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ORIGINAL ARTICLE

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The effect of functional groups on reduction and activation of quinone bioreductive agents by DT-diaphorase

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Abstract *Purpose:* Bioreductive antitumor agents are an important class of anticancer drugs that include the clinically used drug, mitomycin C, and new agents such as EO9 and tirapazamine that have recently been tested in clinical trials. These agents require activation by reductive enzymes such as DT-diaphorase or NADPH:cytochrome P450 reductase. A major focus for improving cancer chemotherapy has been to increase the selectivity and targeting of antitumor drugs to tumor cells. Bioreductive antitumor agents are ideally suited to improving tumor selectivity by an enzyme-directed approach to tumor targeting. However, none of the bioreductive agents developed to date has been specific for activation by a single reductive enzyme. This is in part due to a lack of knowledge about structural factors that confer selectivity for activation by reductive enzymes. The purpose of this study was to investigate the ability of specific functional groups to modify reduction and activation of quinone bioreductive agents by DT-diaphorase. *Methods:* We used a series of model benzoquinone mustard (BM) bioreductive agents and compared the parent compound BM to MBM, which has a strong electron-donating methoxy group, MeBM, which has a weaker electron-donating methyl group, CBM, which has an electron-withdrawing chloro group, and PBM and its structural isomer, meta-PBM (m-PBM), which both have sterically bulky benzene rings attached to the quinone moiety. We determined the rate of reduction of these agents by purified human DT-diaphorase under hypoxic and aerobic conditions. We also measured the cytotoxic activity of these agents in human tumor cell lines with and without the DT-diaphorase inhibitor, dicoumarol. *Results:* Under hypoxic conditions in vitro, the $t_{1/2}$ values for reduction of the analogs by purified DT-diaphorase were 4, 6, 8, 9, 10 and 21 min for BM, MeBM, CBM, MBM, PBM and m-PBM, respectively. Under aerobic conditions the rank order of redox cycling after two-electron reduction by DT-diaphorase was MBM > MeBM > BM≈CBM≈PBM≈m-PBM. The rate of reduction by DT-diaphorase of HBM, a non-alkylating analog of BM, was similar to that of BM under hypoxic conditions, and the rate of redox cycling under aerobic conditions was comparable to that of BM, suggesting that structural changes to the cytotoxic group of these BMs do not affect DT-diaphorase-mediated reduction and redox cycling potential. MBM, MeBM and PBM were more toxic than BM in the NCI-H661 human non-small-cell lung cancer cells and SK-MEL-28 human melanoma cells, while CBM displayed significantly increased cytotoxic activity compared to BM only in the NCI H661 cells. m-PBM had similar cytotoxic activity compared with BM in both cell lines. These cell lines have moderate to high levels of DT-diaphorase activity. When cells were pretreated with the DT-diaphorase inhibitor, dicoumarol, the cytotoxic activity of BM increased while that of MBM decreased in both cell lines, suggesting that BM was inactivated by DT-diaphorase while MBM was activated by this enzyme. Pretreatment of the SK-MEL-28 melanoma cells with dicoumarol resulted in an increased cytotoxic

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activity of MeBM, but pretreatment of the NCI-H661 cells did not affect the cytotoxicity of MeBM. This suggests, that similar to the results with BM, DT-diaphorase is an inactivating enzyme for MeBM in the SK-MEL-28 cell line. Dicoumarol had no significant effect on the cytotoxicity of CBM, PBM or m-PBM in both cell lines. *Conclusions*: These studies demonstrated that functional groups can significantly affect the reduction and activation of bioreductive agents by DT-diaphorase. All the functional groups decreased the rate of reduction of the quinone group by DT-diaphorase. Since MeBM and MBM, with electron-donating functional groups, and CBM with an electron-withdrawing functional group had similar half-lives of reduction by DT-diaphorase, steric rather than electronic effects of the functional groups appear to be more important for modifying the rate of reduction by DT-diaphorase. Steric effects on reduction by DT-diaphorase were also influenced by the position of the functional group on the quinone ring moiety, as the reduction of m-PBM was much slower than the reduction of PBM. The electron-donating methoxy and methyl functional groups increased the ability of the reduced products of MBM and MeBM to undergo redox cycling. DT-diaphorase appeared to be an activating enzyme for MBM. This may have resulted in part from increased formation of reactive oxygen species resulting from the increased redox cycling by MBM. In contrast, DT-diaphorase was an inactivating enzyme for BM, and for MeBM in the SK-MEL-28 melanoma cells, possibly because the hydroquinone product of BM and MeBM may be less cytotoxic than the semiquinone produced by one-electron reduction by NADPH:cytochrome P450 reductase.

Keywords Bioreductive agents · DT-diaphorase · Structure-activity studies

Abbreviations *BM:* 2-[di(chloroethyl)amino]-1,4-benzoquinone · *CBM:* 5-chloro-2-[di(chloroethyl)amino]-1,4-benzoquinone · *DMF:* dimethylformamide · *DT-diaphorase:* NAD(P)H:(quinone acceptor) oxidoreductase · *EO9:* 3-hydroxymethyl-5-aziridinyl-1-methyl-2(1H-indole-4,7-dione)prop-β-en-α-ol · *HBM:* 2-[di(hydroxyethyl)amino]-1,4-benzoquinone · *MBM:* 5-methoxy-2-[di(chloroethyl)amino]-1,4-benzoquinone · *MeBM:* 5-methyl-2-[di(chloroethyl)amino]-1,4-benzoquinone · *MeDZQ:* 2,5-diaziridinyl-3,6-dimethyl-1,4-benzoquinone · *MMC:* mitomycin C · *m-PBM:* 6-phenyl-2-[di(chloroethyl)amino]-1,4-benzoquinone · *PBM:* 5-phenyl-2-[di(chloroethyl)amino]-1,4-benzoquinone · *RED* NADPH:cytochrome P450 reductase

Introduction

Bioreductive drugs have become an important class of antitumor agents [40, 47]. The prototype drug in this class, MMC [40], is used in the treatment of bladder, breast, stomach, head and neck, rectal and other solid tumors [6]. Other agents such as porfiromycin [39] and diaziquone [16] have also been used clinically, and a number of new bioreductive agents such as EO9 [20] and tirapazamine have recently been tested in clinical trials [6]. In addition, other bioreductive agents, such as AQ4N [34], MeDZQ [32] and RH1 [46], have demonstrated good antitumor activity in vitro, and there has been considerable interest in the synthesis of new agents [19].

