STUDIES ON THE MECHANISM OF ANTITUMOR ACTION OF 3'-(3-CYANO-4-MORPHOLINYL)-3'-DEAMINOADRIAMYCIN.

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This thesis is
dedicated to
Tracy Corkum

Studies on the mechanism of antitumor action of 3'-(3-cyano-4-morpholinyl)-3'-deaminoadriamycin

Abstract

3'-(3-Cyano-4-morpholinyl)-3'-deaminoadriamycin (CMA) is a highly potent analog of the antitumor agent, Adriamycin (ADR), being up to 1500 times more cytotoxic than the parent drug both *in vivo* and *in vitro*. In contrast to ADR, CMA and its 5-imino derivative, ICMA, possess alkylating activity, as seen by their ability to produce DNA-DNA crosslinking in tumor cells and isolated DNA. In this study, the cytotoxic activity of CMA was examined in quinone-resistant and alkylator-resistant cell lines, in an attempt to determine the role of the quinone ring and of crosslinking, for antitumor action. There was no cross-resistance to CMA in L5178Y/HBM10, quinone-resistant murine lymphoma cells, which have enhanced levels of free radical protective enzymes, but there was 2-3-fold cross-resistance to CMA in L5178Y/AM6 and L5178Y/HN2 alkylator-resistant cells. In addition, there was no significant difference between the crosslinking produced by CMA in sensitive cells and L5178Y/HN2 cells. These findings suggested that free radical-mediated damage did not contribute to the enhanced cytotoxic activity of CMA, and that crosslinking did not appear to correlate with cytotoxicity in these cells. The pharmacological properties of CMA, ICMA and the alkylating agent, chlorambucil (CHL), were compared to determine the roles of intercalation, the quinone ring, and DNA base composition, in the crosslinking activity of CMA. CMA was 27- and 1000-fold more active than ICMA and CHL, respectively, in crosslinking DNA in L5178Y cells, and 26- and 450-fold more active than ICMA and CHL, respectively, in producing DNA crosslinks in isolated λ-phage DNA. In contrast, the alkylating activity of CMA was only 6-fold greater than that of CHL, suggesting that the interaction of drug with DNA potentiated crosslinking. Indeed, CMA was a better DNA intercalator than ICMA, whereas CHL did not intercalate; and the crosslinking activity of CMA, but not that of CHL, was inhibited by the intercalating agent, ethidium bromide. Both the rates of formation and removal of CMA crosslinks were more rapid than those of CHL, and CMA produced an increasing level of crosslinking, but showed no difference in intercalation, in isolated DNAs of increasing G-C content. As well, both the crosslinking and intercalating, but not the alkylating, activities of CMA and ICMA were decreased by the reducing agent, sodium borohydride. These findings suggested that DNA intercalation contributed to the crosslinking activity of CMA, and that crosslinks formed by CMA might be more easily removed than those of CHL. Like many alkylating agents, CMA had a preference for alkylating G-C bases of DNA. Furthermore, reduction of the quinone group affected the intercalating activity, and indirectly the crosslinking activity, of the anthracycline analogs.

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I. INTRODUCTION AND REVIEW OF LITERATURE

INTRODUCTION

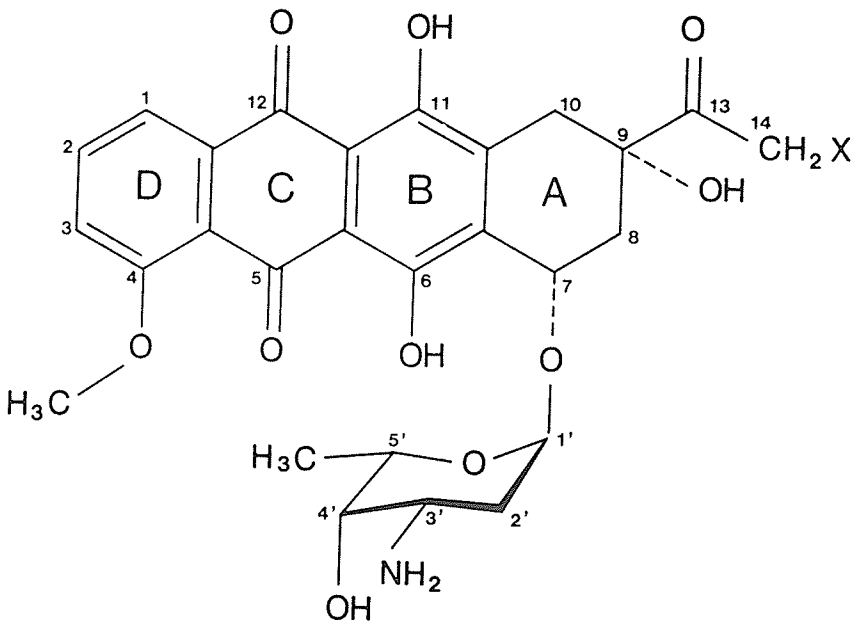
The anthracycline antibiotics, daunorubicin (DAU), and doxorubicin (or Adriamycin, ADR) are important and well established antitumor agents that are effective in the treatment of a broad spectrum of human cancers (20,28). However, the efficacy of these agents is hampered by a number of undesirable side effects, *eg.* cardiotoxicity, and their inactivity against some tumors of clinical importance. Exhaustive studies have thus been undertaken to develop and evaluate analogs of DAU and ADR, in the hope of increasing the antitumor activity and reducing the toxic side effects of these drugs. One such analog, 3'-(3-cyano-4-morpholinyl)-3'-deaminoadriamycin (CMA), has demonstrated increased potency compared to ADR, and has not shown the cardiotoxicity of its parent compound in animal studies (1,125). These findings suggest that CMA has a unique mechanism of antitumor action. In the six years since the initial synthesis of CMA, there have been ongoing efforts to determine the mechanism of action of this analog, but little progress has been made in relating its *in vitro* activities to its enhanced potency. One feature of CMA which is unique among the anthracyclines, is the ability of the drug to produce DNA-DNA crosslinking (7,10,70,151,154). The present study has examined the mechanism by which CMA produces crosslinking, and has attempted to determine the role of this activity in the enhanced potency of the drug.

ANTHRACYCLINE ANTIBIOTICS

Historical

The anthracycline antibiotic, DAU (Fig.1), was originally isolated almost 30 years ago from different *Streptomyces* species (2,3,20). It was structurally unique among anthracyclines, in that it possessed a glycosidic linkage from the tetracycline chromophore to a daunosamine sugar residue. DAU was found to be an effective antileukemic agent, and became the first drug to provide long term remissions in acute leukemia (3). A related compound, ADR, was subsequently isolated from a mutant strain of *Streptomyces peucetius,* and found to have both a wider spectrum of antitumor action and greater therapeutic index than DAU (36). Structurally, ADR only differed from DAU in the hydroxylation of its 14th carbon (Fig.1). ADR is now well established for the treatment of acute leukemias and lymphomas and a wide range of solid tumors (20,28); however, its clinical usefulness is hampered by toxic side effects which include: dose-limiting cardiotoxicity (83), myelosuppression (21), nausea/vomiting, and alopecia (20). As well, some clinically important tumors are not responsive to ADR including pancreatic, renal and colorectal cancers (20,28), and many tumors which initially respond to ADR eventually become resistant to the agent (25,56).

A variety of mechanisms by which ADR exerts its antitumor action have been proposed. At the present time, most evidence suggests that the cytocidal activity of ADR is mediated by the enzyme, DNA topoisomerase II (82,115,139). The sections below will discuss



DAU	X= H
ADR	X= OH

FIGURE 1: Structures of DAU and ADR.

the biochemical activities and proposed mechanisms of antitumor action of the anthracyclines, and will describe mechanisms of ADR toxicity and resistance.

Biochemical activities

Binding to DNA

ADR and DAU have been shown to concentrate in nuclei and stain the chromosomes of cells in culture (35). Structural characteristics of the complex between drug and DNA suggest that the anthracyclines intercalate between DNA strands (19,157), although non-intercalative DNA binding has been observed (19,127). ADR and DAU can also react with RNA (19) and affect the structural organization of chromatin (117,157).

Three-dimensionally, anthracycline intercalation in DNA appears to involve the planer polycyclic ring structure of the drug molecule (Fig.1) inserted between, and hydrogen bonded to, adjacent base pairs of DNA (99,103,107). While many intercalating agents possess varying degrees of specificity for guanine-cytosine (G-C) bases of DNA, the anthracyclines do not show base specificity (42,102), and the intercalative process of these drugs is more of a random nature. Non-intercalative binding of drug to DNA may involve the daunosamine sugar residue, which is also involved in binding the drug to cell membranes (19), and may require metal ions (117).

Binding to Membranes

The anthracyclines are known to bind to the cell membrane and can cause changes in its structural and functional properties (145). ADR binds with the highest affinity to the negatively charged phospholipids, cardiolipin and phosphatidylserine (affinity for cardiolipin is greater than that for phosphatidylserine), and can inhibit cardiolipin synthesis (58,118). Membrane binding involves the protonated amino group of the daunosamine sugar residue (19). Other membrane sites are also capable of binding ADR, including the RBC membrane protein, spectrin, and various membrane glycoproteins (57,92).

Movement of ADR into the cell probably involves binding of drug to the membrane. While Dano and others have shown that efflux of anthracyclines involves an active carrier-mediated process (26,34,131), it is unclear whether influx is carrier-mediated. When ADR is not bound to the membrane, it remains in the protonated form and can not enter the cell (32).

Free Radical Formation

Free radical species, such as the hydroxyl radical, can cause cell damage by producing DNA strand breaks (86,89,119) and lipid peroxidation (94,96). It has been shown that the cytotoxicity of quinone antitumor agents such as ADR can be inhibited by free radical scavengers and by protective enzymes, like superoxide dismutase and catalase (37).

Under aerobic conditions, the quinone moiety of anthracyclines

undergoes one electron reduction to form a semiquinone species, or two electron reduction to form a hydroquinone species. One electron reduction can be catalyzed by such enzymes as cytochrome P-450 reductase or xanthine oxidase, or can take place spontaneously at neutral pH (5,46). The semiquinone can be re-oxidized to the quinone, producing the superoxide radical, which is converted to hydrogen peroxide either spontaneously or by superoxide dismutase. Hydrogen peroxide can be converted to water and oxygen by catalase, or to the highly reactive hydroxyl radical by iron salts or combination with superoxide (116). Myers *et al.* (95) have shown that iron chelation by ADR can mediate hydrogen peroxide-dependent hydroxyl radical formation. In addition, the combination of the ADR semiquinone and hydrogen peroxide can produce the hydroxyl radical without the requirement of metal ions (155).

Two-electron reduction of the quinone to the hydroquinone does not lead to oxygen-dependent production of free radical species. There is little evidence that two-electron reduction of ADR takes place *in vivo*, in normal cells; however, the reaction can be catalyzed by the enzyme, DT-diaphorase, which is present at high levels in some tumor cells (50).

Under anaerobic conditions, both the quinone and semiquinone species of ADR can be enzymatically converted to compounds capable of alkylating DNA and proteins (98). The semiquinone undergoes a rearrangement in which the unpaired electron moves to the C7 position, resulting in cleavage of the daunosamine sugar residue and formation of

the C7-free radical (128). This compound is then capable of covalently binding to DNA or proteins (130). If ADR undergoes a two electron reduction to produce the hydroquinone, there may be an intermolecular electron transfer causing cleavage of the glycosidic bond and formation of an alkylating C7-quinone methide (128).

Metal Ion Chelation

As mentioned previously, ADR can chelate metal ions such as iron, and it has been suggested that this activity is a result of the hydroxyquinone structure of the drug (50). The ADR-Fe(III) complex has been shown to bind to DNA in a non-intercalative fashion (43), and directly catalyze the transfer of electrons from reduced glutathione (GSH) to oxygen, resulting in free radical production and DNA damage (95). This activity is in contrast to the intercalation process, where ADR loses its ability to form free radicals upon association with DNA. Thus, this evidence suggests that it is possible to obtain oxygen free radicals by a process not involving the semiquinone. It has also been demonstrated that the ADR-Fe(III) complex can bind to cell membranes, as seen with erythrocyte ghosts, and cause oxidative destruction (94).

