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MECHANISMS OF ACTION OF QUINONE-CONTAINING ALKYLATING AGENTS I: NQO1-DIRECTED DRUG DEVELOPMENT

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1. ABSTRACT

Alkylating agents have been used to treat cancer since the 1940s. Quinone-containing alkylating agents represent a class of drugs called “bioreductive alkylating agents.” These drugs require reduction of the quinone moiety for activation of their alkylating substituents. Despite active research in this area, mitomycin C is the only bioreductive alkylating agent approved for general use. The “enzyme-directed” approach to bioreductive drug development involves identification of reductases which are overexpressed in tumors when compared to uninvolved tissues. Bioreductive drugs which are substrates for these reductases should be selectively toxic to tumors with high reductase levels. NAD(P)H:quinone oxidoreductase (NQO1, DT-diaphorase, EC 1.6.99.2) is a two-electron reductase found primarily in the cytosol. NQO1 has received considerable attention because of the high levels of this enzyme in tumors particularly in tumors of the lung, colon and breast. In this review, the current state of research on quinone-containing alkylating agents is discussed with the focus on NQO1-directed bioreductive drug development. Recent structure-activity studies on indolequinones, benzoquinones and other novel quinones are reviewed, and the status of drugs which have been studied in clinical trials is discussed. Finally, the limitations and possible future directions in this research area are presented.

2. INTRODUCTION AND HISTORY

Alkylating agents have been used in the treatment of cancer since the first lymphosarcoma patient was treated with nitrogen mustard in 1942 (1). One of the first clinically effective nitrogen mustards, mechlorethamine, is still used today in combination therapy for the treatment of Hodgkin’s lymphoma (2). Other alkylating agents such as cyclophosphamide and the nitrosoureas continue to play an important role as key components of many combination cancer chemotherapy regimens.

There are a number of quinone-containing antitumor agents that are used clinically including the anthracyclines - doxorubicin, daunorubicin and idarubicin - and the anthracenedione, mitoxantrone. These agents bind DNA in a non-covalent manner, and thus they are not considered alkylating agents. The mitomycins from *Streptomyces* sp. are quinone-containing alkylating agents, but only mitomycin C is currently approved for general use. Many mitomycin analogs have been synthesized and studied preclinically over the years, and a few have been tested in clinical situations. Another quinone-containing alkylating agent, the indolequinone EO9, which was modeled after mitomycin C, has undergone clinical trials in Europe under the direction of the European Organization for Research and Treatment of Cancer (EORTC). There has also been much interest in aziridinyl benzoquinones as

potential alkylating agents. One of these, diaziquone (AZQ), has been part of a number of clinical trials. As an investigational treatment for primary brain malignancies, AZQ was selected as the first "orphan drug" by the United States Food and Drug Administration (FDA) in the early 1980s.

In this review, the rationale for the use of quinone-containing alkylating agents, including the origin of the terms "bioreductive alkylation" and "enzyme-directed bioreductive drug development" will be discussed. The current status of quinone-containing alkylating agents in clinical use, clinical trials and preclinical studies will be presented along with a discussion of strategies for drug design based on chemical structures and the properties of the various bioreductive enzymes. Finally, the limitations of the bioreductive approach in the design of quinone-containing alkylating agents and future directions for research will be discussed.

2.1. Bioreductive activation

In anticancer drug design, the goal is to develop drugs which are selectively toxic to tumor cells with minimal toxicity to normal cells. The concept of bioreductive drug activation was based on the premise that hypoxic cells near the necrotic cores of solid tumors should have a greater potential for reductive metabolism than aerobic cells (3). The hypothesis was that drugs requiring reductive activation to become cytotoxic would selectively kill hypoxic cells while regeneration of the less toxic "prodrug" would occur via redox cycling in well-oxygenated cells. Although redox cycling leads to generation of reactive oxygen species, cellular defenses such as superoxide dismutase, catalase and glutathione peroxidase generally limit their toxicity.

The term "bioreductive alkylating agent" was first used by Sartorelli and coworkers (3), and it refers to drugs which generate electrophilic species upon reduction which then bind covalently to cellular macromolecules (4). Intracellular activation of bioreductive alkylating agents requires enzymatic reduction and can be accomplished by both one- and two-electron reductases. The result of bioactivation is the production of both mono- and bifunctional alkylating species (bifunctional alkylating agents can cross-link DNA) and/or generation of reactive oxygen species. Bioreductive alkylating agents include quinone-containing alkylating agents such as mitomycin C, and it is these compounds which are the focus of this review.