Bioreductive agents have varied chemical structures but have a common requirement for reductive activation [47]. They produce their antitumor effects by different mechanisms, and while active under oxygenated conditions, many of these agents are preferentially toxic to hypoxic cells [47]. Bioreductive agents can be activated by one-electron reducing enzymes such as RED (EC 1.6.2.4) [33, 40] and NADH:cytochrome b_5 reductase (EC 1.6.2.2) [27], and by two-electron reducing enzymes such as DT-diaphorase (EC 1.6.99.2) [38, 41] and xanthine dehydrogenase (EC 1.1.1.204) [24]. RED is the most important activating enzyme for many of these agents [26, 38], but DT-diaphorase is a significant contributor in many systems [9, 12, 38, 41]. Drug activation is strongly influenced by the level of oxygen [12, 26] and pH [9, 42]. The relative importance of each activating enzyme varies with the agent, the type and origin of the cell and the tumor environment.

All bioreductive agents contain a bioreductive element and a cytotoxic element. In some agents, for example tirapazamine and AQ4N [34], the bioreductive element may also act as the cytotoxic element, while in others, for example MMC [30, 44], the agents have additional cytotoxic elements that are regulated by the reductive element. Quinone and nitrogen oxide groups are the bioreductive elements most commonly found in current bioreductive antitumor agents. One-electron reduction of these elements results in the formation of radical species that can add across double bonds or can abstract a hydrogen from cellular molecules [45] leading to degradation of cellular components such as proteins, lipids and DNA. In addition, in the presence of oxygen, the radical species can undergo redox cycling resulting in the generation of reactive oxygen species [28] that can also degrade cellular components and produce DNA strand breaks [30, 45]. Direct two-electron reduction of the bioreductive element, or further one-electron reduction of the initial one-electron reduced product, results in a two-electron reduced product that may or may not have cytotoxic activity. Depending on the stability of the two-electron reduced state, it may also undergo redox cycling to generate reactive oxygen species.

Alternatively, reduction of the bioreductive element may result in activation of a cytotoxic element, generally an alkylating group. For example, MMC [30, 44], EO9 [38] and the model bioreductive agent BM [8] all have alkylating groups that are activated by reduction of a quinone bioreductive element. The alkylating groups can bind covalently to cellular components such as DNA and proteins, and can produce DNA crosslinks [8,

30, 38, 44], which can lead to cell death by apoptosis [13]. While the quinone group of these bioreductive agents can also undergo redox cycling [28], and these agents do produce DNA strand breaks [30, 38], the alkylating activity is thought to be most important for their antitumor effect [40]. However, the relative importance of these various mechanisms is highly dependent on the tumor environment including the levels of reductive enzymes within the cells [12, 26], the level of oxygen [12, 26] and the pH [9, 42].

A major focus for improving cancer chemotherapy has been to increase the selectivity and targeting of antitumor drugs to tumor cells. The bioreductive class of antitumor agents is ideally suited to the improvement of tumor selectivity by an "enzyme-directed" approach to tumor targeting [38, 47]. This concept proposes the use of bioreductive agents that are activated by a particular reductive enzyme to treat tumors that have elevated levels of that enzyme. For example, studies have found higher levels of DT-diaphorase in tumor cells than in normal cells [15, 38, 43]. Thus, bioreductive agents that are specifically activated by DT-diaphorase could be used to target tumors that have high levels of this enzyme. This approach is illustrated by the development of bioreductive agents such as EO9, MeDZQ and RH1, which are selectively activated by DT-diaphorase [2, 36, 46]. EO9 has shown very good antitumor activity against tumors that have high levels of DT-diaphorase but causes little bone marrow toxicity [25]. Bone marrow has been shown to have very low levels of DT-diaphorase [10, 14].

An alternative paradigm has been developed for bioreductive agents selectively activated by RED. The activity of these agents is generally lower under aerobic conditions because in the presence of oxygen the initially formed one-electron reduction product can be re-oxidized, resulting in a lower concentration of activated drug [47]. These agents normally have greater activity under hypoxic conditions because the re-oxidation process cannot occur under these conditions. Most solid tumors have regions of hypoxia resulting from poor vascularization, and bioreductive agents that are primarily activated by RED have been used to target these hypoxic cells either as cytotoxic agents or as radiosensitizers [47]. This approach to tumor targeting is illustrated by tirapazamine and AQ4N which are activated by RED and have high hypoxic:oxic cytotoxicity ratios [34].

There has also been considerable interest in the development of new bioreductive agents. Denny et al. [19] have developed new bioreductive molecules and methods for modifying the enzyme selectivity of these agents. Patterson [34] has prepared a number of new bioreductive agents, including AQ4N, based on the aliphatic nitrogen oxide bioreductive element. However, none of the bioreductive agents developed to date has been specific for activation by a single reductive enzyme. Despite a number of structure-activity studies [1, 3, 22, 32, 35], a major problem has been a lack of knowledge of structural factors that produce selectivity for activation of bioreductive agents by reductive enzymes such as DT-diaphorase and RED.

We have shown that BM, which contains a quinone bioreductive element and a nitrogen mustard cytotoxic element, is more toxic to tumor cells than the non-quinone alkylating agent, aniline mustard [4]. BM produces both DNA crosslinks and strand breaks, but the crosslinks are the major contributor to the cytotoxic activity [5, 7]. Both the cytotoxic and crosslinking activities of BM are increased by reduction of the quinone [11]. BM activities are lower in mouse lymphoma cells with elevated DT-diaphorase than in similar cells with low enzyme activity. Dicoumarol, a DT-diaphorase inhibitor, increases BM activity in the cells with high enzyme activity, suggesting that DT-diaphorase acts as a detoxifying enzyme for BM [8]. This result also suggests that BM is primarily activated by one-electron reducing enzymes such as RED.

In the present study, we used analogs of BM to investigate the effect of a number of functional groups on the reduction and activation of these model bioreductive agents by DT-diaphorase in order to identify groups that could be used to develop new agents with increased enzyme selectivity.

Materials and methods

Materials

All media and fetal bovine serum were obtained from GibcoBRL (Grand Island, N.Y.). All reagents for the DT-diaphorase assay, NADH, FAD, dicoumarol, di(chloroethyl)amine hydrochloride, Tris-HCl, vanillin, 1,4-benzoquinone, chromic acid, 2-methyl-1, 4-benzoquinone, 2-phenyl-1,4-benzoquinone and 2-chloro-1,4-benzoquinone were from Sigma-Aldrich (St. Louis, Mo.). Dichloromethane, methanol, hexanes, 95% 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.). DMF was from BDH Laboratory Supplies (Poole, UK). Cupric acetate was from Fisher Scientific (Nepean, ON, Canada). Purified recombinant human wild-type DT-diaphorase with activity of 880 μmol/min per mg protein (as measured by dicoumarol-sensitive reduction of 2,6-dichlorophenolindophenol) was obtained from Dr. D. Ross, University of Colorado Health Sciences Center, Denver, Colo. DT-diaphorase activity was measured using a modification of the method described by Prochaska and Santamaria [21, 37].

Preparation of BM analogs

BM was synthesized using the method developed by Makarova and Berlin [31]. The purple precipitate was collected by filtration, dried and recrystallized from hexanes to give BM. The structure was confirmed and purity was assessed by ^{1}H-NMR analysis and melting point.