In vivo, free iron concentrations are at low levels, as most of the iron is bound to proteins such as ferritin and transferrin or kept in the porphyrin rings of cytochromes and hemoglobin (50). Whether ADR is capable of abstracting iron from these sources is not known.

Mechanisms of antitumor activity

DNA damage

It has been shown that the interaction of anthracyclines and DNA results in the formation of a complex between the drug and DNA topoisomerase II, which manifests as protein-associated double strand breaks and DNA-protein crosslinks (82,115,139). Type II DNA topoisomerases are ATP-dependent nuclear enzymes that introduce transient double strand breaks in DNA, for the purpose of relieving tortional stress on the molecule, and decatenating circular DNA. ADR and other agents inhibit the normally transient stage of the reaction in which topoisomerase II covalently binds to the 5′ terminus of DNA during the strand passage step of the DNA breaking-rejoining reaction (114). For this reason, DNA breaks are unmasked by proteinase K during elution assays. ADR-resistant cells have been shown to have both qualitative and quantitative reductions in topoisomerase II activity (31,104), indicating that this activity may play an important role in the antitumor activity of ADR.

Anthracyclines also damage DNA via free radical mechanisms. DNA single strand breaks can result from free radicals produced by either the ADR semiquinone, or the ADR-Fe(III) complex. Recent evidence has shown that exogenous free radical scavengers and detoxifying enzymes reduce the antitumor activity of agents like ADR (37), and depletion of cellular glutathione levels by L-buthionine sulfoximine results in increased ADR antitumor effect (41,64,84). Sinha *et al.* (129) have shown that there are significantly reduced levels of the hydroxyl

radical generated by ADR in resistant MCF-7 breast cancer cells. In contrast, it has been reported that Chinese hamster ovary (CHO) cells containing elevated levels of superoxide dismutase and catalase are not cross-resistant to ADR (79), and when free radical scavengers and iron chelators have been used to decrease the cardiotoxicity of ADR, there has been no corresponding reduction in antitumor activity (38,45,108). Thus, it appears that free radicals may play a role in the antitumor activity of anthracyclines, however, this may vary between cell types.

Interactions with DNA and RNA

Another activity which has been examined with anthracyclines, is the inhibition of both DNA and pre-ribosomal RNA synthesis. This activity appears to involve direct interactions of the agents with the DNA template (3,27) and has no base specificity (42,102). For ADR and DAU, inhibition of nucleic acid synthesis requires drug concentrations which are higher than those required for cytotoxicity, suggesting that this activity may not be important to their mechanism of action. However, some anthracycline analogs can cause inhibition of pre-ribosomal RNA synthesis at drug concentrations which are lower than that required for cytotoxicity (27,76,150). Thus, the role that this action plays in the cytotoxic activity of these analogs is not clear.

Interactions with Membranes

Some evidence exists that the membrane may be involved in ADR-induced cytotoxicity. Tritton *et al.* (145) reported that when ADR was

linked to polymer beads preventing it from entering cells, cytotoxicity was not altered. While these experiments may not have been physiological, they suggested that cytotoxicity could be achieved without the interaction of drug with DNA or other cellular organelles. It has been proposed that ADR could modulate the surface of tumor cells and cause cell death through alterations in membrane fluidity, ion flux, or the expression of growth factor receptors (67); however, at the present time there is little evidence to support these mechanisms. If the membrane is involved in the antitumor action of ADR, it would suggest that there is a common basis to the cytotoxicity and the cardiotoxicity of the drug, as the latter activity does appear to be a result of membrane interactions (96).

Mechanisms of toxicity

Cardiotoxicity

A major limitation to the therapeutic efficacy of ADR is the occurance of a chronic dose-dependent cardiotoxicity, which is characterized histologically by dilated sarcoplasmic reticulum, and electron dense mitochondria (20). In advanced stages, cardiac myocytes can become fibrotic. The most common biochemical lesion seen in this form of cardiomyopathy is a defect in calcium transport (147). The two most accepted mechanisms of ADR cardiotoxicity involve free-radical mediated lipid peroxidation, and direct effects of ADR on cell membranes.

Experimentally, cardiac mitochondria and sarcosomes, sites of both

calcium handling and ADR damage, have been shown to produce superoxide in the presence of the drug (40,140). As well, ADR-stimulated hydrogen peroxide production has been observed in perfused rat hearts, and can be inhibited by superoxide dismutase, catalase, and the iron chelator, ICRF-187 (108). Free radical scavenging agents, such as α-tocopherol, and reducing agents like ascorbic acid, have been shown to decrease ADR cardiotoxicity in animal models (47,96). These findings suggest a role for free radicals in ADR-induced cardiomyopathy, and support a mechanism of a membrane bound ADR-Fe(III) complex which is selectively toxic to cardiac cells.

Free radical inactivating mechanisms have been studied in cardiac tissues in an attempt to understand why ADR toxicity is selective to the heart and not observed in other organs where free radical generating mechanisms are present. Doroshow *et al.* (39) reported that the levels of superoxide dismutase and catalase were significantly less in cardiac tissue than in liver, and that the level of glutathione peroxidase, which conjugates reduced glutathione (GSH) with hydrogen peroxide, was decreased in the heart after ADR treatment. Exogenous administration of the sulfhydryl-containing compound, N-acetylcysteine, markedly decreased the lethality of ADR in mice (38), whereas GSH depletion by 1,3-bis(2-chloroethyl)-1-nitrosurea (BCNU) resulted in ADR-induced lipid peroxidation in isolated hepatocytes (4). These findings suggested that free sulfhydryl content could be a determinant of ADR toxicity, and as the heart had no means of removing hydrogen peroxide during ADR exposure, free radical damage and lipid

peroxidation were able to proceed.

Recent evidence has indicated that lipid peroxidation can enhance phospholipase activity and thus cause degradation of mitochondrial membranes, leading to cardiac dysfunction (97). This finding supports a free radical mechanism of ADR-induced cardiomyopathy.

A direct membrane action of ADR has also been suggested as a possible mechanism for its cardiotoxicity since cardiac mitochondrial membranes are rich in cardiolipin, and can therefore bind large amounts of ADR (145). ADR and its major metabolite, doxorubicinol, have been shown to produce negative inotropic effects in cardiac muscle as a result of their ability to inhibit membrane-associated ion pumps (15). Doxorubicinol was shown to be a potent inhibitor of ATPase activity in the sarcoplasmic reticulum and sarcolemma and in cardiac mitochondria (15). ATPase inhibition could lead to high levels of intracellular calcium, which would explain the dilated sarcoplasmic reticulum and electron dense mitrochondria seen in cardiac tissue following ADR treatment.

The bipyridines and methylxanthines are cardiotonic drugs which act independently of β-receptors and sodium-potassium ATPases. They exert their effect by the inhibition of phosphodiesterases, although methylxanthines are only active at very high concentrations, and thus increase cAMP and the calcium sensitivity of the cell. Two such drugs, amrinone and sulmazole, have recently been shown to reduce the negative inotropic effects of ADR in isolated guinea pig atria (14), suggesting that calcium handling plays an important role in ADR cardiotoxicity.

It is unclear at the present time whether ADR-induced cardiotoxicity is a result of free radical-mediated lipid peroxidation, or altered calcium handling.

Genotoxicity

ADR has been shown to be mutagenic in that it damages DNA by producing sister chromatid exchanges and point mutations (105). Mutations have been observed in cells following treatment with agents which inhibit topoisomerase II (33,105), suggesting a mechanism for the mutagenic action of these drugs. ADR is also a carcinogen, as increases in tumor incidence have been observed in rats treated with the drug for prolonged periods (90,133).

Other Toxicities

Most cancer chemotherapeutic agents function by inhibiting the growth of proliferating cells, and as a result toxic effects are usually seen on bone marrow, hair, and gastrointestinal mucosal cells. The anthracyclines cause myelosuppression, nausea/vomiting, and alopecia in most patients (20), and these effects can be attributed to the antiproliferative action of the drugs.

Another toxicity of ADR can result from its administration. As ADR is given intravenously, subcutaneous leakage of the drug into the perivascular space can result in necrosis and ulceration of the tissue (20,50), and treatment often requires plastic surgery. Free radical scavengers have been shown to decrease ADR-induced tissue

damage (137), suggesting a role for free radicals in this form of toxicity.

Mechanisms of drug resistance

The development of drug resistance is one of the major limitations to the efficacy of anthracycline therapy. In many circumstances, resistance to anthracyclines is associated with cross-resistance to a number of other natural product drugs (13,110). This phenomenon is known as multidrug resistance (MDR). The mechanisms which have been proposed to explain resistance to anthracyclines include altered drug transport, decreased topoisomerase II activity, and changes in drug detoxifying systems.

Alterations in drug transport

The reduced ability of a tumor cell to take up and retain cytotoxic drugs would provide an obvious means for drug resistance. It has been demonstrated that the cellular accumulation of DAU in DAU-resistant Ehrlich ascites tumor cells is less than that in sensitive cells, as a result of increased drug efflux (26). Skovsgaard (132) has also observed that active extrusion of both DAU and the mitotic spindle poison, vincristine, takes place in DAU-resistant cells, suggesting a common mechanism of resistance to these drugs.

Biedler and Riehm (13,110) were the first to describe the phenomenon of MDR in P388 murine leukemia and Chinese hamster cells which had been selected for resistance to actinomycin D. It was shown

that these cells were cross-resistant to a wide range of natural product drugs such as DAU and vinblastine. Ling *et al.* (77,78,112) later demonstrated that the MDR phenotype was associated with decreased intracellular drug accumulation, and with the presence of a M_r 170,000 membrane glycoprotein, p-glycoprotein (p-gp). The gene for MDR (*mdr*-1), which encodes p-gp, can be either amplified or over expressed in resistant cell lines (111,120,124), and recent experiments have shown that transfection of *mdr*-1 into drug-sensitive cells produces the MDR phenotype and p-gp expression (29,61). It has also been reported that the coding sequence for *mdr*-1 is homologous with specific bacterial membrane proteins involved in periplasmic transport (48,60). These findings suggest that p-gp acts to remove drug from inside the cell and provides a mechanism for resistance to ADR and other agents.

P-gp has been shown to contain two ATP binding sites on the cytoplasmic side of the protein (24,48). The calcium antagonist, verapamil, can inhibit active efflux of drug and reverse the MDR phenotype (146) and has been shown to increase ATPase activity and ATP consumption in MDR cells (16,63). Since there is no calcium requirement for drug extrusion, the above findings suggest that verapamil competes with ADR and other drugs for binding to an energy-dependent extrusion pump.

Other mechanisms of drug resistance

The role of DNA topoisomerase II in the antitumor activity of the anthracyclines has been discussed, and it has been reported that

topoisomerase II levels are low in leukemic cells which are unresponsive to ADR (106). Furthermore, two groups have shown that there are quantitative and qualitative changes in the activity of topoisomerase II in ADR-resistant cells (31,104). Deffie *et al.* (31) demonstrated that both the catalytic and cleavage activities of the enzyme were reduced in resistant cells, and by western blot analysis it was shown that the level of topoisomerase II enzyme was decreased. A previous report had shown that the same resistant cells had increased levels of p-gp, increased repair of topoisomerase-mediated double strand breaks, increased glutathione-S-transferase (GST) activity, and a decreased level of ADR uptake (30). These results suggest that resistance to ADR in P388 cells is multifactorial, and may involve more than alterations of drug transport.