2.2. Enzyme-directed bioreductive drug development

Another approach to achieving selective toxicity through bioreductive activation is to identify reductase enzymes which are overexpressed in tumor cells when compared to normal cells. This "enzyme-directed" approach was first proposed by Workman and Walton (5), and the principal elements of enzyme-directed bioreductive drug development have been detailed (6). One element involves the identification of compounds which are substrates for and bioactivated by a particular reductase. Much work has been done on structure-based drug design for specific reductases, and this work will be described in detail later in this review. A second element suggests the need for enzyme profiling for patients to document that the selected reductase is elevated in the targeted tumor (6).

The two-electron reductase, NAD(P)H:quinone oxidoreductase (NQO1, DT-diaphorase, EC 1.6.99.2), has received considerable attention in the area of enzyme-directed bioreductive drug development. Markedly elevated levels of this enzyme have been reported in a range of tumor types, and the role of NQO1 in the activation of quinone-containing alkylating agents has been documented (7-9). Other reductase enzymes have also been identified as activators of quinone-containing alkylating agents. They include NADPH:cytochrome P-450 reductase (P450R, EC 1.6.2.4), NADH:cytochrome b_5 reductase (b5R, EC 1.6.2.2), xanthine oxidase (XO, EC 1.2.3.2) and xanthine dehydrogenase (XDH, EC 1.1.1.204) among others. Evidence for the role of each of these reductases in bioreductive drug activation will be discussed later in this review. A comprehensive analysis of the many factors which contribute to the activation of quinone-containing alkylating agents - including the relative importance of reductase activity, pH, oxygen tension and other microenvironmental factors - is beyond the scope of this review. Several recent reviews have been published which include discussions on this issue (8-10).

2.3. Clinical agents

Discovery of novel quinone-containing alkylating agents and their subsequent development as anticancer drugs has been an active area of research for many years. Since the isolation of the mitomycins from Streptomyces sp. in the late 1950s (11), their structure has formed the foundation for the synthesis of hundreds of analogs. For many of these, the basic structure of the mitomycins has been retained, but for many others a more simplified indolequinone framework has been used. Other research groups have focused on the development of benzoquinone alkylating agents, and some work has been done with benzimidazolequinones. Several analogs of mitomycin C have been studied in a clinical trial in the U.S., Europe or Japan (12-15) along with the indolequinone EO9 (16) and the benzoquinone diaziquone (AZQ) (17,18). Examples of indolequinone and benzoquinone alkylating agents which have been approved for use or evaluated in clinical trials are shown in Figure 1.

2.3.1. Mitomycin C and its analogs

Mitomycin C is considered the prototype bioreductive drug (19). It is the only quinone-containing alkylating agent that is approved for general use. Mitomycin C has shown activity against tumors of the stomach, pancreas, colon and breast (20). It has been used clinically with success in combination therapy (20) and has been called the most active single agent for the treatment of non-small cell lung cancer (21). It has also been used as a local treatment for papillary cancer of the bladder (2). Mitomycin C is a very toxic drug (2), and its principal toxicity, bone marrow suppression, is related to the total cumulative dose (20). The search for active analogs of mitomycin C has been due, at least in part, to its high toxicity.