MBM was synthesized using a combination of the method described by Crosby and Lutz [18] for oxidative amination of 1,4-benzoquinones and Makarova and Berlin [31] for synthesis of BM. Di(chloroethyl)amine hydrochloride, potassium fluoride and cupric acetate were added to a solution of 2-methoxy-1,4-benzoquinone in 95% ethanol. 2-Methoxy-1,4-benzoquinone was prepared as previously described [17]. The reaction mixture was stirred at room temperature for 3 days with minimum light exposure. The mixture

was filtered and the filtrate was washed four times with ethyl acetate. The combined organic phases were washed with an equal volume of 0.1 *M* HCl to remove any remaining cupric acetate. The ethyl acetate layer was separated, dried with magnesium sulfate and rotary evaporated in vacuo to dryness to give a red solid. The red solid was recrystallized using 95% ethanol to give MBM. The structure was confirmed and purity was assessed by ^{1}H-NMR analysis and by melting point.

CBM was synthesized using the methods of Crosby and Lutz [18] and Makarova and Berlin [31] starting from 2-chloro-1,4-benzoquinone, as described above for MBM. The resulting purple solid was recrystallized using methanol to give CBM. The structure was confirmed and purity was assessed by ^{1}H-NMR analysis and melting point.

MeBM was synthesized using the methods of Crosby and Lutz [18] and Makarova and Berlin [31] starting from 2-methyl-1,4-benzoquinone, as described above for MBM. The mixture was filtered using vacuum and the resulting precipitate was washed with ethyl acetate (4×20 ml). The combined organic phases were washed with an equal volume of 0.1 *M* HCl and then dried with anhydrous sodium sulfate. Concentration afforded a crude solid which was recrystallized from methanol affording MeBM. The structure was confirmed and purity was assessed by ^{1}H-NMR and X-ray analysis.

m-PBM and PBM were synthesized using the methods of Crosby and Lutz [18] and Makarova and Berlin [31] starting from 2-phenyl-1,4-benzoquinone, as described above for MBM. The mixture was filtered using vacuum and the resulting precipitate was washed with ethyl acetate (4×25 ml). The combined organic phases were washed with water, with an equal volume of 0.1 *M* HCl and then dried with anhydrous sodium sulfate. Concentration afforded a crude mixture of isomeric phenyl benzoquinone mustards. Recrystallization from ethyl acetate/petroleum ether afforded the major isomer, m-PBM as red crystals. The structure was confirmed and purity was assessed by ^{1}H-NMR and X-ray analysis. Concentration of the crude mother liquor from above afforded a red solid enriched in the 5-phenyl isomer. About 200 mg of this mixture was dissolved in ethyl acetate (2.5 ml) and the mixture separated using radial chromatography with a Chromatotron apparatus on a 4-mm silica plate. Ethyl acetate/hexanes (2:3) was used as a solvent. PBM was less polar than the m-PBM isomer. Concentration and drying under vacuum afforded the minor isomer PBM as red crystals. The structure was confirmed and purity was assessed by ^{1}H-NMR, X-ray analysis and melting point.

Cells

NCI-H661 human non-small-cell lung carcinoma cells were obtained from American Type Culture Collection (Rockville, Md.) and were grown in RPMI-1640 plus 10% fetal bovine serum. SK-MEL-28 human malignant melanoma cells were obtained from American Type Culture Collection and were grown in DMEM/F12 1:1 plus 10% fetal bovine serum. The DT-diaphorase activities in these cell lines were 112.7 ± 12.4 nmol/min per mg protein and 586.7 ± 19.6 nmol/min per mg protein, respectively [21].

Reduction of BM analogs by purified DT-diaphorase

Reductions were carried out in 1.5-ml microfuge tubes in 1 ml of a 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 100 μ*M* and 0.5 μ*M*, respectively. The tube was sealed and the cover was perforated twice with a needle to create an inlet and outlet hole. A 1.5-inch stainless steel needle was placed through the inlet into the reaction buffer. Using this needle, the reaction buffer was purged with nitrogen gas or air for 3 h at 37°C. DT-diaphorase at a final concentration of 0.2 μg/ml, followed by the BM analog at a final concentration of 50 μ*M*, were added through the outlet hole to the reaction buffer. DT-diaphorase was prepared fresh on the day of the experiment from –80°C frozen stock and activated with 0.01% Tween 20. BM analogs were prepared fresh on the day of the experiment in DMF. The final concentration of DMF in the reaction buffer was 1%. Nitrogen or air was bubbled into the reaction vessel for the entire time course. At various times, 10 μ*M* dicoumarol was added through the outlet hole to stop the reaction, and the reaction solution was immediately frozen at –80°C for HPLC analysis. Samples were kept frozen prior to analysis for no longer than 5 days. Reduction of the BM analogs was determined by measuring consumption of NADH. An aliquot of the reaction solution was removed and consumption of NADH was quantified by HPLC, as described by Gibson et al. [23].

Cytotoxicity studies

Cells were incubated with or without 100 μ*M* dicoumarol for 20 min at 37°C and then with BM analog for 1 h. Cytotoxicity was determined by MTT assay [29], 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 [21]. The cytotoxic activities of CBM, MBM, MeBM, PBM and m-PBM were compared with that of BM by *t*-tests which assessed differences between the slopes of the linear regression lines of the optical density versus drug concentration curves. The effect of dicoumarol 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 between the mean surviving cell fraction of cells treated without or with dicoumarol.

The dose of each BM analog that reduced the surviving cell fraction to 0.1 (D_{10} value) was calculated for the NCI-H661 and the SK-MEL-28 cell lines respectively. The D_{10} value 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. Data are expressed as the means ± SEM of four or more experiments.

Results

BM analogs

We prepared a series of BM analogs having different functional groups attached to the quinone bioreductive element (Fig. 1). MeBM has a weak electron-donating methyl group near the quinone, MBM has an electron-donating methoxy group, CBM has an electron-withdrawing chloro group, while PBM and m-PBM both have sterically bulky benzene rings at the C5 and C6 position of the quinone, respectively. These analogs were used to investigate the effect of functional groups on the reduction of the quinone group by DT-diaphorase and on the cytotoxic activity of the reduced agents. HBM is an analog of BM in which the chlorines on the nitrogen mustard group have been replaced by hydroxyl groups making the mustard unable to alkylate cell components.