The GSTs are a group of isozymes which are involved in detoxifying cells by conjugating electrophilic compounds with GSH in the cytoplasm (62). The enzymes can be involved in conjugating GSH with alkylating agents, like BCNU and melphalan (44), and may play an important role in ADR resistance, since GSH can act as a free radical scavenger (30,123). It has been shown that cells which contained enhanced levels of the free radical detoxifying enzymes, superoxide dismutase and catalase, were not cross-resistant to ADR (79), suggesting that these enzymes are not involved in anthracycline resistance. However, cells which had been treated with L-buthionine sulfoximine, to reduce cellular GSH, were hypersensitive to ADR (41,64,84). Sinha and others have shown that GSH can have an indirect role in ADR resistance, as altered

glutathione redox cycling is associated with the MDR phenotype (41,80,129). The glutathione redox cycle is involved in removing hydrogen peroxide from the cell through the action of glutathione peroxidase (41,80). It has also been reported that glutathione esters may play a role in repairing oxidative damage to DNA (152). These findings suggest that GSH, and its associated enzymes can play a role in anthracycline resistance by protecting the cell from oxidative damage.

Summary

DAU and ADR are complex agents with multiple biochemical effects. The mechanism of antitumor action of these drugs may involve damaging DNA through topoisomerase II inhibition or free radical mechanisms, and may be dependent on cell type. Most of the toxicities of these drugs are a result of their cytocidal effects on proliferating cells; however, the chronic cardiotoxicity appears to be the result of a different mechanism. Resistance to the anthracyclines can involve alterations in drug transport, changes in topoisomerase II, or modifications in drug detoxifying systems.

ALKYLATING AGENTS

Historical

Alkylating agents are a highly reactive group of antitumor drugs which exert their effects by covalently modifying nucleophilic sites

within cells. They are effective in the treatment of some leukemias and lymphomas, and a broad range of solid tumors (17). The use of these agents in cancer chemotherapy developed from observations of the myelosuppressive and lymphocytolytic effects of the sulfur mustard gases used in World War I (53). Methyl-bis(β-chloro-ethyl)amine (HN2), a member of the nitrogen mustard group of alkylating agents and a structural analog of mustard gas, was eventually used in the treatment of malignant diseases following World War II. Since that time a number of polyfunctional alkylating drugs have been developed in the search for more stable and less toxic compounds. These include nitrogen mustards like chlorambucil (CHL), melphalan and cyclophosphamide, nitrosoureas like BCNU, and alkylsulfonates, aziridines, mitomycins, and platinum compounds.

The therapeutic efficacy of polyfunctional alkylating agents is limited by the development of drug resistance, and in many cases cross-resistance to a number of alkylating drugs. For this reason, these agents play an important role in synergistic combination therapy with other anticancer drugs. The toxic effects of alkylating agents are generally dose-related, and occur in proliferating tissues such as bone marrow and the gastrointestinal tract (17). As the most commonly used agents are given orally, they do not result in vesicant effects on the skin. Chronic toxicities include alopecia, myelosuppression, and peripheral neuropathies (17).

Mechanism of antitumor activity

The alkylating agents become reactive via the formation of carbonium ion intermediates which alkylate nucleophilic sites within normal and neoplastic cells (53). Tumor specificity of these agents may be a result of altered pharmacokinetic and metabolic patterns within different tumor types (17). The observation of cross-resistance to multiple alkylating agents suggests that these drugs share similar mechanisms of antitumor action (138), and some agents, such as mitomycin C (MMC), appear to require more complicated mechanisms of activation than others (69).

The cytotoxic action of alkylating agents is assumed to relate to their ability to covalently alkylate DNA bases (66). The vast majority of alkylating agents bind to DNA at the N^7 and O^6 positions of guanine, however, all oxygen and nitrogen residues on DNA bases are potential alkylation sites (126), and it has been suggested that some sites may be more critical to cytotoxicity than others. The most effective alkylating drugs are bifunctional, in that they have two reactive groups and can thus bind to complementary strands of DNA forming interstrand (DNA-DNA) crosslinks (66,141). It has been shown that this type of DNA-drug interaction can be lethal to cells by interfering with the DNA template and inhibiting DNA replication (113). Interstrand crosslinking correlates well with the cytotoxicity of bifunctional alkylating agents (66), and enhanced repair of these crosslinks may explain alkylator-resistance in some cells (6). As well, reduced repair of crosslinking has been associated with hypersensitivity to MMC

(68), providing further evidence that interstrand crosslinking is the mechanism by which these agents exert their antitumor effects. In contrast, while DNA intrastrand and DNA-protein crosslinking can be produced by alkylating drugs, these effects do not correlate with cytotoxicity (65). Miscoding and depurination have also been shown to result from the alkylation of DNA bases, but likely only play an important role in the antitumor action of the monofunctional alkylating agents (65,66).

Mechanisms of drug resistance

Several mechanisms of resistance to alkylating agents have been observed, including decreased drug uptake (9,11,52,55), enhanced drug inactivation, *eg.* by GSH and GST (18,59,136,149), drug metabolism (59), and increased capacity to repair DNA damage (6,49,91,113). In addition, it has been shown that resistance to alkylating agents is often due to more than one of these mechanisms.

Alterations in drug transport

It was reported by Goldenberg *et al.* (55), that L5178Y murine lymphoma cells which were resistant to HN2, L5178Y/HN2, had both a reduced capacity for drug uptake, and increased sulfhydryl levels. While influx of HN2 was not altered in these cells, efflux was enhanced by the presence of an active carrier-mediated transport system. The endogenous substrate for the transporter was choline, a close structural analog of HN2, suggesting that there was specificity for HN2

transport (54). However, as enhanced efflux could not explain all of the resistance to HN2, it was suggested that resistance might be due to more than one mechanism. In contrast to the 20-30-fold resistance to HN2, the cells were 2-3-fold cross-resistant to the alkylating agents CHL, melphalan, BCNU and MMC, and these drugs did not competitively inhibit HN2 transport (52). These findings suggested that cross-resistance might be a result of different transport systems, elevated sulfhydryl levels, or other mechanisms.

In L5178Y cell murine lymphoma cells, uptake of the amino acid nitrogen mustard, melphalan, was shown to involve an active carrier-mediated process, utilizing two separate amino acid carriers (11). However, the cytotoxic activity of melphalan was reduced by the presence of leucine and glutamine (148), suggesting that altered transport of melphalan could be a mechanism of resistance to the drug. It was later shown that melphalan-resistant CHO cells had reduced drug uptake, increased sulfhydryl levels, and decreased melphalan crosslinking (9). As reduced drug uptake could not account for all of the melphalan resistance in these cells, and it did not appear that decreased crosslinking activity was a result of repair, it was suggested that resistance was multifactorial.

Drug detoxification mechanisms

GSH can play the same role in inactivating alkylating agents as it does with free radicals. It has been shown that there is a good correlation between tumor sulfhydryl levels and resistance to

alkylating agents (18,136). Furthermore, Vistica *et al.* (148) have shown that exogenous thiol-containing amino acids can produce resistance to melphalan, and it has been reported that depletion of cellular GSH by incubating cells in cysteine-free medium or in the presence of L-buthionine sulfoximine can enhance the cytotoxic action of melphalan and other alkylating agents (59,64).

The GST isozymes are involved in conjugating a wide range of electrophilic compounds with GSH (62). Although many alkylating agents can readily react with GSH, the GSTs may increase both the rate and efficiency of the interaction and thus play an important role in removing reactive alkylating agent metabolites from the cell before they reach critical target sites (123). Elevated GST levels have been associated with resistance to alkylating agents like HN2, CHL, melphalan and BCNU *in vitro* (44,149), suggesting a role in drug resistance.

Alterations in DNA repair

Repair of the DNA lesions produced by bifunctional alkylating agents was first reported by Roberts *et al.* (113), in murine lymphoma cell lines which showed differing sensitivities to mustard gas. Cells of the lowest sensitivity had the highest ability to repair DNA damage, suggesting that resistance was a result of enhanced crosslink removal. In a recent study, Ozols *et al.* (91) have shown that mutant A2780 human ovarian cancer cells selected for resistance to the alkylating agent, cisplatin, had an enhanced ability to repair DNA damage. The specific

inhibitor of DNA polymerase α, Aphidicolin, produced a dose-dependent inhibition of "excision repair" and a corresponding increase in cisplatin cytotoxicity, indicating a direct correlation between repair of DNA damage and cytotoxicity.

Other studies have shown that resistance to alkylating agents in some cell types, can correlate with increased activity of enzymes like O^6-alkylguanine-DNA alkyltransferase (49,122), which remove the DNA adducts formed by alkylating agents. In addition, Gerson *et al.* (49) have shown that the alkyltransferase inhibitor, O^6-methylguanine, can sensitize BCNU-resistant HL-60 cells to nitrosoureas, indicating that resistance in these cells is due an elevated ability to repair DNA damage.

Summary

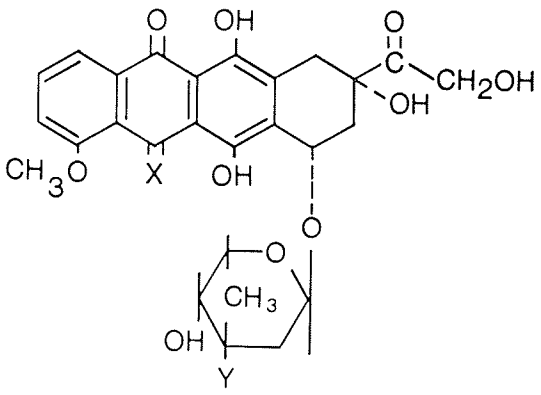
In summary, it appears that most alkylating agents possess similar mechanisms of antitumor action. Damage to DNA in the form of interstrand crosslinking results from the alkylation of DNA bases by bifunctional drugs and correlates well with cytotoxicity. Cross-resistance to these agents has been demonstrated in a number of tumors, and the mechanisms of resistance can involve decreased drug uptake, increased levels of drug detoxifying compounds, and enhanced repair of DNA damage.

CYANOMORPHOLINYL ADRIAMYCIN

Historical

A large number of structural analogs of DAU and ADR have been synthesized to improve the therapeutic efficacy of this class of drugs. Some analogs, such as epirubicin (4'-epidoxorubicin), idarubicin (4-demethoxydaunorubicin), and esorubicin (4'-deoxydoxorubicin) have gone into clinical trials, but improvements in potency, spectrum of activity, and toxicity have been limited (2,156).

Acton *et al.* (1,93,144) have synthesized a number of analogs of ADR, in which the C3'amines are N-alkylated. This form of chemical modification was predicted to produce more lipid soluble compounds which would retain their C3' basicity and thus their ability to interact with DNA and membrane phospholipids. One such analog, 3'-(4-morpholinyl)-3'-deaminoadriamycin (MA) (Fig.2), was found to be 4 times more potent than ADR against P388 murine leukemia *in vivo* (93). However, a related analog, CMA (Fig.2), in which the C3'amine was replaced by a cyanomorpholinyl ring, was found to be 700-fold more potent than ADR in P388 leukemia *in vivo* (1), 100-fold more active than ADR in HT-29 human colon carcinoma cells *in vitro* (70,75,76) and 40- to 1500-fold more potent than ADR in a variety of other cell lines (7,125,135,151). In addition, CMA produced no cardiotoxicity at concentrations which caused antitumor effects in mice and rats (1,125), and ADR-resistant human ovarian sarcoma and MDR P388 leukemia cells were not cross-resistant to either CMA or MA *in vitro* (72,125,134). It has been suggested that the reason CMA does not produce cardiotoxic



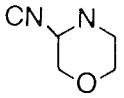
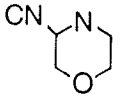
DRUG	X	Y
ADR	O	NH_2
CMA	O	
ICMA	NH	
MA	O	

FIGURE 2: Structures of ADR, MA, CMA, and ICMA.

effects, is because it effectively kills tumor cells at concentrations well below those required for cardiotoxicity (101,125). The observation that CMA and MA are effective against ADR-resistant cells, indicates that they may have a unique mechanism of antitumor action. Sikic *et al.* (121) have suggested that non-cross-resistance to CMA in MDR P388 cells may be due to the enhanced lipophilicity of the analog or to reduced affinity for p-gp as a result of C3'substitution.

