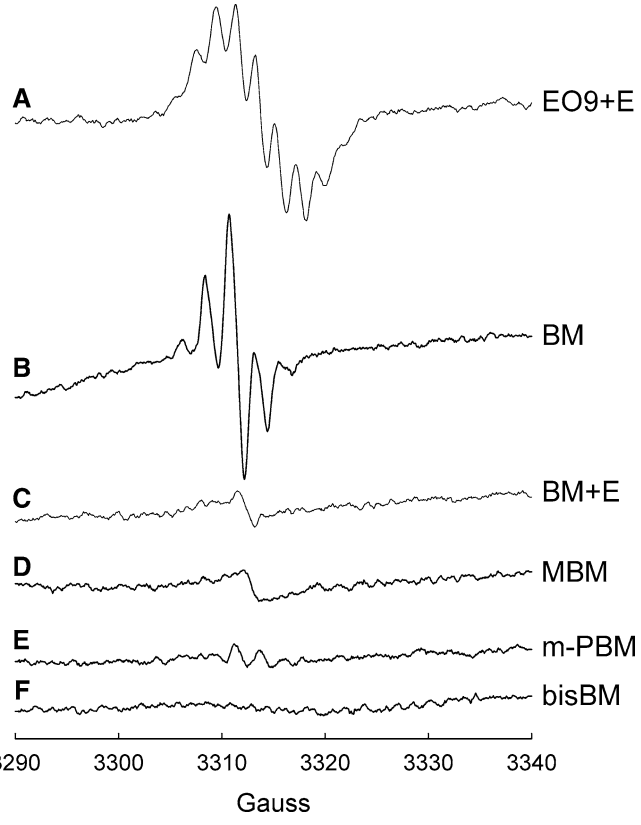


Fig. 7 EPR spectra of BM and its analogs produced under hypoxic conditions by 10 mM of the compound in 100 mM Tris buffer (pH 8.0) in the presence or absence of cytochrome P450-reductase/NADPH showing semiquinone-free radical formation. **a** EO9 plus cytochrome P450-reductase/NADPH; **b** BM; **c** BM plus cytochrome P450-reductase/NADPH; **d** MBM; **e** m-PBM; and **f** bisBM. A total of 10 spectra were accumulated over 7 min and averaged. The EO9 and two BM spectra were recorded at 25°C and the others at 37°C. +E indicates that the drug was treated with the cytochrome P450-reductase/NADPH system

its reaction with oxygen, produce a variety of damaging ROS [8, 30, 32], it was decided to examine whether the benzoquinone mustard compounds could be reductively activated by NADPH cytochrome P450-reductase. In preliminary EPR experiments the BM compounds themselves were studied in the absence of NADPH cytochrome P450-reductase because quinones are known to spontaneously form semiquinone radicals [45].

As shown in Fig. 7b, d and e BM, MBM and m-PBM produced a detectable free radical EPR signal at $g \sim 2.0$ under hypoxic conditions (pH 8.0) in the absence of the cytochrome P450 reductase enzyme system. The EPR spectra shown were all collected over a fixed time interval of 7 min so that a useful measure of relative semiquinone radical-anion level could be obtained. In the case of BM, the amount of semiquinone produced was pH dependent, as the signal (as measured by its peak-to-peak signal height) at pH 7.4 was approximately 40% of that at pH 8.0. The signal decreased with time by about half upon recording a second set of ten scans 10 min later. Upon the introduction of air to the sample, the BM semiquinone signal increased by about 40% over that seen initially under hypoxic conditions. Similar formation of equilibrium concentrations of semiquinones under aerobic conditions have been reported [45]. This may be due to the quinone undergoing an hydrolytic reduction to produce the hydroquinone QH_2 (or its corresponding anion) or that hydroquinone is a contaminant [45]. The quinone Q and the hydroquinone may then undergo a equilibrium disproportionation reaction ($Q + QH_2 \rightleftharpoons 2Q^{\cdot -} + 2H^+$) to yield low steady-state levels of the semiquinone $Q^{\cdot -}$ [33, 40]. Semiquinones react very quickly with oxygen to produce the superoxide radical anion $O_2^{\cdot -}$, which in turn dismutates to produce H_2O_2. Thus, the EPR results suggest that the BM analogs may, in part, also be growth inhibitory through the production of ROS without having to undergo biological reductive activation. Quinones with electron-donating methyl or methoxy groups would have a less favorable stability constant due to an increase in the pK_a for the hydroquinone and would produce less of the semiquinone [40] as seen for MBM compared to BM (Fig. 7).