Reduction of BM analogs by purified DT-diaphorase

Reduction of the BM analogs by purified DT-diaphorase in vitro was carried out under hypoxic or aerobic conditions in solutions containing 0.2 μg/ml purified DT-diaphorase, 100 μ*M* NADH, 0.5 μ*M* FAD and 50 μ*M* BM analog. At various time-points, 10 μ*M* dicoumarol was added to stop the reaction, and the extent of reduction was measured using HPLC to follow the

loss of the enzyme cofactor, NADH, which acts as the electron donor (Fig. 2). Under hypoxic conditions, approximately half (one equivalent) of the NADH was consumed with each of the BM analogs, and the rate of reduction of the analogs was BM > MeBM > CBM≈ MBM≈PBM > > m-PBM, with $t_{1/2}$ values of 4, 6, 8, 9, 10 and 21 min, respectively. When the BM analogs were reduced by purified DT-diaphorase under aerobic conditions, greater than one equivalent of NADH was consumed for all the BM analogs (Fig. 2). Initial loss of NADH under aerobic conditions was rapid with MBM and MeBM, but was slower with BM, CBM, PBM and m-PBM.

HBM and BM had identical rates of reduction by DT-diaphorase under hypoxic conditions ($t_{1/2}$ of reduction of 4 min), as well as a similar rate under aerobic conditions (Fig. 2).

Cytotoxic activity of BM analogs

NCI-H661 human non-small-cell lung cancer cells, which have a moderate level of DT-diaphorase, were pretreated at 37°C for 20 min without or with 100 μ*M* dicoumarol, and then were incubated with BM, CBM, MBM, MeBM, PBM or m-PBM for 1 h. Cytotoxic activity was determined by MTT assay (Fig. 3a). m-PBM had a cytotoxic activity similar to that of BM in the NCI-H661 cells in the absence of dicoumarol, while CBM, MBM, MeBM and PBM had significantly greater activity in these cells ($P < 0.05$; Fig. 3a, Table 1). Pretreatment with dicoumarol significantly increased the cytotoxic activity of BM ($P < 0.001$; Table 2) and decreased the cytotoxic activity of MBM ($P < 0.05$; Table 2). Dicoumarol did not significantly affect the cytotoxicity of MeBM, CBM, PBM and m-PBM in these cells (Table 2).

SK-MEL-28 human melanoma cells, which have a high level of DT-diaphorase, were pretreated at 37°C for 20 min without or with 100 μ*M* dicoumarol, and then were incubated with BM, CBM, MBM, MeBM, PBM or m-PBM for 1 h. The surviving cell fractions were determined by MTT assay (Fig. 3b). CBM and m-PBM had cytotoxic activities similar to that of BM in the SK-MEL-28 cells in the absence of dicoumarol, while MBM, MeBM and PBM had significantly greater activity in these cells ($P < 0.05$; Fig. 3b, Table 1). Pretreatment with dicoumarol significantly increased the cytotoxic activity of BM and MeBM in these cells ($P < 0.05$; Table 2). In contrast, pretreatment with dicoumarol decreased the cytotoxic activity of MBM ($P < 0.05$; Table 2). Dicoumarol did not significantly affect the cytotoxicity of CBM, PBM and m-PBM in these cells (Table 2).

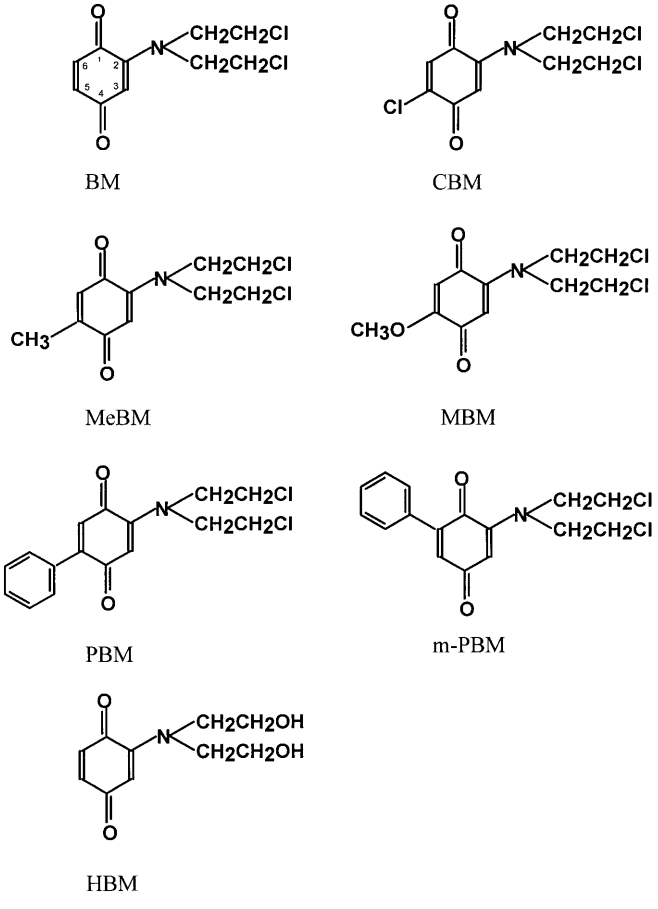

Fig. 1 Structures of BM analogs

Discussion

Enzyme-directed targeting of antitumor agents is a new strategy for increasing the effectiveness of cancer chemotherapy. This approach is based on the premise that the selectivity of antitumor agents can be increased by the use of agents that are activated by specific activating enzymes in tumors that have high levels of these enzymes [38, 47]. This would result in increased tumor kill and decreased toxicity to normal tissues which do not have elevated levels of the activating enzyme. Bioreductive agents are ideally suited for enzyme-directed tumor targeting since they require activation by reductive enzymes. Although there has been considerable effort to develop new bioreductive agents with greater enzyme selectivity [19, 34], none of the bioreductive agents developed to date has been specific for activation by a single reductive enzyme. A major problem encountered in developing agents with specificity of activation has been a lack of knowledge of structural factors that produce selectivity for activation of bioreductive agents by reductive enzymes such as DT-diaphorase and RED. In the present study, we investigated structure-activity relationships to identify the effect of functional groups on reduction and activation of bioreductive agents by DT-diaphorase.

We have previously investigated the antitumor activity and mechanisms of action of the model bioreductive agent, BM [4, 5, 7, 8, 11]. We showed that BM is

600-fold more toxic to mouse lymphoma cells than the non-quinone alkylating agent, aniline mustard, and is 30,000-fold more toxic to these cells than HBM, a benzoquinone analog with an inactive nitrogen mustard group [4]. BM produces both DNA crosslinks and strand breaks, but the crosslinks are the major contributor to the cytotoxic activity [5, 7]. Both the cytotoxic and crosslinking activities of BM are increased by reduction of the quinone [11]. The cytotoxic activity and DNA damage produced by BM are lower in mouse lymphoma cells which have high DT-diaphorase activity than in similar cells which have low enzyme activity. The DT-diaphorase inhibitor, dicoumarol, increases BM activity in the cells with high enzyme activity, suggesting that DT-diaphorase acts as a detoxifying enzyme for BM [8].