The 5-imino derivatives of the anthracyclines have also been synthesized (1,142). It was shown that this type of modification of the quinone ring resulted in less free radical forming capacity (87) and less cardiotoxicity (142). While 5-imino DAU retained its antitumor activity compared to DAU and ADR *in vivo* (142) and *in vitro* (51), the 5-imino derivative of CMA, ICMA, was more potent than ADR and MA, but less cytotoxic than CMA *in vivo* (1) and *in vitro* (70). These findings suggested that the quinone ring might play a role in the antitumor activity of CMA. Because of its structural modifications, ICMA has also been a useful tool for investigating the mechanism of action of CMA.

Mechanism of action

It has been shown that CMA, ICMA, and the cyanomorpholinyl analogs of DAU, can act as alkylating agents, producing DNA-DNA crosslinking *in vitro*. Westendorf *et al.* (154) were the first to observe this activity with CMA and cyanomorpholinyl DAU in L1210 murine leukemia and V79 murine fibroblast cell lines. Begleiter *et al.* (10,70-72) have since

shown that both CMA and ICMA can produce DNA crosslinking in HT-29 human colon carcinoma cells and nuclei, L5178Y murine lymphoblast cells, and isolated λ-phage DNA, while other groups have reported this activity for CMA as well (121,151). In contrast, DNA-DNA crosslinking has not been observed with ADR, MA, or any other anthracyclines which do not contain a cyanomorpholinyl group. Crosslinking may account for the enhanced potency of CMA and ICMA, and as only those analogs which contain the cyanomorpholinyl moiety produce the activity, it appears that the cyanide group is essential for crosslinking and that the quinone ring is also involved. Recent reports have shown that there is facile exchange of the cyanide group of CMA (100,153) suggesting that it may act as a leaving group in the alkylation process. This activity may resemble that of the potent alkylating antibiotic, Saframycin A, which is structurally similar to CMA in that it has a cyanide group linked to a heterocyclic ring and a quinone (76,88). Saframycin A binds covalently to DNA following elimination of the cyanide, and this activity is potentiated by reduction of the quinone.

In a previous study it was shown that neither CMA, nor ICMA produced DNA-protein crosslinking at cytotoxic doses, suggesting that these analogs did not form topisomerase II cleavable complexes (70). Furthermore, as crosslinking occured in isolated HT-29 nuclei and λ-phage DNA, metabolic activation of the analog did not appear to be required for alkylating activity (70).

The role of the quinone ring of the morpholinyl anthracyclines in free radical production, has been examined by Peters *et al.* (101). It

was shown that the redox activities of ADR, MA, and CMA in P388 and rat liver microsomes did not correlate with cytotoxicity. In fact, it was noted that the concentration of CMA required to augment oxygen consumption was well above that required for cytotoxicity. As this was not the case for MA and ADR, it appeared that oxygen free radical production was not involved in the antitumor action of CMA (101).

Other studies have shown that uptake of MA and CMA in P388 and HT-29 cells was greater than that of ADR, and was related to the greater lipophilicity of the analogs (73,74,76,134). Thus, the increased potency of MA compared to ADR may be due to differences in drug uptake. The uptake of MA and CMA was reduced in ADR-resistant P388 cells compared to sensitive cells, and verapamil could reverse this effect (134). This finding suggested that resistance to MA and CMA involved similar mechanisms. Since the order of uptake of these drugs was MA > CMA > ADR, the enhanced cytotoxicity of CMA could not be explained on a basis of drug transport alone. It was also shown that the level of efflux of CMA was less than that of the other analogs, suggesting that the drug remained bound to sites within the cell (76,134).

CMA has been shown to be a much more potent inhibitor of nucleic acid synthesis than either MA or ADR, in L1210 murine leukemia, KBM human leukemia, and HT-29 cells (1,76,151). While these reports indicated that the inhibition of RNA synthesis was achieved at lower concentrations of the analogs than DNA synthesis, the DNA inhibitory effects of CMA correlated better with cytotoxicity than the RNA

inhibitory effects. The effects of the analogs on the inhibition of polymerase activity was also examined, and at low doses CMA was only slightly more potent than the other analogs in inhibiting DNA and RNA polymerases from *E. coli* and chicken leukemia cells (23,75). Furthermore, inhibition of polymerase activity appeared to result more from drug interactions with the DNA template than with the enzymes (23,150), providing an explanation for the poor correlation between cytotoxicity and polymerase inhibition.

Summary

CMA is an extremely potent analog of the antitumor agent, ADR, being up to 1500 times more potent both *in vivo* and *in vitro*. As well, the analog is effective against ADR-resistant cells and does not produce the chronic cardiotoxicity associated with anthracycline therapy. In contrast to non-cyanomorpholinyl containing anthracyclines, CMA is able to produce DNA-DNA crosslinking *in vitro*. The following evidence supports DNA crosslinking as a mechanism of antitumor activity for CMA: (i) analogs which contain the cyano and the quinone moieties and are able to crosslink DNA are also highly cytotoxic; (ii) CMA is a more potent inhibitor of nucleic acid synthesis than other intercalators and this effect correlates with antitumor activity. However, there has been no direct correlation between the antitumor and the crosslinking activities of this drug, and the mechanism by which CMA produces crosslinking has not been examined.

OBJECTIVES

An essential requirement to directly correlate the crosslinking and the cytotoxic activities of CMA, would be to show that crosslinking is reduced in cells which are resistant to the drug. For this purpose, the cytocidal activity of CMA has been examined in cell lines which were selected for resistance to other alkylating agents. In those cell lines which showed cross-resistant to CMA, the crosslinking activity of the drug was measured to determine if it was altered.

It has been demonstrated that the quinone group of CMA plays a role in both the enhanced potency and the crosslinking activity of the analog. To examine this role, the cytotoxic activity of CMA was measured in a cell line which was selected for resistance to the model quinone antitumor agent, hydrolyzed benzoquinone mustard, a drug which produces its antitumor activity by causing free-radical induced DNA damage. This cell line had previously been shown to have enhanced activity of the free radical protective enzymes, catalase and superoxide dismutase, as well as elevated levels of GSH and of DT-diaphorase activity (12).

The mechanism by which CMA produces crosslinking is not known. However, since anthracyclines are known to be good intercalators (18,157), and there is evidence that 5-imino substituted anthracyclines intercalate less than their parent compounds (1), it is possible that prior intercalation into DNA could potentiate the crosslinking activity of CMA and might explain its enhanced potency compared to ICMA. Thus, this study has compared the crosslinking, alkylating, and DNA

intercalating activities of CMA to those of ICMA and the clinically used alkylating agent, CHL (Fig.3), in order to determine the roles of intercalation, the quinone ring, and DNA base composition in the crosslinking activity of CMA.

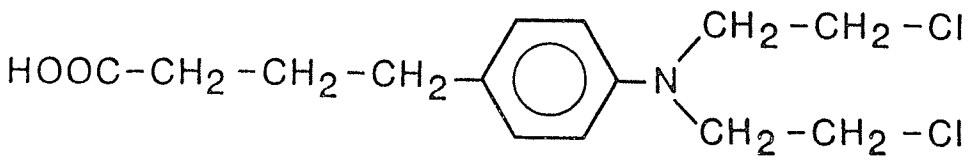


FIGURE 3: Structure of CHL.

II. MATERIALS AND METHODS

Materials

CMA and ICMA were kindly supplied by Dr. Edward M. Acton of the NIH, Bethesda, MD, dissolved in N,N-dimethyl formamide and stored in the dark at -20°C. CHL, ethidium bromide, acridine orange, NBP, sodium borohydride, and ultrapure *Clostridium perfringens, Escherichia coli,* and calf thymus DNAs were obtained from Sigma Chemical Co., St. Louis, MO. CHL was freshly prepared in 1% acidified ethanol. Isolated λ-phage DNA was obtained from Boehringer Mannheim, Dorval, Quebec, Canada. Fischer's medium and horse serum, were obtained from Gibco Laboratories, Grand Island, NY. [^{14}C]Thymidine (specific activity, 50 mCi/mmol) and [^{3}H]thymidine (specific activity, 50 to 80 Ci/mmol) were obtained from New England Nuclear, Boston, MA. Proteinase K was obtained from E. Merck, Darmstadt, West Germany; tetrapropylammonium hydroxide was obtained from Eastman Kodak Co., Rochester, NY, and the 0.8 μm polycarbonate filters were purchased from Nucleopore Corp., Pleasanton, CA. Swinnex polyethylene filter holders were obtained from Millipore Corp., Bedford, MA.

Tissue culture

L5178Y murine lymphoma cells arose from a spontaneous neoplasm in a DBA/2 mouse, and were grown as a suspension culture at 37°C in Fischer's medium supplemented with 12% horse serum (8,22). Exponentially growing cells had a doubling time of 12 hours. Hydrolyzed benzoquinone mustard-resistant L5178Y/HBM10 cells and aniline mustard-resistant L5178Y/AM6 cells, were developed by growing

L5178Y cells in media containing the respective drug (12). The drug concentration was increased in a stepwise fashion every 2 months, reaching final concentrations of 1 mM for hydrolyzed benzoquinone mustard and 6 μM for aniline mustard. The cells were cloned in soft agar (8,22), and single colonies were selected and grown continuously in the presence of drug. L5178Y/HBM10 and L5178Y/AM6 cells grew exponentially with doubling times of 18-22 hours (12). The L5178Y/HN2 nitrogen mustard-resistant cells were obtained from Dr. G.J. Goldenberg, University of Manitoba, grown in Fischer's medium supplemented with 15% horse serum, and had a doubling time of 16.5 hours (55).

Cell viability

Cell viability was measured using the soft agar clonogenic assay as described previously (8,22). L5178Y parental or resistant cells were incubated *in vitro* with various concentrations of CMA for 2 hours at 37°C. The drug was added in a 1:100 dilution, to 1.25×10^6 cells suspended in medium containing 15% horse serum. After the incubation, the cells were washed twice with cold medium, and counted in a Coulter Z_{B1} electronic particle counter. The cells were diluted, to concentrations ranging from 50 to 100,000 cells/tube, in Fischer's medium containing 15% horse serum and 2.6 mg/ml noble agar, and aliquoted into culture tubes in quadruplicate. Following a 10-14 day incubation at 37°C in 95% air:5% CO_2, the colonies were counted and the surviving cell fraction was calculated, as described (8,22). The

cloning efficiencies ranged from 33 to 88%. Cytotoxicity was expressed as an LD_{90}, the concentration of drug required to reduce cell viability by 90%.

DNA crosslinking in whole cells

DNA crosslinking was measured in L5178Y and L5178Y/HN2 cells using a modification of the alkaline elution assay described by Kohn (9,80). Cellular DNA was labeled with either [^{14}C]thymidine or [^{3}H]thymidine by growing the cells for one day in the presence of 1 μM labeled thymidine. On the day of the experiment, [^{14}C]-labeled cells were washed twice to remove excess radioactivity, resuspended in medium at 2 x 10^5 cells/ml, and divided into 2.5 ml aliquots. L5178Y or L5178Y/HN2 cells were incubated for 2 hours at 37°C with varying concentrations of CMA, ICMA, or CHL. After the incubations, the cells were washed with cold medium to remove exogenous drug, and irradiated on ice with 600 rads, using a ^{60}Co source with a dose rate of 89 rads/min. Irradiated cells were then poured onto 0.8 μm polycarbonate filters in Swinnex polyethylene filter holders modified to hold 20 ml (9). [^{3}H]-labeled parental cells were used as an internal control to standardize the flow rate through the filters. The same number of [^{3}H]-labeled cells were washed, irradiated with 150 rads, and added to the filters with the [^{14}C]-labeled cells. The cells were washed once on the filters with PBS (pH 7.2) and exposed to 3 ml of SDS lysing solution (2% SDS, 20 mM Disodium EDTA, 0.1 M glycine, pH 10.0), containing 0.5 mg/ml proteinase K, for 1 hour. The cells were washed

with 5 ml of 20 mM EDTA (pH 10.0), and 10 ml of alkaline eluting solution (20 mM EDTA, pH adjusted to 12.2 with 10% tetrapropylammonium hydroxide) was then carefully layered onto the lysed cells. The alkaline pH caused the DNA to become single stranded, and labeled DNA flowed through the filters at a rate dependent on its size. The eluting solution was slowly pumped into the filter holder at a rate of 2-3 mls/hour, and fractions were collected from each filter over 1½ hour periods. After 18 hours the pump was stopped and any remaining DNA was hydrolyzed from the filters at 60°C. The filter holders were washed with 4 ml of 0.25 N NaOH and the wash was collected. For each filter, the hydrolysate, the wash sample, and the 12 elution fractions were analyzed for [^{14}C] and [^{3}H] radioactivity by liquid scintillation spectrometry. The counting efficiencies were 15% and 75% for [^{3}H] and [^{14}C], respectively. DNA crosslinking was calculated, as described by Kohn (80), by plotting the fraction of [^{14}C] DNA remaining on the filter against the fraction of [^{3}H] DNA remaining on the filter after each 1½ hour time period. The elution profiles of irradiated and non-irradiated control filters provided a means of expressing the DNA crosslinking as rad equivalents (80).