The anticancer indolequinone EO9 (Fig. 1) has been shown by EPR to be reduced by NADPH cytochrome P450-reductase to its semiquinone-free radical [3]. Thus, we decided to investigate if the BM compounds could likewise be reduced to the semiquinone. BM (10 mM at 25°C in Tris buffer, pH 7.4) under hypoxic conditions in the presence of the enzyme system (2 mM NADPH, 1.6 μg protein/ml of NADPH cytochrome P450-reductase) produced only a small EPR signal (Fig. 7c) that was approximately one-sixth of that seen in the absence of the enzyme system (Fig. 7b). EO9 in the presence of the enzyme system served as a positive control and produced the previously observed EPR spectrum [3] (Fig. 7a). Under similar conditions bisBM also only produced a small signal in the presence of NADPH cytochrome P450-reductase that was approximately one-quarter of that seen in the absence of the enzyme system. The small EPR signal produced by MBM in the presence of the enzyme system was about the same as that seen in the absence of the enzyme system (Fig. 7d). Thus, these results indicate that the BM compounds compared to EO9 were not good substrates for NADPH cytochrome P450-reductase. In fact the decrease in the small EPR signals seen in the presence of the enzyme system for BM and bisBM suggest that NADPH cytochrome P450-reductase may have slowly reduced the semiquinone to a hydroquinone.

QSAR analysis on the BM analogs

The data of Table 1 was subjected to linear correlation analysis to further determine whether the growth inhibitory effects of the BM compounds were due to inhibition of topoisomerase II. Linear correlation analysis of the cell growth inhibitory log IC_{50} versus topoisomerase II log IC_{50} was both negatively and poorly correlated for both CHO ($r^2 = 0.13$, $P = 0.55$) and DZR ($r^2 = 0.07$, $P = 0.66$) cells. When benzoquinone and menadione were included in the data set, the correlations were not improved ($r^2 = 0.09$ and 0.07, and $P = 0.51$ and 0.58, respectively). Similarly, correlation analysis on the BM compounds with our previously determined growth inhibitory data [14] for the NCI-H661 and SK-Mel-28 cell lines were both negatively and poorly correlated ($r^2 = 0.007$ and 0.03, and $P = 0.92$ and 0.82, respectively). Thus, on the basis of the QSAR analysis, it can be concluded that topoisomerase II was not a target for the growth inhibitory effects of the BM compounds.

Quinones undergo electrophilic substitution by nucleophiles, most likely at the most electropositive vinylic carbon [1, 8, 32]. Thus, an attempt was made to correlate the log IC_{50} of the CHO and DZR cell lines with the most electropositive MOPAC Wang-Ford calculated charge on the quinone vinylic carbon. For the complete data set of Table 1, log IC_{50} was positively and poorly correlated for both CHO ($r^2 = 0.46$, $P = 0.094$) and DZR ($r^2 = 0.57$, $P = 0.051$). Exclusion of menadione and benzoquinone from the data set did not result in significance being achieved. If ease of electrophilic substitution was correlated with increased cell growth inhibitory effects, then a negative correlation would have been expected. Therefore, this analysis indicated that electrophilic substitution was not a determinant of cell growth inhibitory activity.