Because of their relatively simple structure, analogs of BM are good models for investigating the effects of various functional groups on the reduction of the quinone group by DT-diaphorase and on the activity of the reduced products. MBM and MeBM were used to study the effects of strong and weak electron-donating groups, respectively. CBM was used to study the effect of an electron-withdrawing group. Both the electron-donating and -withdrawing functional groups would be expected to affect the electron density around the quinone, and thus might alter the ability of DT-diaphorase to reduce this bioreductive element. These functional groups could also influence the reactivity of the nitrogen mustard group after reduction of the quinone. PBM and its positional isomer m-PBM were used to study the effect of a sterically bulky group on the reduction by DT-diaphorase, as the size and position of such a group may also influence the substrate-enzyme affinity.

We measured reduction of the BM analogs by purified DT-diaphorase in vitro to determine the effect of the functional groups on the rate of reduction of the quinone under hypoxic and aerobic conditions. Reduction under hypoxic conditions provides a true measure of the rate of reduction of the analogs since redox cycling is prevented. Under these conditions, the $t_{1/2}$ values for reduction of the analogs by DT-diaphorase were 4, 6, 8, 9, 10 and 21 min for BM, CBM, MBM, MeBM, PBM and m-PBM, respectively. Overall, the functional groups decreased the rate of reduction of the quinone compared to the parent compound BM. The electron-donating methoxy and methyl groups in MBM and MeBM, and the electron-withdrawing chloro group in CBM, had

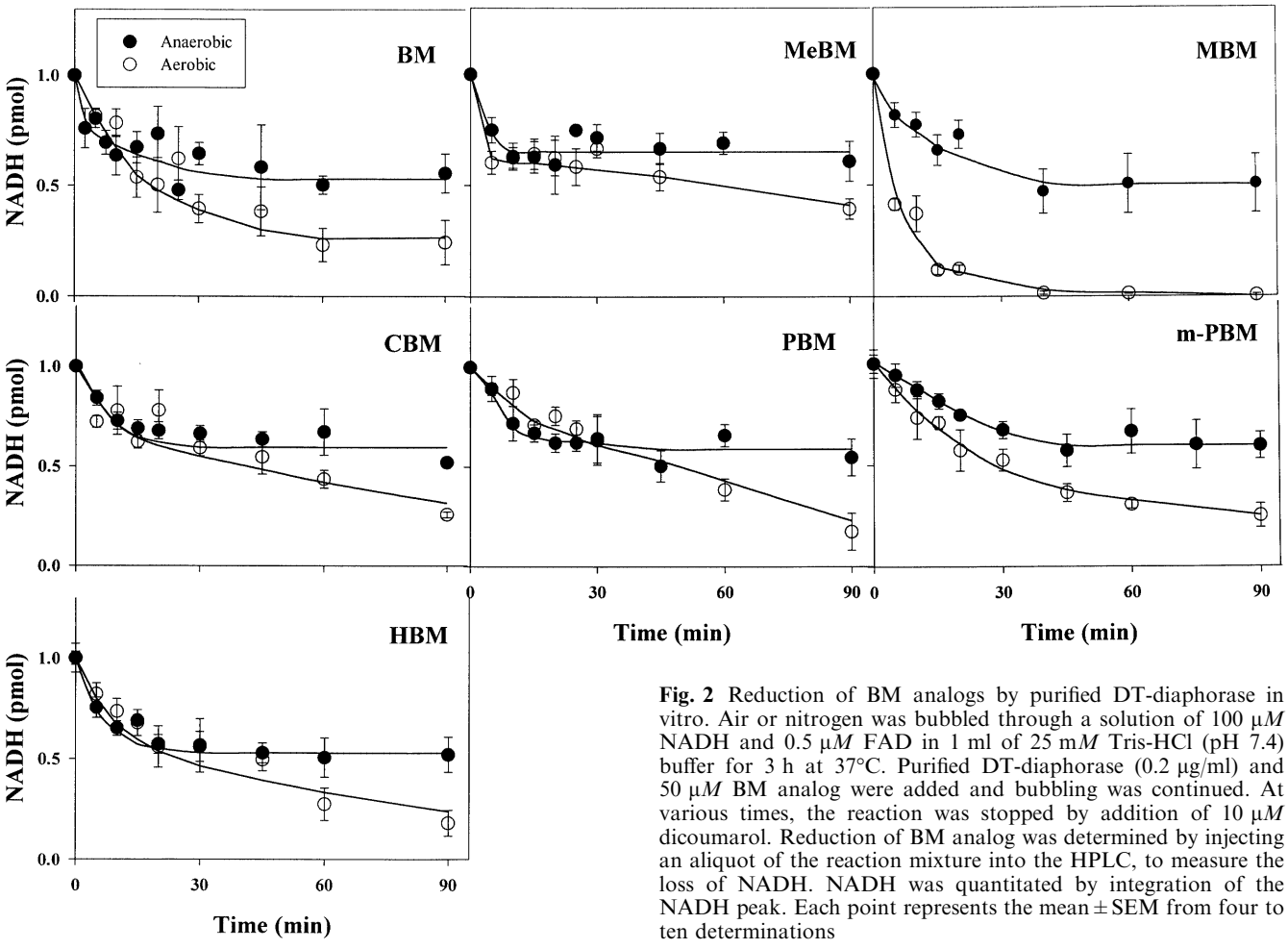

Fig. 2 Reduction of BM analogs by purified DT-diaphorase in vitro. Air or nitrogen was bubbled through a solution of 100 μ*M* NADH and 0.5 μ*M* FAD in 1 ml of 25 m*M* Tris-HCl (pH 7.4) buffer for 3 h at 37°C. Purified DT-diaphorase (0.2 μg/ml) and 50 μ*M* BM analog were added and bubbling was continued. At various times, the reaction was stopped by addition of 10 μ*M* dicoumarol. Reduction of BM analog was determined by injecting an aliquot of the reaction mixture into the HPLC, to measure the loss of NADH. NADH was quantitated by integration of the NADH peak. Each point represents the mean ± SEM from four to ten determinations