Repair of DNA crosslinking

[^{14}C]-Labeled L5178Y cells were incubated *in vitro* for 2 hours at 37°C with either 60 nM CMA or 120 μM CHL. The cells were washed twice with cold medium to remove exogenous drug and resuspended in Fischer's medium containing 12% horse serum. Aliquots were removed and placed on

ice, and the remaining cells were incubated at 37°C. At time intervals up to 18 hours, additional aliquots were removed and placed on ice, at which time all of the samples were analyzed for DNA crosslinking by the alkaline elution assay with proteinase K (80). Irradiated and non-irradiated control cells were not incubated with the respective drugs.

DNA crosslinking in isolated DNA

Isolated λ-phage DNA (2.5 units/ml) was incubated *in vitro* at 37°C with CMA, ICMA, and CHL, in the presence or absence of the reducing agent, sodium borohydride. For each drug, the concentration of reducing agent used was 10 times that of the highest drug concentration. To confirm that the drug was reduced, the absorbance of sodium borohydride treated CMA was measured on a Beckman DU-8 spectrophotometer using a wavelength scan from 220 nm to 600 nm. Crosslinking was measured by the ethidium bromide fluorescence assay described by Lown (86). At timed intervals, aliquots of the DNA-drug mixture were removed and added to a buffer solution (pH 11.8) containing 20 mM potassium phosphate, 0.8 mM EDTA, and 0.5 μg/ml ethidium bromide (86). The fluorescence of the samples was measured using a Gilson Spectro/Glo Filter fluorimeter with an excitation wavelength of 525 nm and an emission wavelength of 600 nm. The samples were denatured at 100°C for 5 minutes using an aluminum module heating block, rapidly cooled on ice, equilibrated to room temperature, and the fluorescence measured again. Crosslinking was calculated as previously described (86). The maximum percentage of crosslinking

produced by a concentration of drug was determined from the maximum percent of return of fluorescence after heat denaturation. All three drugs produced the maximum level of crosslinking after 100 minutes of incubation with DNA.

To determine the effect of DNA base content on CMA crosslinking, isolated DNAs which contained different percentages of G+C residues were incubated with CMA and crosslinking was measured by the ethidium bromide fluoresence assay (86). *Clostridium perfringens*, calf thymus, and *Escherichia coli* DNA (5 units/ml) contained 26.5, 42, and 50 percent G+C, respectively.

Inhibition of CMA crosslinking was measured in λ-phage DNA by the addition of various concentrations of ethidium bromide to the DNA-drug mixture. The increase in fluorescence caused by the inhibitor was corrected for by controls at each ethidium bromide concentration. A control study was carried out with the non-intercalating alkylating agent, CHL, to determine if intercalation of ethidium bromide would interfere with crosslinking by distortion of the DNA.

Alkylation of p-nitrobenzyl pyridine

The alkylating activities of CMA and CHL, in the presence or absence of sodium borohydride, were determined as described by Linford (85), using a colorimetric measurement of the alkylation product of the drug with the nucleophile, p-nitrobenzyl pyridine (NBP). NBP was incubated with drug for 20 minutes at 56°C in the presence of 1 M acetic acid and then cooled to room temperature. The addition of

triethylamine caused the sample to turn a blue color as a result of the loss of a proton from the initial alkylation product. The absorption spectrum of the sample was recorded using a Beckman DU-8 spectophotometer and the absorbance peaks for the alkylation products from both drugs were at 578-594 nm. Linear regression analysis of the concentration-alkylation curves for CMA and CHL were obtained, and the alkylating activity of the drugs was compared on the basis of the slopes of the regression curves.

Melting temperature of DNA

Various concentrations of CMA, ICMA, or CHL were added to calf thymus DNA (100μg/ml) and the melting temperature (Tm) was determined, as described by Tong (143), using a Beckman DU-8 spectrophotometer and Tm compuset module to monitor the absorbance of DNA at 260 nm. Tm is the temperature at the mid-point of the denaturing transition of DNA. Specifically, this occurs at the maximum rate of increase in absorbance per change in degree temperature. When a drug intercalates into DNA, it causes a change in the Tm as it interferes with DNA unwinding. In these experiments, the change in Tm was calculated by subtracting the Tm of DNA which had not been exposed to drug, from the Tm of DNA which had been exposed to drug.

Displacement of acridine orange

Various concentrations of CMA, ICMA, or CHL, in the presence or absence of sodium borohydride, were added to 10 mM sodium cacodylic

acid buffer (pH 6.7) containing 0.5 μM of the intercalating agent acridine orange. Using a Gilson Spectra/Glo Filter fluorimeter with an excitation wavelength of 480 nm and an emission wavelength of 520 nm, the fluorescence of the samples were determined both before and after the addition of 0.05 units calf thymus, *C. perfringens*, or *E. coli* DNA. The displacement of acridine orange from DNA was determined as described previously (51,109) and the change in fluorescence after the addition of DNA was expressed as a percent of control. For each drug, an IC_{50} (concentration of drug required to reduce the change in fluorescence to 50% of the control) was determined.

III. RESULTS

Cytocidal activities

L5178Y, L5178Y/HBM10, L5178Y/AM6, and L5178Y/HN2 cells were incubated with CMA *in vitro* at 37°C for 2 hours and analyzed for viability by the soft agar clonogenic assay described in "Materials and Methods". In all cell lines, CMA produced concentration-dependent decreases in viability (Figs.4,5) with LD_{90} of: 0.160 nM in the L5178Y cells, 0.175 nM in the L5178Y/HBM10 cells, 0.310 nM in the L5178Y/AM6 cells, and 0.510 nM in the L5178Y/HN2 cells. A comparison of the LD_{90} of CMA in the L5178Y cells to that of CMA in the other cell lines showed that there was no cross-resistance to CMA in the L5178Y/HBM10 cells, but that the L5178Y/AM6 and L5178Y/HN2 cells were 1.94-fold and 3.19-fold resistant to CMA, respectively (Table 1). Unpaired t-test analysis showed that the cell viability of the resistant cells was only significantly different from the parental cells at CMA concentrations up to 0.5 nM for the L5178Y/AM6 cells, and up to 1.0 nM for the L5178Y/HN2 cells.

DNA crosslinking in whole cells

L5178Y and L5178Y/HN2 cells were incubated with CMA *in vitro* for 2 hours at 37°C and the cells were then analyzed for DNA crosslinks by the alkaline elution assay with proteinase K (Fig.6). CMA caused concentration-dependent crosslinking reaching a level of 450 rad equivalents at 100 nM. A comparison of the slopes of the concentration-response curves from the L5178Y parental cells and the L5178Y/HN2 alkylator-resistant cells showed that CMA produced 1.5-fold

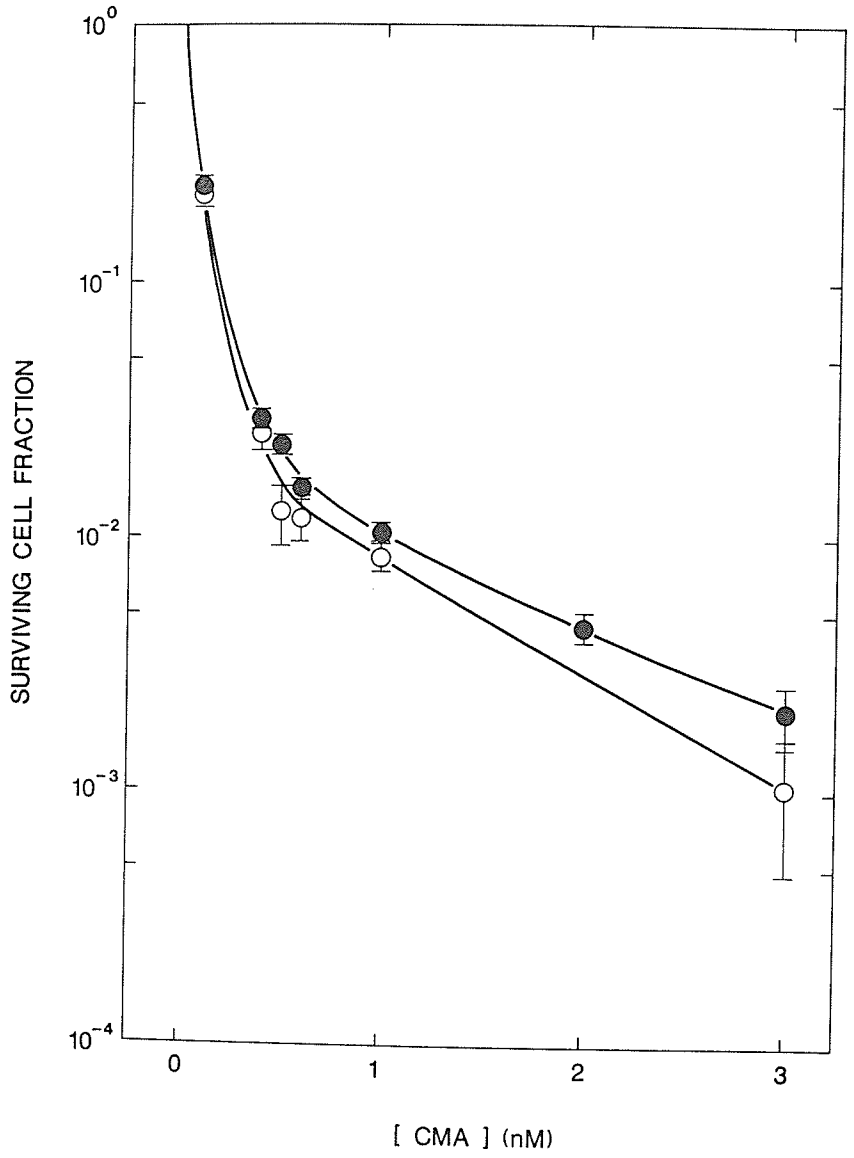


FIGURE 4: Cytotoxicity of CMA in L5178Y and L5178Y/HBM10 cells. L5178Y (○) or L5178Y/HBM10 (●) cells were incubated for 2 hours at 37°C with various concentrations of CMA. Cell viability was measured by the soft agar clonogenic assay described in "Materials and Methods" and expressed as the surviving cell fraction. *Points*, mean of 4-10 determinations; *bars*, standard error.