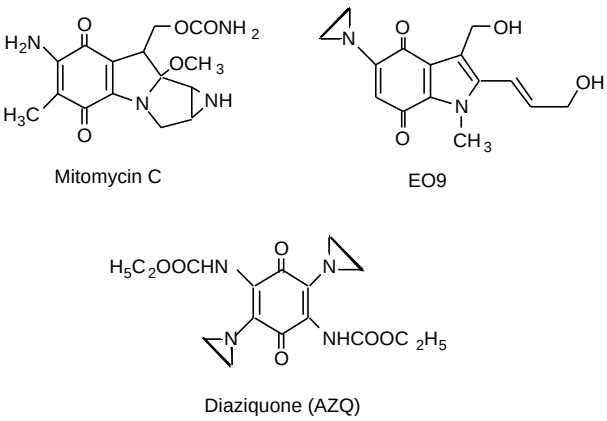

Figure 1. Clinical agents

As of the late 1980s, there had been more than 600 mitomycin derivatives involved in some kind of preclinical testing (13). Several compounds have made it to early clinical trials, but none have been approved for use. KW-2149 (7-*N*-[2-(gamma-L-glutamylamino) ethyldithioethyl] mitomycin C, which appears to be activated by non-protein thiols rather than bioreductive enzymes, has undergone phase I studies (15). Unfortunately, serious pulmonary toxicity developed (22,23), and this effect was not suppressed by pretreatment with steroids (23). BMS-181174 (previously known as BMY 25067; 7-*N*-[2-(4-nitrophenyl)dithioethyl]mitomycin C) was also examined in a phase I trial (24). No further trials were planned with this compound due to pneumonitis, thrombophlebitis and other serious toxicities (24). Porfiromycin (*N*-methylmitomycin C) was studied clinically in the 1970s (25) and most recently was the subject of a phase III trial for treatment of head and neck cancer in combination with radiation therapy (26). Results from the latter study showed that porfiromycin had acceptable toxicity and encouraging efficacy, but the study was still ongoing (26). Another mitomycin C analog, KW-2083 (7-*N*-[4-hydroxyphenyl]mitomycin C) was investigated clinically in Japan in the 1980s (27). In a phase II study, KW-2083 was used to treat advanced non-small cell lung cancer. Only a few partial responses were noted and pronounced myelosuppression was a dose-limiting problem (27).

2.3.2. EO9

EO9 is a synthetic indolequinone which was designed as an analog of mitomycin C. EO9 became a candidate for clinical trials because of its excellent performance in preclinical studies. It showed excellent activity in solid tumor animal models, was effective against hypoxic tumor cells and showed no significant bone marrow toxicity in animal toxicology studies (28). EO9 was selected by the EORTC for clinical trials, and partial responses were observed in phase I studies (29). However, it showed no antitumor activity in phase II trials of breast, colon, pancreatic and gastric cancer (16) and no activity against non-small cell lung cancer in another phase II trial (30). The reason for EO9's failure in the clinic are unknown, but several possibilities exist. First, it may be that the trials were not designed to measure appropriate endpoints for evaluation of bioreductive agents (31). The second possibility relates to drug delivery to the tumor. EO9 has a very short half-life and exhibits poor tissue penetration (9). This apparent shortcoming (poor drug delivery) may actually be an advantage for treatment of bladder cancer by intravesical administration since systemic toxicity would be minimized. EO9 is now being considered for this application (32). Work is also proceeding on analogs of EO9 and other indolequinones with emphasis on retaining solid tumor and hypoxic cell activity while improving drug delivery characteristics. These studies will be discussed later.

2.3.3. Diaziquone (AZQ)

Diaziquone (AZQ) is an aziridinylbenzoquinone which was designed for tumors of the central nervous system (20,33) and has shown some activity against primary malignant brain tumors (34). Myelotoxicity, especially thrombocytopenia, was the primary toxicity in this study (34), and this is generally the case with AZQ (20). More recently in a phase II study, AZQ, mitoxantrone and etoposide were evaluated in two-drug combinations in patients with acute myeloid leukemia (17). Based on some promising results, combination therapy with AZQ and mitoxantrone was selected as the focus for further testing (17). At least two other aziridinylbenzoquinones, trenimon (triaziquone) and carbazilquinone, have been studied clinically in Europe and Japan (33). Development of new aziridinylbenzoquinones has been an active area of research and will be discussed in a later section.

3. NQO1-DIRECTED DRUG DEVELOPMENT

Enzyme-directed drug development involves the identification of compounds which are substrates for and bioactivated by a particular reductase. Much of the recent work in this area has focused on NQO1 due to the high levels of this enzyme in some tumors.