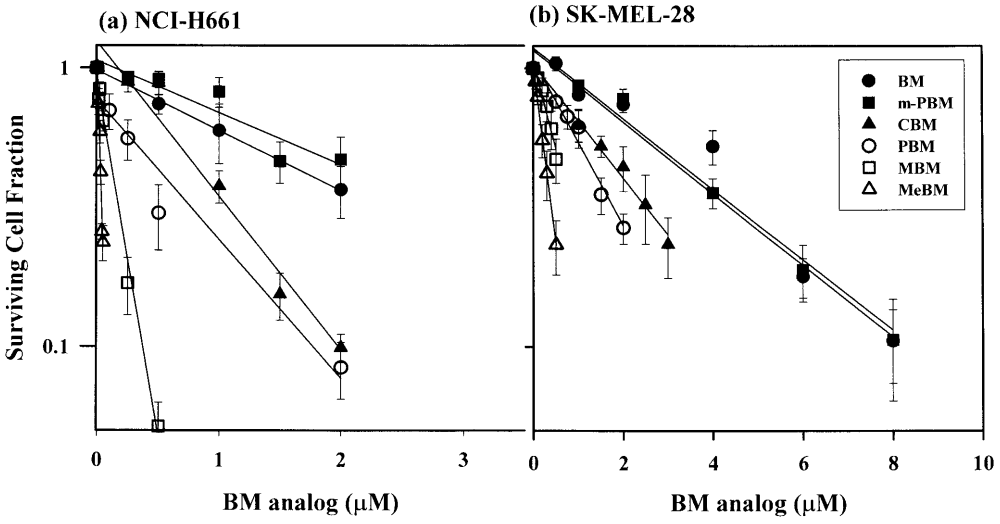

Fig. 3a, b Cytotoxic activity of BM, CBM, MBM, MeBM, PBM and m-PBM in NCI-H661 human non-small-cell lung cancer cells (**a**), and SK-MEL-28 human melanoma cells (**b**). Cells were incubated without or with 100 μ*M* dicoumarol for 20 min and then with BM analog for 1 h. Cytotoxicity was determined by 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). Points represent the means ± SEM of 4 to 16 determinations, and lines are linear regression lines. Slopes of the linear regression lines of the surviving cell fraction versus concentration of BM analog were compared by two-tailed *t*-tests comparing the differences in the slopes between BM and its analogs

approximately the same effect on the rate of reduction, suggesting that the electron density around the quinone was not a major factor influencing reduction by DT-diaphorase. The half-life of reduction of PBM was slower than that of the other BM analogs with smaller functional group substituted at the C5 position, but this was not as slow as that observed with m-PBM. This suggests that sterically bulky groups at the C5 position may not interfere with the substrate-enzyme interaction as much as sterically bulky groups at the C6 position. The relatively small effect of the benzene ring at C5 on the reduction of the quinone may be due to the ability of the ring to rotate and take on a position that would interfere minimally with the enzyme-substrate interaction when substituted at the C5 position, but this may not be possible at the more critical site for the enzyme-substrate interaction at the C6 position.

When the BM analogs were reduced by purified DT-diaphorase under aerobic conditions, there was a greater loss of NADH for all compounds compared with the loss under hypoxic conditions, indicating that the initially produced reduction product could undergo redox cycling. The initial loss of NADH (indicated by the initial slope of the NADH versus time plots under aerobic conditions) was faster with MBM and MeBM than with BM, CBM, PBM and m-PBM, which produced similar but slower losses of NADH. This suggests that the methoxy and methyl groups may enhance the rate of redox cycling.

The rate of reduction or redox cycling of the quinone is not affected by small structural alterations to the cytotoxic element. The structure of the BM analog, HBM, is identical to that of BM except that the chloro groups on the cytotoxic mustard have been replaced by hydroxyl groups. Reduction of HBM by DT-diaphorase and its ability to redox cycle were similar to that of BM.

The cytotoxicity studies in human tumor cell lines with moderate and high levels of DT-diaphorase suggested that the phenyl group of m-PBM had little effect

Table 1 Cytotoxic activity of BM, CBM, MBM, MeBM, PBM and m-PBM 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 (dose 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 drug 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 the means ± SEM of 4 to 16 experiments (*NS* non-significant)

BM analog	NCI-H661 cell line		SK-MEL-28 cell line	
	D_{10} (μ*M*)	*P*-value	D_{10} (μ*M*)	*P*-value
BM	4.87 ± 0.14		7.57 ± 0.76	
CBM	1.67 ± 0.15	< 0.05	4.71 ± 0.45	0.1
MBM	0.40 ± 0.02	< 0.001	1.39 ± 0.04	< 0.05
MeBM	0.56 ± 0.18	< 0.05	0.76 ± 0.02	< 0.001
PBM	2.16 ± 2.65	< 0.05	3.11 ± 0.27	< 0.05
m-PBM	5.05 ± 1.37	NS	7.32 ± 0.37	NS

Table 2 Cytotoxic activity of BM, CBM, MBM, MeBM, PBM and m-PBM 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. Cytotoxicity was determined by 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 represent 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* non-significant)

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

on the overall cytotoxic activity of the bioreductive agent, since BM and m-PBM produced similar tumor cell kill in both cell lines. In contrast, the methoxy, methyl and phenyl groups at the C5 position significantly increased the cytotoxic activity, with MBM, MeBM and PBM showing greater activity than BM in both cell lines. The chloro group at the C5 position significantly increased the cytotoxic activity of the bioreductive agent in the NCI-H661 cell line, but the cytotoxicity of CBM in the SK-MEL-28 cell line only indicated a trend towards an increased cytotoxic activity of CBM compared to BM ($P = 0.1$).

As we had observed earlier in mouse lymphoma cells with high DT-diaphorase activity [8], dicoumarol increased the cytotoxicity of BM in both NCI-H661 and SK-MEL-28 cells, suggesting that DT-diaphorase was an inactivating enzyme for this agent. Similarly, dicoumarol increased the cytotoxicity of MeBM in the SK-MEL-28 cell line, but this did not occur in the NCI-H661 cell line. On the other hand, dicoumarol decreased the cytotoxic activity of MBM in both human tumor cell lines, suggesting that DT-diaphorase was an activating enzyme for MBM. The enzyme inhibitor did not significantly affect the cytotoxic activities of CBM, PBM and m-PBM in both cell lines, suggesting that DT-diaphorase does not play an important role in activating these agents.

The effect of DT-diaphorase on the cytotoxic activity of the BM analogs may, at least in part, be explained by the effect of the functional groups on reduction of the quinone by DT-diaphorase and the subsequent redox cycling of the reduced products. Reduction of BM by DT-diaphorase produces a hydroquinone product which may be less active than the semiquinone produced by one-electron reduction by RED. Hence, DT-diaphorase-mediated reduction of BM may decrease the amount of drug available for reduction by RED leading to an overall detoxifying effect. We have previously demonstrated that BM does produce DNA crosslinks and strand breaks in tumor cell lines [7, 8], and therefore, one-electron reduction via enzymes such as RED appears to be most important for activating this agent.

The increased cytotoxicity observed with MBM may be due to the electron-donating group conferring an increased cytotoxicity through activation of the cytotoxic elements of the reduced products, as the increased electron density within the hydroquinone may facilitate the loss of the chloride leaving groups of the cytotoxic mustard group. Furthermore this compound has an increased rate of redox cycling compared to BM, CBM, PBM and m-PBM, which may allow elevated formation of reactive oxygen species leading to increased DNA and other cellular damage. MeBM had an increased cytotoxicity compared to BM, but unlike MBM, DT-diaphorase did not appear to be important in activating this compound, and thus this agent may be preferentially activated by RED. Compared to MBM, MeBM did not undergo as much redox cycling, as not all the NADH was consumed over 90 min, as was observed with MBM. This may reflect the weaker electron-donating character of the methyl group compared to the methoxy group. The methoxy group may therefore allow fast redox cycling and quantitatively more reactive oxygen species to form compared to the weaker electron-donating methyl group.