An attempt was also made to correlate growth inhibitory effects of the quinones with the MOPAC-calculated LUMO energy of the quinones of Table 1, as this is a parameter that often correlates well in QSAR studies, and is a parameter that can be used in the place of Hammett substituent σ values for more complex molecules where these parameters are lacking [29]. LUMO energies, which are the negative values of the electron affinity, also correlate well with one-electron reduction potentials, and have been shown to correlate with the hepatocyte cytotoxicity of a series of quinones

[39]. For both CHO and DZR cells, LUMO was negatively and poorly correlated with log IC_{50} ($r^2 = 0.0007$ and 0.01, and $P = 0.95$ and 0.82, respectively) for all the quinones of Table 1. Topoisomerase II log IC_{50}, however, was just barely significantly correlated with LUMO ($r^2 = 0.52$, $P = 0.044$). The failure to obtain a good correlation here may be due to several factors. In the case of the hepatocyte study [39], cytotoxicity was measured after a 2 h drug treatment in a medium that did not contain cysteine [39], whereas in the present study, cell growth inhibition was measured after a 72 h drug treatment in a medium containing cysteine that would have reacted with the BM compounds (Fig. 3) and changed their reactivity through their reactions as shown in Fig. 6. The lack of a correlation might also be due to the fact that the BM compounds have a bifunctional alkylating group, and thus have a more complex mechanism of cell growth inhibition than the simple quinones.

Discussion

Quinones are thought to be cytotoxic and growth inhibitory both through their ability to covalently bind to peptides, proteins, DNA and RNA and through their ability to be reductively activated to semiquinone-free radicals and hydroquinones, which can then generate ROS resulting in oxidative stress [8, 32]. The BM analogs may also have additional growth inhibitory mechanisms, with or without reductive activation, mediated by the reactivity of their bifunctional alkylating groups as suggested by the poor growth inhibitory effects of the non-alkylating HBM analog (Table 1).

Like several other studies that have shown that a variety of quinones interact with and inhibit the catalytic activity of topoisomerase II [17, 18, 24, 41], we determined that the BM compounds likewise potently inhibited topoisomerase II (Table 1). The electrophilic reaction of the quinone with critical sulfhydryl groups on the topoisomerase IIα monomer which contains 13 cysteine residues, are thought, in part, to be responsible for the inhibition of topoisomerase II [18, 24, 41]. We have recently shown in a proteomics mass spectrometry study that topoisomerase IIα contains at least 5 free sulfhydryl groups per monomer and that the antitumor agent cisplatin may be inhibiting topoisomerase IIα by reacting with critical free sulfhydryl groups [23]. Other amino acid targets on topoisomerase II are also possible as quinones can also react, though more slowly, with nitrogen-containing nucleophiles [1, 2]. The fact that BM was able to slowly react with BSA (Fig. 3d) suggests that reaction of BM with critical sulfhydryl groups on topoisomerase II could be responsible for its inhibition. The fact that HBM inhibited topoisomerase II more weakly than the BM compounds suggests that alkylation of topoisomerase II may be important in its inhibition.

Our results showed that while the BM compounds produced protein–DNA complexes with K562 cells and nuclei, a result that is often indicative of a drug acting as a topoisomerase II poison, the similar profile of activity in etoposide-resistant K/VP.5 cells and nuclei that contain reduced levels of topoisomerase IIα [11, 35, 36], was not consistent with the BM compounds acting as topoisomerase II poisons. Likewise, BM compounds induced very little or no formation of linear DNA in a topoisomerase II-catalyzed pBR322 DNA cleavage assay (Fig. 5), suggesting that the BM compounds did not act as topoisomerase II poisons. The lack of cross resistance to cell growth inhibition by the BM compounds towards the DZR cells, compared to the parental CHO cells that contain one-half the topoisomerase II level [22], also suggests that these compounds do not inhibit cell growth by acting as topoisomerase II poisons. Reports on whether various other quinones are able to act as topoisomerase II poisons are mixed in their conclusions [4, 7, 10, 12, 15–18, 24, 26, 31, 41]. In addition, the fact that the growth inhibitory effects of the BM compounds were not significantly correlated with the inhibition of topoisomerase II suggests that these compounds did not exert their growth inhibitory effects by targeting topoisomerase II. Finally, in a previous study [13] we showed that the BM compounds induced DNA cross links and strand breaks which may be the primary mechanism by which they exerted their growth inhibitory effects.