3.1. Properties of NQO1

The discovery of NQO1, known to many as DT-diaphorase, was reported by Ernster and Navazio in 1958 (35). NQO1 is a flavoenzyme; a cytosolic (>90%), two-electron reductase that is characterized by its capacity for utilizing either NADH or NADPH as reducing cofactors and by its inhibition by dicumarol (36). NQO1 is usually categorized as a detoxification enzyme, and it can protect the cell from a broad range of chemically reactive metabolites (37). NQO1 reduces quinones to hydroquinones bypassing the potentially toxic semiquinone radical intermediates (38,39). Not all hydroquinones are redox-stable, however. Redox-labile hydroquinones can react with molecular oxygen to form semiquinones and generate reactive oxygen species, or semiquinones can be generated via comproportionation reactions (40). The dual role of NQO1 as both a prooxidant and antioxidant enzyme in quinone metabolism has been comprehensively reviewed (40).

Reduction by NQO1 can lead to bioreductive activation of the quinone-containing alkylating agents. Among the compounds that can be activated by NQO1 are

the clinical agents discussed in the previous section; mitomycin C (41), AZQ (42) and EO9 (43). Correlations between NQO1 activity and cytotoxicity of antitumor quinones have been reported for tumor cell lines from a range of tissues including breast, lung, colon, CNS and ovary (44-46). A comprehensive study using the NCI's panel of 69 tumor cell lines compared enzyme levels of NQO1, P450R and b5R with sensitivity to mitomycin C and EO9 (47). NQO1 levels significantly correlated with drug sensitivity, and in general, NQO1 was expressed at higher levels relative to the other reductive enzymes measured (47).

NQO1 can be induced by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and polycyclic aromatic hydrocarbons (48). Induction by procarcinogens and the capacity of NQO1 for detoxifying reactive metabolites suggest that changes in expression of NQO1 are likely to occur during carcinogenesis. Elevations in NQO1 activity and gene expression have been documented in both preneoplastic tissues and established tumors. Tumors in which above normal NQO1 expression has been observed include those from the lung, liver, breast and colon (49-52). In addition, one recent study demonstrated that expression of NQO1 increased with malignant progression in colon cancer (53). The high levels of NQO1 in certain tumor types and the potential activation of quinone-containing alkylating agents by NQO1 has prompted an active search for compounds that can be efficiently bioactivated by NQO1.

3.2. Structure-based drug design

NQO1 catalyzes the reduction of a broad range of substrates including *p*-quinones, *o*-quinones, quinone epoxides, glutathionyl quinones, aromatic nitro compounds, conjugated dialdehydes, quinone imines and azo dyes (36). For this reason, it has historically been problematic to identify those compounds which are the most efficient substrates for NQO1. There have been a number of attempts to identify key characteristics of antitumor quinones that are required for metabolic activation, but defining a single variable that ultimately leads to a potential clinical agent has remained elusive. It is known that chemical reduction potential and *in vitro* substrate specificity do not correlate for naphthoquinones (54), aziridinylbenzoquinones (55) or indolequinones (56,57). In the naphthoquinone paper, rates of reduction by NQO1 did not correlate with octanol-water partition coefficients which further limits the ability to predict substrate specificity for NQO1 (54).

The relationship between enzyme activation and toxicity is a complex issue. Values for substrate specificity derived from cell-free metabolism systems have generally been useful as a first estimate for screening new compounds. Metabolism by NQO1 appears to correlate loosely with toxicity to NQO1-containing cells, and this has been shown in structure activity studies for aziridinylbenzoquinones (55) and indolequinones (56). Ultimately, toxicity to the cell is likely influenced by the nature of the toxic metabolite that is generated and microenvironmental factors such as pH and oxygen tension.

3.2.1. Indolequinones

Indolequinones, mitosenes and cyclopropamitosenes have been the most popular structures for NQO1-directed anticancer drug discovery (Figure 2). Based on the structures of mitomycin C and EO9, many analogs have been synthesized, characterized and tested in *in vitro* metabolism and toxicity systems.

Mitomycin C is a poor substrate for NQO1 (44,58), and metabolism is pH dependent (59) due to pH-dependent inactivation of NQO1 by reduced mitomycin C (60). We have suggested that the presence of a leaving group at the indole-3-position (9-position in mitosene numbering system) - mitomycin C has a carbamate leaving group at this position - may lead to generation of a reactive electrophile which could alkylate the enzyme (56). Indolequinones and mitosenes with carbamate or acetate leaving groups at this position were not reduced by NQO1, and NADH-dependent inactivation of NQO1 was observed for these same compounds (56). These observations were confirmed by Phillips and coworkers in a later study (61). Interestingly, Naylor *et al.* determined that leaving group elimination at the indole-3-position occurred predominantly from the hydroquinone (2-electron reduction product) rather than the semiquinone (1-electron reduction product) (62).