PBM displayed an increased cytotoxicity compared to BM, but reduction by DT-diaphorase did not appear to be important in the activation of PBM. Other reductases such as RED may therefore be important in the activation of this compound. The cytotoxicity of CBM was increased compared to that of BM in the NCI-H661 cell line, and there was a trend towards increased cytotoxicity of CBM compared to that of BM in the SK-MEL-28 cell line. As in the case of PBM, reduction by DT-diaphorase did not appear to be important in the activation of this compound, and therefore other reductases may be more important in the activation of CBM. Furthermore, the chloro group, and the other functional groups, may also affect the alkylating and crosslinking activities of the DT-diaphorase reduction metabolites, and this may further influence the cytotoxic

activity of the BM analogs. In the case of m-PBM, the sterically bulky phenyl group significantly decreased the rate of reduction. Thus, this activation pathway may be relatively unimportant compared with reduction by other enzymes such as RED.

The results of the present study are consistent with those of earlier structure-activity studies in which reduction of a quinone group by DT-diaphorase was examined [1, 3, 22, 32, 35]. Studies with indoloquinones [1, 3, 35] and quinolinequinones [22] have indicated that functional groups at the 5-position in these compounds, which is equivalent to the C5 position of the functional groups in the current study, influence reduction of the quinone by DT-diaphorase through a steric effect. However, our results suggest that steric hindrance at the C6 position is more important in changing the rate of reduction by DT-diaphorase. In addition, similar to our finding that the methoxy group increased redox cycling of reduced MBM, it has been suggested that a 5-methoxy group in the indoloquinone models may increase the rate of re-oxidation of the reduced quinone group [32]. However, in contrast to some of our findings, those indoloquinones that were most readily reduced by DT-diaphorase appear to have the greatest cytotoxic activity under aerobic conditions [1] and an aziridinyl group appears to be particularly advantageous for increasing cytotoxic activity [1, 3, 35]. These discrepancies likely result from differences in the activities of the reduced benzoquinone and indoloquinone compounds and demonstrate that the effects of specific functional groups on the cytotoxicity of these compounds can be very complex.

Taken together, these findings demonstrate that functional groups can significantly affect the reduction and activation of bioreductive agents by DT-diaphorase. The methoxy, methyl, chloro and phenyl functional groups decreased the rate of reduction of the quinone group by DT-diaphorase. Steric, rather than electronic, effects of the functional groups appeared to be more important for modifying the rate of reduction by DT-diaphorase. DT-diaphorase appeared to be an inactivating enzyme for BM and MeBM (in the SK-MEL-28 cell line). It was an activating enzyme for MBM but had little effect on the cytotoxic activity of MeBM (in the NCI-H661 cell line), CBM, PBM and m-PBM. This may result, in part, from the formation of a less-reactive hydroquinone product following reduction of BM by DT-diaphorase, an effect of the methoxy group on the ability of the reduction product of MBM to redox cycle, and the slow rate of reduction of m-PBM by DT-diaphorase. Electron donating groups also increased the rate of redox cycling of the analog. The increased cytotoxic effects of MeBM, CBM and PBM may be due to activation of the compounds by other reductases, redox cycling of the reduced product, as well as direct effects of the functional groups on the activity of the cytotoxic elements of the reduced products. Further studies are required to identify other mechanisms through which the functional groups exert their effects on cytotoxic activity.

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References

1. Bailey SM, Suggett N, Walton MI, Workman P (1992) Structure-activity relationships for DT-diaphorase reduction of hypoxic cell directed agents: indoloquinones and diaziridinyl benzoquinones. Int J Radiat Oncol Biol Phys 22:649
2. Beall HD, Murphy AM, Siegel D, Hargreaves RHJ, Butler J, Ross D (1995) Nicotinamide adenine dinucleotide (phosphate):quinone oxidoreductase (DT-diaphorase) as a target for bioreductive antitumor quinones: quinone cytotoxicity and selectivity in human lung and breast cancer cell lines. Mol Pharmacol 48:499
3. Beall HD, Hudnott AR, Winski S, Siegel D, Swann E, Ross D, Moody CJ (1998) Indoloquinone antitumor agents: relationship between quinone structure and rate of metabolism by recombinant human NQO1. Bioorg Med Chem Lett 8:545
4. Begleiter A (1983) Cytocidal action of the quinone group and its relationship to antitumor activity. Cancer Res 43:481
5. Begleiter A (1986) The contribution of alkylation to the activity of quinone antitumor agents. Can J Phys Pharmacol 64:581
6. Begleiter A (2000) Clinical applications of quinone-containing alkylating agents. Front Biosci 5:E153
7. Begleiter A, Blair GW (1984) Quinone-induced DNA damage and its relationship to antitumor activity in L5178Y lymphoblasts. Cancer Res 44:78
8. Begleiter A, Leith MK (1990) Activity of quinone alkylating agents in quinone-resistant cells. Cancer Res 50:2872
9. Begleiter A, Leith MK (1993) Role of NAD(P)H:(quinone acceptor) oxidoreductase (DT-diaphorase) in activation of mitomycin C under acidic conditions. Mol Pharmacol 44:210
10. Begleiter A, Robotham E, Lacey G, Leith MK (1989) Increased sensitivity of quinone resistant cells to mitomycin C. Cancer Lett 45:173
11. Begleiter A, Leith MK, Pan SS (1991) Mechanisms for the modulation of alkylating activity by the quinone group in quinone alkylating agents. Mol Pharmacol 40:454
12. Begleiter A, Robotham E, Leith MK (1992) Role of NAD(P)H:(quinone acceptor) oxidoreductase (DT-diaphorase) in activation of mitomycin C under hypoxia. Mol Pharmacol 41:677
13. Begleiter A, Lee K, Israels LG, Mowat MRA, Johnston JB (1994) Chlorambucil induced apoptosis in chronic lymphocytic leukemia (CLL) and its relationship to clinical efficacy. Leukemia 8[Suppl 1]:S103
14. Begleiter A, Leith MK, Curphey TJ (1996) Induction of DT-diaphorase by 1,2-dithiole-3-thione and increase of antitumour activity of bioreductive agents. Br J Cancer 74[Suppl 27]:S9
15. Belinsky M, Jaiswal AK (1993) NAD(P)H:quinone oxidoreductase1 (DT-diaphorase) expression in normal and tumor tissues. Cancer Metastasis Rev 12:103
16. Chamberlain MC, Prados MD, Silver P, Levin VA (1988) A phase I/II study of 24 hour intravenous AZQ in recurrent primary brain tumors. J Neurooncol 6:319
17. Corral C (1957) Reaction of 4-vinylindan and other styrenes with substituted p-benzoquinones. Rev Real Acad Cienc Exact Fis Y Mat Madrid 51:10
18. Crosby AH, Lutz RE (1956) A study of an oxidative-amination method for the synthesis of aminoquinones. J Med Chem 90:1233
19. Denny WA, Wilson WR, Hay MP (1996) Recent developments in the design of bioreductive drugs. Br J Cancer 74[Suppl 27]:S32
20. Dirix LY, Tonnesen F, Cassidy J, Epelbaum R, Huinink WWT, Pavlidis N, Sorio R, Gamucci T, Wolff I, Te Velde A, Lan J, Verweij J (1996) EO9 phase II study in advanced breast, gastric, pancreatic and colorectal carcinoma by the EORTC early clinical studies group. Eur J Cancer 32A:2019