Our spectrophotometric results showed that some of the quinones reacted very quickly with GSH and cysteine (Fig. 3), with MBM being the only exception. Our culture medium in which the growth inhibition experiments were carried out contained 0.57 mM cysteine, and thus the cysteine-reactive BM compounds would have been modified even before they entered the cell. The reaction of GSH with benzoquinone is unstable with an intramolecular cyclization, which occurs by involving the α-amino group [1]. In addition, benzoquinones can form bi- and tri-GSH conjugates [8, 32]. The fact that the growth inhibitory effects of BM were increased approximately 10-fold when the medium contained no cysteine (Fig. 6) suggests that the BM-cysteine reaction products were less cytotoxic than BM itself. Interestingly MBM, which did not measurably react with GSH, was the most potent of the BM compounds (Table 1). It is possible that because MBM did not react with cysteine in the medium, it would have entered the cell as the unmodified quinone and could have undergone enzymatic activation and/or reaction in the cell leading to an increase in cell damage compared to the other BM analogs. We previously showed that of the BM compounds tested, MBM underwent the fastest redox cycling by NQO1 under aerobic conditions [14], which would also likely contribute to its more potent growth inhibitory effects. Quinones with electron-donating groups such as MBM, upon reduction, produce a hydroquinone that is more easily oxidized [14, 30], and which would thus produce a higher flux of more damaging ROS.

Quinones typically can be activated by NADPH cytochrome P450-reductase to their semiquinone-free

radical form [34]. However, the EPR results of Fig. 7 showed that the BM compounds were not reductively activated by NADPH cytochrome P450-reductase. In fact the EPR results (Fig. 7) showed that BM and the other analogs studied produced the semiquinone-free radical in the absence of any added reducing agent or enzyme. Because the semiquinone reacts quickly with oxygen to produce ROS, this mechanism also provides a way in which the BM analogs may exert their growth inhibitory effects. The fact that the semiquinone EPR signal was decreased by the presence of NADPH cytochrome P450-reductase system suggests that the semiquinone may, in fact, be enzymatically reduced to the hydroquinone. Our previous studies showed that NQO1 caused BM-mediated DNA cross link and strand break formation [13, 14]. The BM analogs with electron-donating groups such as MBM were the most potent and also the most growth inhibitory [13, 14] (Table 1). However, the NQO1-mediated rate of reduction did not correlate with potency [13, 14]. Thus, the mechanism of DNA strand break and cross link formation may be due to NQO1-mediated reduction to the hydroquinone which then oxidizes to form ROS.

In conclusion these studies have shown that even though the BM compounds were potent catalytic inhibitors of topoisomerase II, this inhibition was not a significant mechanism by which these compounds exert their growth inhibitory effects. Even though the BM compounds with chloroethyl groups produce protein–DNA complexes in K562 nuclei and cells, the formation of these protein–DNA complexes was independent of topoisomerase II levels. In addition, the BM compounds had little activity in a topoisomerase-II-mediated DNA cleavage assay. Hence, the BM compounds are not likely to act as topoisomerase II poisons in a cellular context. Several of the BM compounds reacted very quickly with glutathione, cysteine and DTT to produce several products. The fact that the growth inhibitory effect of BM was increased 10-fold in the absence of cysteine suggested that BM reacted with cysteine even before it entered the cells. Thus cysteine and sulfhydryl-containing plasma proteins would also be able to modulate the activity of these compounds. Taken together with earlier reports of the activity of BM compounds [13, 14], we conclude that these quinone mustards exert their action through DNA cross-linking and/or by inducing oxidative stress. Although DNA topoisomerase II is evidently not a direct target of these agents in the cells, this enzyme may play a role in processing the consequences of direct DNA adduction and/or oxidative DNA damage.

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