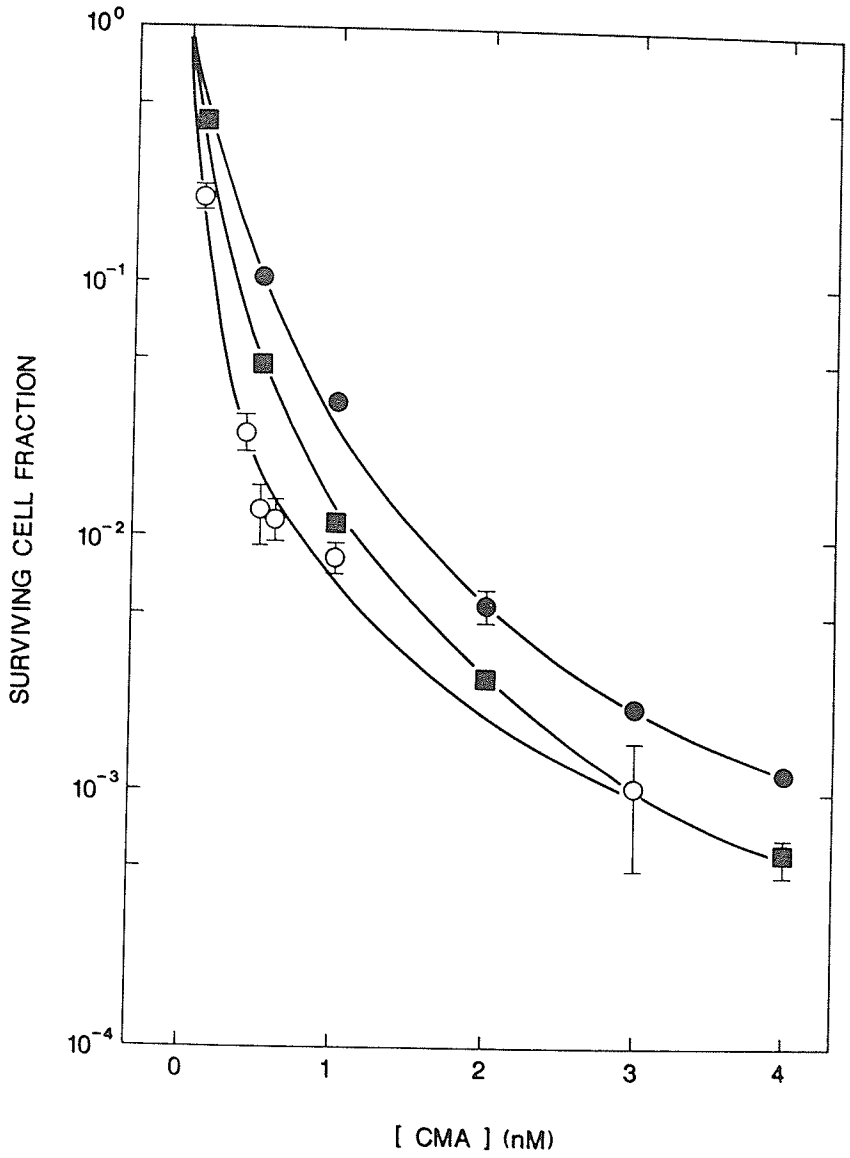


FIGURE 5: Cytotoxicity of CMA in L5178Y, L5178Y/AM6, and L5178Y/HN2 cells.

L5178Y (○), L5178Y/AM6 (■), or L5178Y/HN2 (●) cells were incubated with various concentrations of CMA at 37°C for 2 hours. Cell viability was measured by the soft agar clonogenic assay described in "Materials and Methods" and expressed as the surviving cell fraction. *Points*, mean of 2-10 determinations; *bars*, standard error. On occasion the standard error was too small to be shown.

TABLE 1. Cytotoxic activities of CMA in L5178Y cell lines.

CELL LINE	LD_{90} (nM)[a]	RELATIVE CYTOTOXICITY[b]
L5178Y	0.160	1.00
L5178Y/HBM10	0.175	1.09
L5178Y/AM6	0.310	1.94
L5178Y/HN2	0.510	3.19

[a] The cell lines were treated with CMA and cytotoxicity was measured as described in "Materials and Methods". LD_{90}, the drug concentration required to reduce cell viability by 90%, taken from Figs. 4-5.

[b] The relative cytotoxicity was obtained from the ratio of the LD_{90} of CMA in the resistant cell line, to that of CMA in the parental cell line.

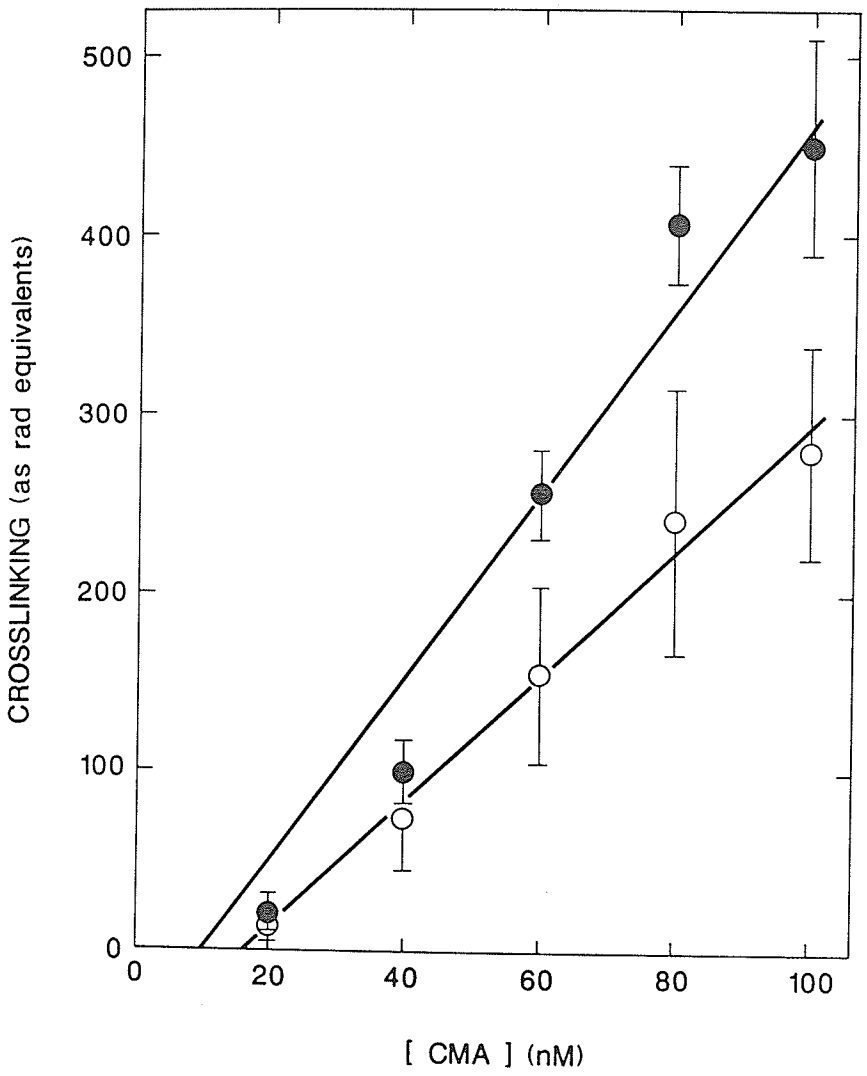


FIGURE 6: DNA crosslinking by CMA in L5178Y and L5178Y/HN2 cells. L5178Y (○) or L5178Y/HN2 (●) cells were incubated for 2 hours at 37°C with CMA at the concentrations shown. Crosslinking was measured by the alkaline elution technique described in "Materials and Methods" and expressed as rad equivalents. *Points*, mean of 5-16 determinations; *bars*, standard error; *lines*, linear regression of concentration *versus* crosslinking.

more crosslinking in the resistant cells. While a t-test of the significance of the difference of slopes between the two cell lines showed that they were significantly different ($p < 0.01$), unpaired t-test analysis of crosslinking at each concentration, demonstrated that none of the points was significantly different between the two cell lines.

L5178Y cells were incubated with either CMA, ICMA, or CHL for 2 hours and analyzed for DNA crosslinks by the alkaline elution assay with proteinase K (Fig.7). All three drugs produced concentration-dependent DNA-DNA crosslinking, with crosslinking reaching a level of 280 rad equivalents at 100 nM CMA, a level of 262 rad equivalents at 1.5 μM ICMA, and a level of 638 rad equivalents at 200 μM CHL. A comparison of the slopes of the concentration-response curves showed that CMA was 27-fold more active than ICMA and 1000-fold more active than CHL in producing DNA crosslinks.

Repair of DNA crosslinking

L5178Y cells were incubated with CMA or CHL at 37°C for 2 hours, and following removal of the drug, were again incubated at 37°C. At various timed intervals cell samples were cooled on ice and crosslinking was measured by the alkaline elution assay (Fig.8). CMA crosslinking began to decrease immediately following drug removal, and by 4 hours crosslinking had been reduced to 22% of the initial level. In contrast, CHL crosslinking continued to increase after drug removal, reaching a maximum after an additional 8 hours. Thereafter, CHL

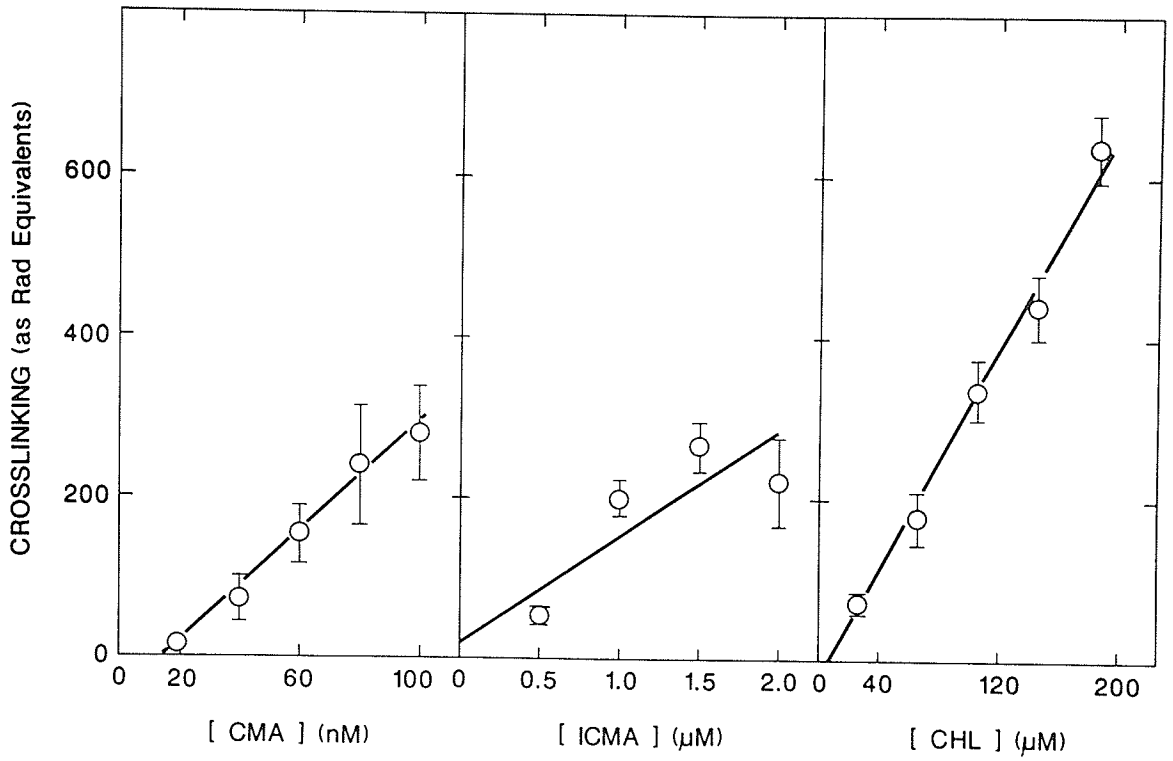


FIGURE 7: DNA crosslinking by CMA, ICMA, and CHL in L5178Y cells. L5178Y cells were incubated with drug at 37°C for 2 hours at the concentrations shown. Crosslinking was measured by the alkaline elution assay described in "Materials and Methods" and expressed as rad equivalents. *Points*, mean of 6-16 determinations; *bars*, standard error; *curves*, linear regression of concentration *versus* crosslinking.