In contrast to mitomycin C, EO9 is a good substrate for NQO1 (44). Several structure-activity studies have been published for compounds closely related to EO9 (57,61,63) and for indolequinones in general (56,61,64,65). A common conclusion was that the presence of an aziridine or methylaziridine group at the indole-5-position was consistently desirable in eliciting a toxic response (56,57,61,63-65) and for achieving selective toxicity to NQO1-rich cell lines (56,61). The 2-methyl group on the aziridine ring decreases metabolism by NQO1 and toxicity to aerobic cells relative to aziridine alone (56,61), but interestingly, it increases selectivity against hypoxic cells by more than 10-fold (65). An hydroxymethyl group at the indole-3-position appears to optimize the potency and selectivity gained from the 5-aziridine or 5-methylaziridine substituents (56,57). As an example, 5-(aziridin-1-yl)-3-hydroxymethyl-1,2-dimethylindole-4,7-dione was over 500-fold more toxic to the NQO1-rich H460 non-small cell lung cancer (NSCLC) cell line than to the NQO1-deficient H596 NSCLC cell line (56). Some modifications have also been made at the indole-2-position. Substituting phenyl for methyl at this position has produced mixed results for substrate specificity (56,61), but the 2-methyl derivative is substantially more selective to NQO1-rich cells than the 2-phenyl derivative (56,61).

3.2.2. Benzoquinones

AZQ is a benzoquinone and is reduced by NQO1 (58). In studies of analogs of AZQ, several aziridinylbenzoquinones were found to be better substrates for NQO1 than AZQ (55). One of these (MeDZQ, Figure 3) was an excellent substrate for NQO1 (44,58) was 100-

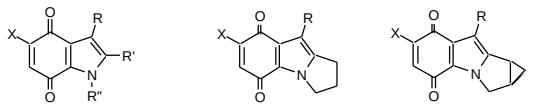

Figure 2. Indolequinones

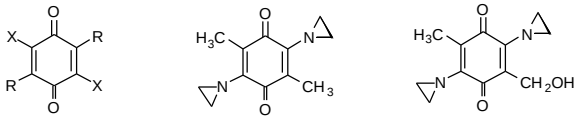

Figure 3. Benzoquinones

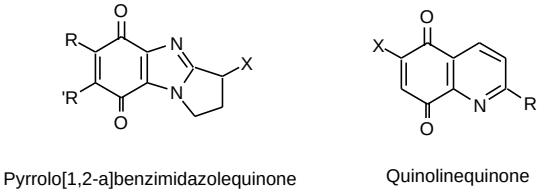

Figure 4. Other novel quinones

fold more toxic to the NQO1-rich HT-29 colon carcinoma cell line than AZQ (55), and required enzymatic activation by NQO1 in order to generate DNA cross-links (66). In two studies comparing MeDZQ toxicity in NQO1-deficient cell lines which had been transfected with human NQO1 cDNA, MeDZQ was four-fold more toxic to NQO1-transfected Chinese hamster ovary (CHO) cells than to parental CHO cells (67) and seven-fold more toxic to NQO1-transfected BE human colon carcinoma cells than to parental BE cells (68).

Despite these impressive numbers, MeDZQ has potential formulation problems due to its low solubility. To address this, a water soluble analogue (RH1, Figure 3) has been prepared and is currently under consideration for clinical trials by the National Cancer Institute and Cancer Research Campaign (69). RH1 demonstrated increased metabolism by purified recombinant human NQO1 compared to MeDZQ, and it was more selective against NSCLC and colon cancer cell lines overexpressing NQO1 compared to NQO1 null cell lines (68). RH1 was also 17-fold more toxic to the NQO1-transfected BE cell line compared to parental BE cells which do not express NQO1 (68). RH1 had a short plasma half-life in mice ($t_{1/2}$alpha = 4.8 min, beta = 9.6 min) (70), but due to its highly toxic nature in cell culture (68) the levels of drug that are achieved are expected to be sufficient to exert a toxic response in target tissues. In support of this, RH1 was observed to confer significant antitumor efficacy in three of four human lung tumor xenograft models using conventional intraperitoneal bolus daily treatments (71). Subsequent testing in one of these models and in one human ovarian tumor model revealed that continuous infusions of RH1 conferred greater efficacy and reduced the potential for drug-related toxicity (71).