21. Doherty GP, Leith MK, Wang X, Curphey TJ, Begleiter A (1998) Induction of DT-diaphorase by 1,2-dithiole-3-thiones in human tumor and normal cells and effect on antitumor activity of bioreductive agents. Br J Cancer 77:1241
22. Fryatt T, Goroski DT, Nilson ZD, Moody CJ, Beall HD (1999) Novel quinolinequinone antitumor agents: structure-metabolism studies with NAD(P)H:quinone oxidoreductase (NQO1). Biorg Med Chem Lett 9:2195
23. Gibson NW, Hartley JA, Butler J, Siegel D, Ross D (1992) Relationship between DT-diaphorase-mediated metabolism of a series of aziridinylbenzoquinones and DNA damage and cytotoxicity. Mol Pharmacol 42:531
24. Gustafson DL, Pritsos CA (1992) Bioactivation of mitomycin C by xanthine dehydrogenase from EMT6 mouse mammary carcinoma tumors. J Natl Cancer Inst 84:1180
25. Hendriks HR, Pizao PE, Berger DP, Kooistra K, Bibbey MC, Boven E, Dreef-Van der Muelen HC, Henrar REC, Fiebig HH, Double JA, Hornstra HW, Pinedo HM, Workman P (1993) EO9, a novel bioreductive alkylating indoloquinone with preferential solid tumour activity and lack of bone marrow toxicity in pre-clinical models. Eur J Cancer 29A:879
26. Hoban PR, Walton MI, Robson CN, Godden J, Stratford IJ, Workman P, Harris AL, Hickson ID (1990) Decreased NADPH:cytochrome P-450 reductase activity and impaired drug activation in a mammalian cell line resistant to mitomycin C under aerobic but not hypoxic conditions. Cancer Res 50:4692
27. Hodnick WF, Sartorelli AC (1993) Reductive activation of mitomycin C by NADH:cytochrome b5 reductase. Cancer Res 53:4907
28. Kappus H (1986) Overview of enzyme systems involved in bioreduction of drugs and redox cycling. Biochem Pharmacol 35:1
29. Kirkpatrick DL, Duke M, Goh TS (1990) Chemosensitivity testing of fresh human leukemia cells using both a dye exclusion and a tetrazolium dye (MTT) assay. Leuk Res 14:459
30. Lown JW, Begleiter A, Johnson D, Morgan AR (1976) Studies related to antitumor antibiotics. Part V. Reaction of mitomycin C with DNA examined by ethidium fluorescence assay. Can J Biochem 54:110
31. Makarova AN, Berlin AY (1967) Di-(2-chloroethyl)-amino-p-benzoquinones and their derivatives. Zhural Obshchei Khimii 37:637
32. Naylor MA, Jaffar M, Nolan J, Stephens MA, Butler S, Patel KB, Everett SA, Adams GE, Stratford IJ (1997) 2-Cyclopropylindoloquinones and their analogues as bioreductively activated antitumor agents: structure-activity in vitro and efficacy in vivo. J Med Chem 40:2335
33. Pan SS, Andrews PA, Glover CJ, Bachur NR (1984) Reductive activation of mitomycin C and mitomycin C metabolites catalyzed by NADPH-cytochrome P-450 reductase and xanthine oxidase. J Biol Chem 259:959
34. Patterson LH (1993) Rationale for the use of aliphatic N-oxides of cytotoxic anthraquinones as produg DNA binding agents: a new class of bioreductive agent. Cancer Metastasis Rev 12:119
35. Phillips RM, Naylor MA, Jaffar M, Doughty SW, Everett SA, Breen AG, Choudry GA, Stratford IJ (1999) Bioreductive activation of a series of indolequinones by human DT-diaphorase: structure-activity relationships. J Med Chem 42:4071
36. Plumb JA, Gerritsen M, Milroy R, Thomson P, Workman P (1994) Relative importance of DT-diaphorase and hypoxia in the bioactivation of EO9 by human lung tumor cell lines. Int J Radiat Oncol Biol Phys 29:295
37. Prochaska HJ, Santamaria AB (1988) Direct measurement of NAD(P)H:quinone reductase from cells cultured in microtiter well: a screening assay for anticarcinogenic enzyme inducers. Anal Biochem 169:328
38. Riley RJ, Workman P (1992) DT-diaphorase and cancer chemotherapy. Biochem Pharmacol 43:1657
39. Rockwell S, Keyes SR, Sartorelli AC (1988) Preclinical studies of porfiromycin as an adjunct to radiotherapy. Radiat Res 116:100
40. Rockwell S, Sartorelli AC, Tomasz M, Kennedy KA (1993) Cellular pharmacology of quinone bioreductive alkylating agents. Cancer Metastasis Rev 12:165
41. Ross D, Siegel D, Beall H, Prakash AS, Mulcahy RT, Gibson NW (1993) DT-diaphorase in activation and detoxification of quinones. Cancer Metastasis Rev 12:83
42. Siegel D, Gibson NW, Preusch PC, Ross D (1990) Metabolism of mitomycin C by DT-diaphorase: role in mitomycin C-induced DNA damage and cytotoxicity in human colon carcinoma cells. Cancer Res 50:7483
43. Smitskamp-Wilms E, Giaccone G, Pinedo HM, Van der Laan BFAM, Peters GJ (1995) DT-diaphorase activity in normal and neoplastic human tissues; an indicator for sensitivity to bioreductive agents? Br J Cancer 72:917
44. Tomasz M, Lipman R, Chowdary D, Pawlak L, Verdine GL, Nakanishi K (1987) Isolation and structure of a covalent cross-link adduct between mitomycin C and DNA. Science 235:1204
45. Trush MA, Mimnaugh EG, Gram TE (1982) Activation of pharmacological agents to reactive intermediates. Biochem Pharmacol 33:3335
46. Winski SL, Hargreaves RHJ, Butler J, Ross D (1998) A new screening system for NAD(P)H:quinone oxidoreductase (NQO1)-directed antitumor quinones: identification of a new aziridinylbenzoquinone, RH1, as a (NQO1)-directed antitumor agent. Clin Cancer Res 4:3083
47. Workman P, Stratford IJ (1993) The experimental development of bioreductive drugs and their role in cancer therapy. Cancer Metastasis Rev 12:73