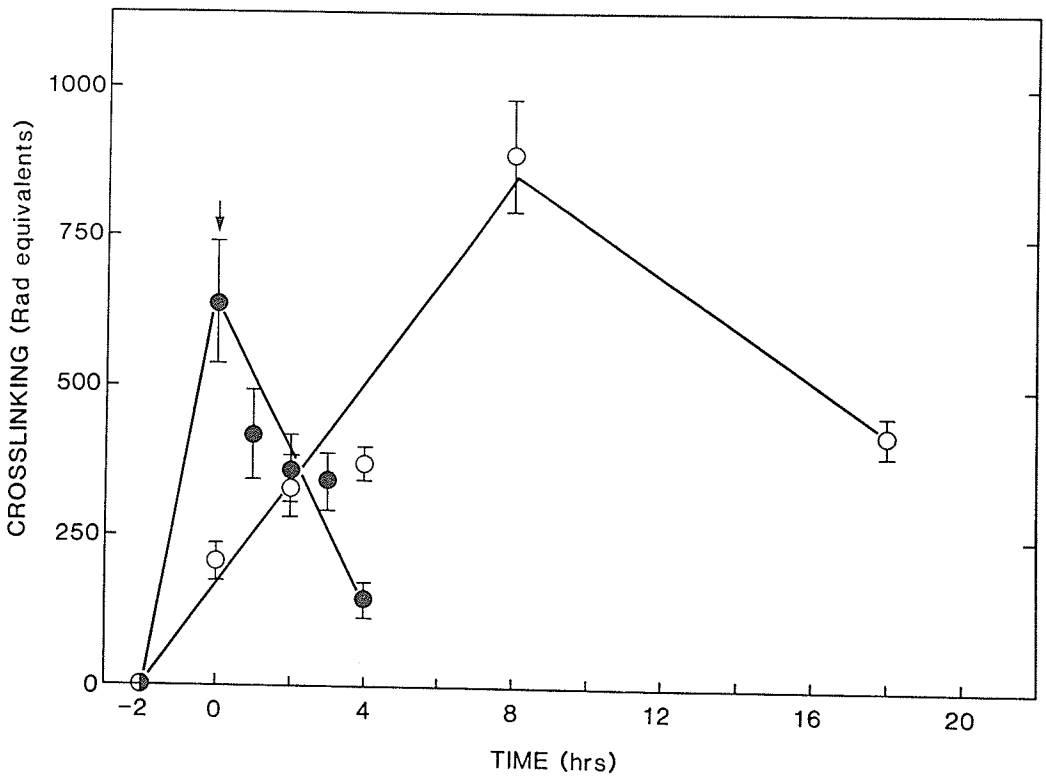


FIGURE 8: Formation and removal of DNA crosslinks by CMA and CHL in L5178Y cells.

L5178Y cells were incubated for 2 hours at 37°C with 60 nM CMA (●) or 120 μM CHL (○). The drug was removed and the cells were incubated at 37°C for the time intervals shown. Crosslinking was measured by the alkaline elution assay described in "Materials and Methods" and expressed as rad equivalents. *Points*, mean of 4-6 determinations; *bars*, standard error; *arrow*, time when drug was removed.

crosslinking began to decrease reaching a level of 48% of the maximum level after 18 hours.

DNA crosslinking in isolated DNA

Isolated λ-phage DNA was incubated with CMA, ICMA, or CHL at 37°C in the presence or absence of a 10-fold excess of sodium borohydride. Addition of the reducing agent to CMA caused the loss of the broad absorbance peak of the quinone group between 450 nm and 550 nm; whereas, dithiothreitol had no effect on this peak. DNA crosslinking was measured by the ethidium bromide fluorescence assay (Fig.9). All three drugs produced concentration-dependent crosslinking. In the absence of the reducing agent, CMA produced a level of 68% crosslinking at 7 μM, ICMA a level of 76% crosslinking at 200 μM, and CHL a level of 59% crosslinking at 2.5 mM. A comparison of the slopes of the concentration-response curves showed that CMA was 26-fold more potent than ICMA, and 453-fold more potent than CHL in producing crosslinks in isolated DNA. The addition of sodium borohydride to the reaction mixture reduced CMA crosslinking by 55%, ICMA crosslinking by 41%, but had no significant effect on the level of CHL crosslinking.

CMA crosslinking in λ-phage DNA was inhibited by the addition of ethidium bromide to the DNA-drug mixture (Fig.10). The crosslinking produced by 5 μM CMA was decreased to 27% of control by 8 μM ethidium bromide; whereas, concentrations of ethidium bromide as high as 80 μM did not decrease the crosslinking produced by 1.5 mM CHL.

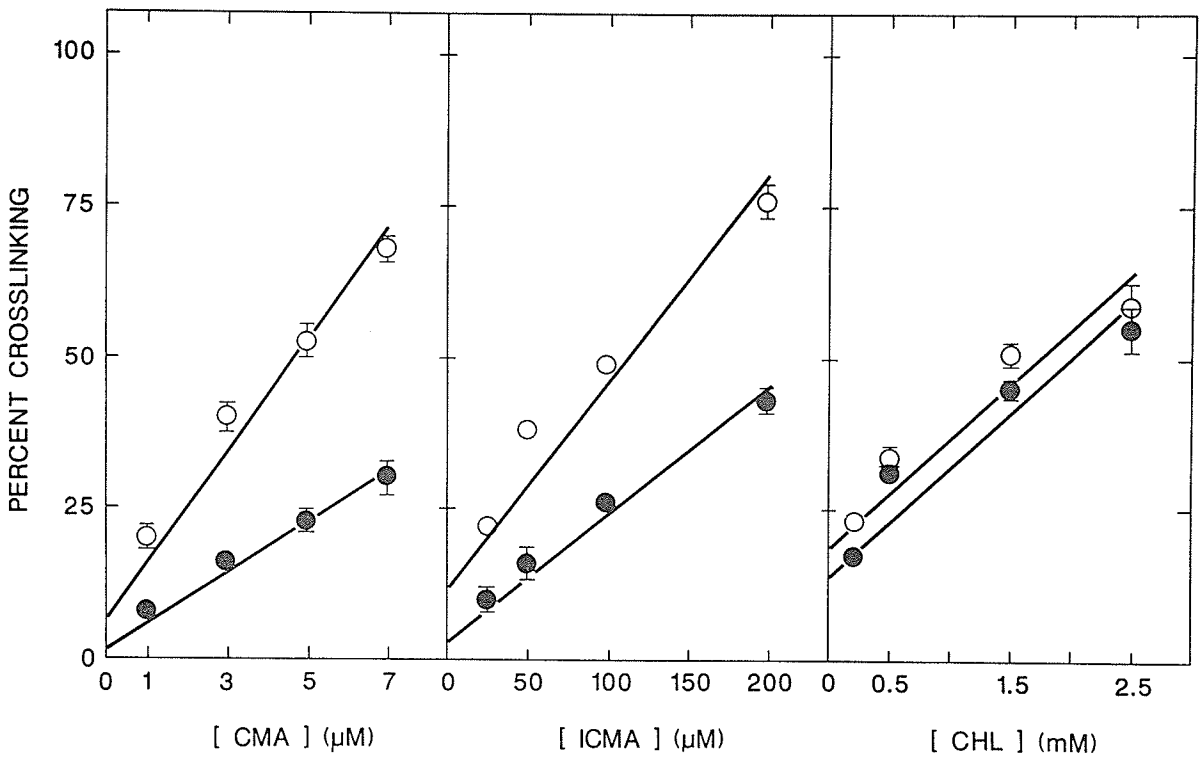


FIGURE 9: Crosslinking by CMA, ICMA, and CHL, in λ-phage DNA. λ-phage DNA was incubated at 37°C with CMA, ICMA, or CHL, in the presence (●) or absence (○) of 70 μM, 2 mM, or 25 mM sodium borohydride, respectively. Crosslinking was measured using the ethidium bromide fluorescence assay described in "Materials and Methods" and expressed as percentage crosslinking (maximum % return of fluorescence after heat denaturation). *Points*, mean of 6 determinations; *bars*, standard error; *curves*, linear regression of concentration *versus* crosslinking. On occasion the confidence intervals were too small to be shown.

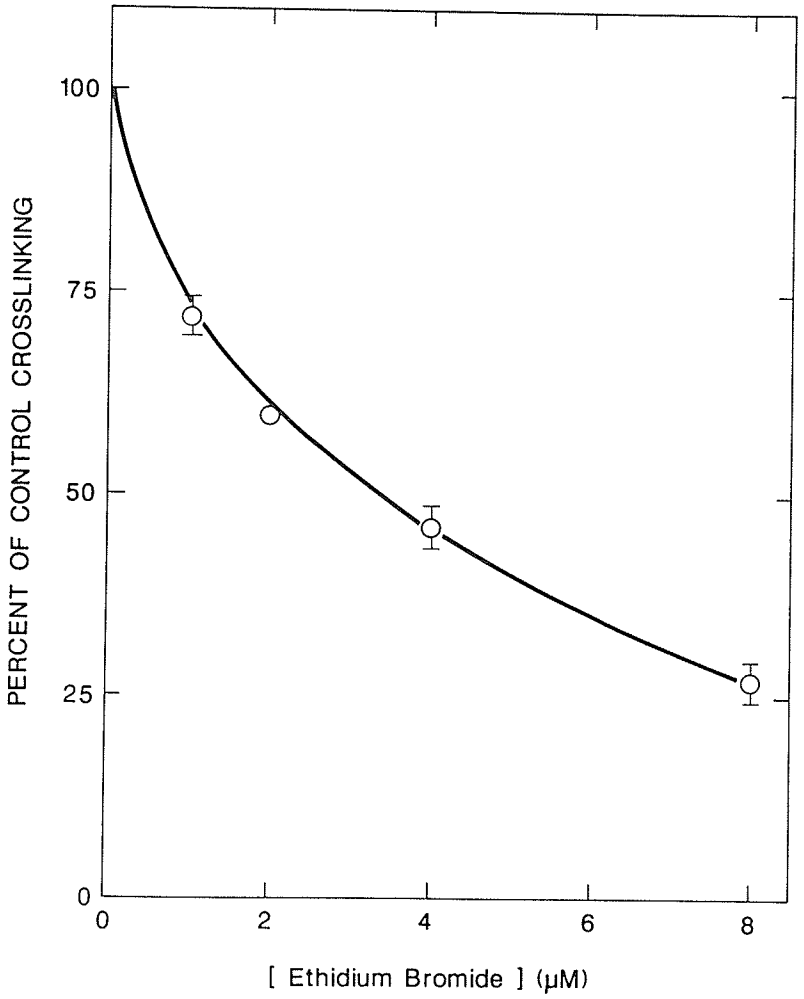


FIGURE 10: Inhibition of CMA crosslinking by ethidium bromide. λ-phage DNA was incubated at 37°C with 5 μM CMA, and ethidium bromide at the concentrations shown. Crosslinking was measured, using the ethidium bromide fluorescence assay described in "Materials and Methods" and expressed as percentage crosslinking (maximum % return of fluorescence after heat denaturation). *Points*, mean of 7 determinations; *bars*, standard error. On occasion the confidence intervals were too small to be shown.

Alkylating activity

The alkylating activities of CMA, with or without sodium borohydride, and of CHL, were determined by measuring the ability of the drugs to bind to the nucleophile, NBP (Fig.11). Both drugs produced concentration-dependent alkylation. A comparison of the concentration *versus* NBP-binding curves showed that CMA produced a 6-fold higher level of alkylation than CHL. When CMA was reduced, there was little or no change in the level of alkylation.

Intercalation into DNA

CMA, ICMA, or CHL was added to calf thymus DNA and the Tm of the DNA was determined (Table 2). In the absence of drug, the Tm of calf thymus DNA was 64°C. CMA caused a dose-dependent increase in the Tm, reaching a maximum of 76°C at 15 μM, for a change in Tm of 12°C. At the same concentration, ICMA produced a change of 5.5°C, whereas CHL produced no change in Tm.