A series of benzoquinone mustards has also been synthesized and tested for their ability to be activated by NQO1 and their toxicity to SK-MEL-28 human melanoma cells (72). This structure-activity study showed that the nature of the substituents on the benzoquinone ring affected both metabolism by NQO1 and cytotoxicity, but the results were complicated by the formation of a cyclic intermediate in some cases (72).

3.2.3. Other novel quinones

Although the indolequinones and benzoquinones have been the most studied structures for development of quinone-containing alkylating agents, other heterocyclic quinones have been proposed as substrates for NQO1. Skibo and coworkers have studied a range of pyrrolo[1,2-*a*]benzimidazolequinones (PBIs) which are substrates for NQO1 (Figure 4) (73). They demonstrated both the activation and detoxification properties of NQO1. Non-alkylating PBIs which are inhibitors of topoisomerase II in the quinone form are inactivated by NQO1. Conversely, alkylating PBIs are reductively activated by NQO1 (73). Further studies with selected alkylating PBIs showed very good *in vivo* activity in the NCI hollow fiber tumor assay (74).

There has also been some interest in quinolinequinones as the basic structure for development of quinone-containing alkylating agents (Figure 4) (75). Quinolinequinones are generally much better substrates for NQO1 than analogous indolequinones (75).

3.3. NQO1 crystal structure

Resolution of the three-dimensional structure of rat NQO1 was reported in 1995 (76), and the structure of human NQO1 has recently been resolved to 2.3 angstroms (77) and 1.7 angstroms (78). Molecular modeling studies based on these structures will be important for the design of novel quinone-containing alkylating agents.

3.4. NQO1 polymorphism

A C to T base change at position 609 of human NQO1 cDNA results in a loss of NQO1 enzymatic activity (79,80). The incidence of this polymorphism ranges from 4% to 22% depending on ethnicity of the sample (81). Obviously, this becomes important for NQO1-directed chemotherapy with quinone-containing alkylating agents. Genotype-phenotype characterization of this polymorphism in individuals is accomplished most conveniently by analysis of blood and saliva samples (81). Ideally, a tumor biopsy sample would also be taken for enzyme profiling prior to initiation of drug treatment with quinone-containing alkylating agents.

3.5. NQO1 induction

Another approach to NQO1-directed cancer chemotherapy involves the use of an inducer of NQO1 to increase NQO1 activity in the target tumor. Begleiter *et al.* used 1,2-dithiole-3-thione to increase NQO1 activity in murine lymphoma cells. Exposure to 1,2-dithiole-3-thione did not increase NQO1 activity in normal mouse bone

marrow cells. Toxicities of mitomycin C and EO9 to the tumor cells were enhanced after exposure to 1,2-dithiole-3-thione with no corresponding increase in toxicity of mitomycin C to marrow cells and minimal increase in EO9 toxicity to marrow cells (82). In a subsequent study, similar results were obtained using a panel of human tumor cell lines (83).

4. OTHER BIOREDUCTIVE ENZYMES

It has been suggested that there should be two distinct strategies for development of bioreductive drugs; one which targets NQO1-rich aerobic cells and one which targets NQO1-deficient hypoxic cells (9). There is ample evidence supporting the involvement of bioreductive enzymes other than NQO1 in the activation of quinone-containing alkylating agents.

Apart from NQO1, cytochrome P450 reductase (P450R) has probably received the most attention as a target enzyme for activation of quinone-containing alkylating agents. While it is clear that mitomycin C is a substrate for P450R (84,85), no relationship between P450R activity and chemosensitivity to mitomycin or EO9 was reported in the NCI tumor cell line study (47). However, a study with P450R-transfected CHO cells did show that the transfectants were 2-3-fold more sensitive to mitomycin C than the wild-type CHO cells (86). In contrast to what has been reported for NQO1, a recent study found no significant differences in P450R activity in 17 lung and 18 breast tumor samples when compared to corresponding normal tissues (87).

Other one- and two-electron reductases may also play a role in the activation of quinone-containing alkylating agents. Another one-electron reductase, cytochrome b5 reductase (b5R), can also reduce mitomycin C (88). Like P450R, b5R does not appear to be overexpressed in tumors (89), and no relationship between mitomycin C or EO9 chemosensitivity and b5R activity was observed in the NCI tumor cell line study (47). Xanthine oxidase (XO), acting as a one-electron reductase, can bioactivate mitomycin C (84) and EO9 (90). Mitomycin C can also be reduced by xanthine dehydrogenase (XDH), and reduction is predominantly by two electrons (91). Carbonyl reductase (CR) can catalyze the reduction of a variety of quinone substrates (92), and it was recently demonstrated that CR activity was increased between 2- and 40-fold in lung tumor tissue when compared to normal tissue (87). Finally, there have been reports of unique cytosolic (93) and mitochondrial (94) factors which are capable of metabolizing mitomycin C.

For the one electron reductases such as P450R and b5R, the focus for bioreductive activation of quinone-containing alkylating agents is on the hypoxic fraction of tumors. In the NCI study, the chemosensitivity studies were performed under aerobic conditions, and it was suggested that the results may have been different had they been carried out under hypoxia (47). If hypoxic tumor cells are the target for selective toxicity, then the lack of evidence for over-expression of enzymes such as P450R and b5R may be irrelevant.

5. PERSPECTIVE

Despite the very high level of research on the design, synthesis and preclinical/clinical testing of quinone-containing alkylating agents, mitomycin C remains the only drug approved for general use as an anticancer agent. Compounds that have seemed promising in preclinical studies have either been too toxic or have been ineffective in the clinic.

The use of *in vitro* assays to predict *in vivo* efficacy has produced mixed results. Although one report did demonstrate a relationship between NQO1 activity and responsiveness to mitomycin C in human lung tumor xenografts in athymic nude mice (50), other studies have been less successful. Nishiyama *et al.* showed an inverse relationship between NQO1 activity and xenograft responsiveness to mitomycin C (95). Cummings and coworkers demonstrated no statistically significant correlation between reductase activity (NQO1, P450R or b5R) and antitumor activity of EO9, but mouse and human tumor xenografts (two of each) were grouped together in this study (96). Within each species, the data suggested that there may be a relationship between NQO1 activity and xenograft response (96), and this was also reported for mitomycin C in the mouse tumor xenografts (97). Mitomycin C and EO9 metabolism data from these two studies suggest that overall metabolic capability may be more important than individual reductase activities (96,97).

In those cases where promising *in vitro* studies have failed to translate into *in vivo* efficacy, there are a number of possible explanations. Tumor heterogeneity is certainly an important factor that cannot be duplicated through the use of tumor cell line studies. Cells within tumors will likely have different enzymology and consequently variable abilities to activate quinone-containing alkylating agents. Microenvironmental factors such as pH, oxygen tension and blood flow can also vary within a tumor, and these factors cannot be easily duplicated in studies with cell lines.

Positive *in vivo* animal data doesn't necessarily translate into success in the clinic. EO9 was very effective in solid tumor xenografts, and it showed some selectivity for tumors with high NQO1 activity (28). As indicated earlier, however, EO9 failed to show activity in a range of tumors in phase II clinical trials (16,30). The importance of proper study design (31) and drug delivery considerations (9) cannot be overemphasized since problems in these areas may have contributed to the failure of EO9 in clinical trials.

Future work should continue to focus on identification of novel quinone-containing alkylating agents. Molecular modeling studies should benefit from the recently resolved crystal structure of human NQO1 (77,78). The experience with EO9 should lead to more drug penetration and pharmacokinetic studies. Drug penetration models such as those of Phillips *et al.* (98) could be very important for selecting promising compounds early in the drug development process. Studies using animal models for *in vivo* efficacy and toxicology testing must continue to be

done despite their limitations. Finally, mechanistic studies are essential to understanding how quinone-containing alkylating agents cause damage to tumor cells. Knowledge from these studies will provide insights into selectivity mechanisms and facilitate development of more selective and effective anticancer agents.

6. ACKNOWLEDGMENTS

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