CMA, ICMA, and CHL, in the presence or absence of sodium borohydride, were examined for their ability to displace acridine orange from intercalation sites in calf thymus DNA (Table 2 and Fig.12). In the absence of sodium borohydride, CMA produced a dose-dependent displacement of acridine orange with an IC_{50} of 0.18 μM, while ICMA had an IC_{50} of 0.65 μM. CHL did not cause any displacement of acridine orange at concentrations as high as 750 μM. In the presence of sodium borohydride, displacement of acridine orange by CMA was decreased 63% with the IC_{50} increasing to 0.49 μM, while

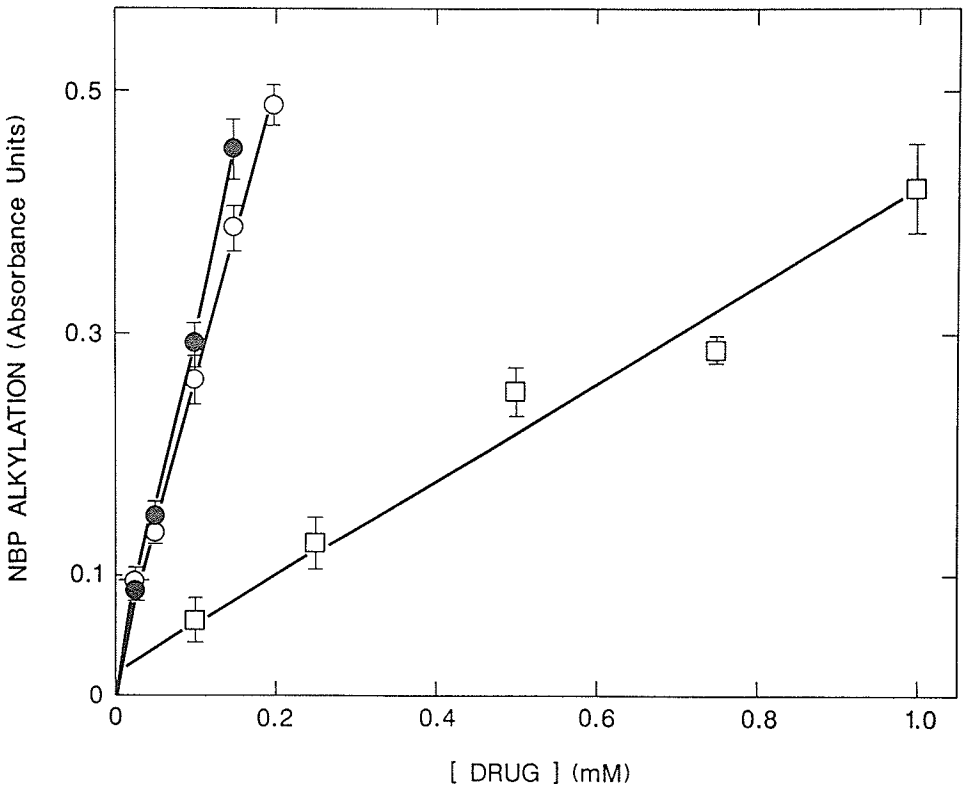


FIGURE 11: Alkylation of NBP by CMA and CHL.
NBP was incubated at 56°C for 20 minutes with CMA, in the presence (●) or absence (○) of sodium borohydride, or with CHL (□). The alkylating activity was measured as described in "Materials and Methods" and expressed in absorbance units. *Points*, mean of 3-4 determinations; *bars*, standard error; *curves*, linear regression of concentration *versus* NBP alkylation.

TABLE 2. Intercalating activities of CMA, ICMA, and CHL in calf thymus DNA.

DRUG	$NaBH_4$	CHANGE IN Tm[a] (°C)			RELATIVE INTERCALATING ACTIVITY[b]	ACRIDINE ORANGE DISPLACEMENT[c] IC_{50} (μM)	RELATIVE INTERCALATING ACTIVITY[d]
		5μM	10μM	15μM			
CMA	-	4	8.3	12	1.00	0.18	1.00
CMA	+	-	-	-	-	0.49	0.37
ICMA	-	2	4.3	5.5	0.47	0.65	0.28
ICMA	+	-	-	-	-	1.06	0.17
CHL	-	0	0	0	0	>750	0

[a] Drug was added to calf thymus DNA (100μg/ml) and the change in Tm was determined as described in "Materials and Methods". Numbers represent means of 2-4 determinations.

[b] The relative intercalating activity was obtained from the ratio of the slope of the change in Tm *versus* concentration curve of the drug, to that of CMA.

[c] The ability of the drug to displace acridine orange from calf thymus DNA was determined, as described in "Materials and Methods". The IC_{50} is the drug concentration required to reduce acridine orange-DNA binding to 50% of the control. IC_{50} values were obtained from Fig. 12.

[d] The relative intercalating activity was obtained from the ratio of the IC_{50} of CMA, to that of the drug.

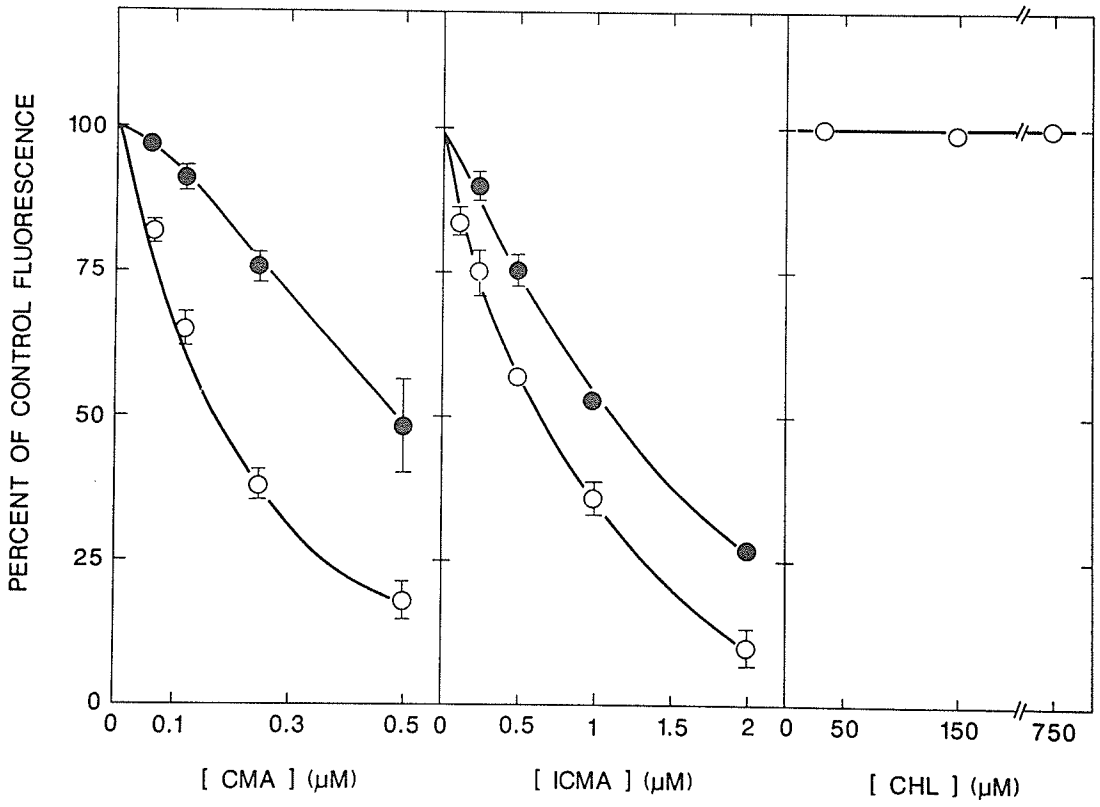


FIGURE 12: Displacement of acridine orange from calf thymus DNA by CMA, ICMA, and CHL.

The ability of CMA, ICMA and CHL to displace acridine orange from DNA was measured in the presence (●) or absence (○) of sodium borohydride as described in "Materials and Methods". The results are expressed as a percent of control fluorescence. *Points*, mean of 6-9 determinations; *bars*, standard error. On occasion the confidence intervals were too small to be shown.

displacement of acridine orange by ICMA was 39% lower with the IC_{50} increasing to 1.06 μM.

The role of DNA base content

CMA crosslinking in isolated DNAs having different percentages of G+C residues was measured using the ethidium bromide fluoresence assay (Fig.13). A concentration of 30 μM CMA produced 32, 53, and 75% crosslinking in calf thymus, *C. perfringens* and *E. coli* DNAs, respectively. At 50 μM, CMA produced 40, 71, and 90% crosslinking, respectively, in the same DNAs.

The intercalating ability of CMA was measured, by the displacement of acridine orange from calf thymus, *C. perfringens* or *E. coli* DNA (Fig.14). There was no significant difference in the acridine orange displacing activities of 125 nM, or 250 nM CMA in the three DNAs.

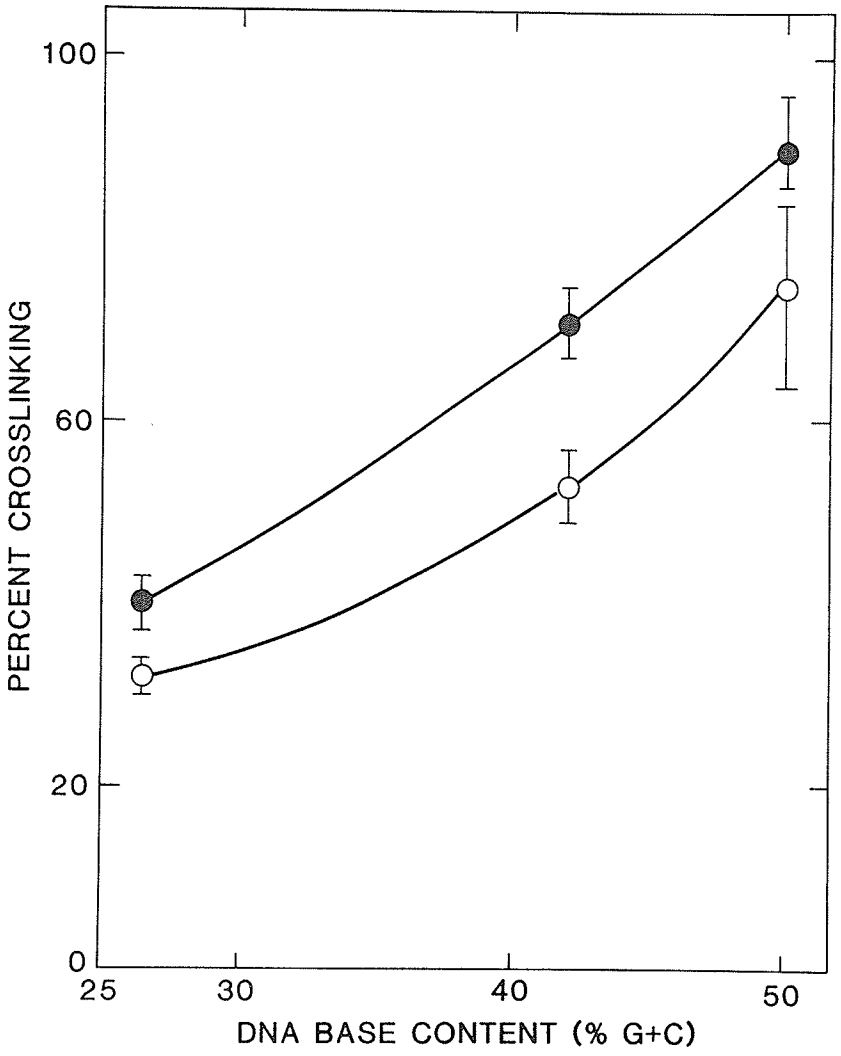


FIGURE 13: Effect of DNA base content on crosslinking by CMA. Isolated DNA from *C. perfringens* (26.5 % G+C), calf thymus (42 % G+C), or *E. coli* (50 % G+C), was incubated at 37°C with 30 μM (○) or 50 μM (●) CMA. Crosslinking was measured, using the ethidium bromide fluorescence assay described in "Materials and Methods" and expressed as percentage crosslinking (maximum % return of fluorescence after heat denaturation). *Points*, mean of 4 determinations; *bars*, standard error.

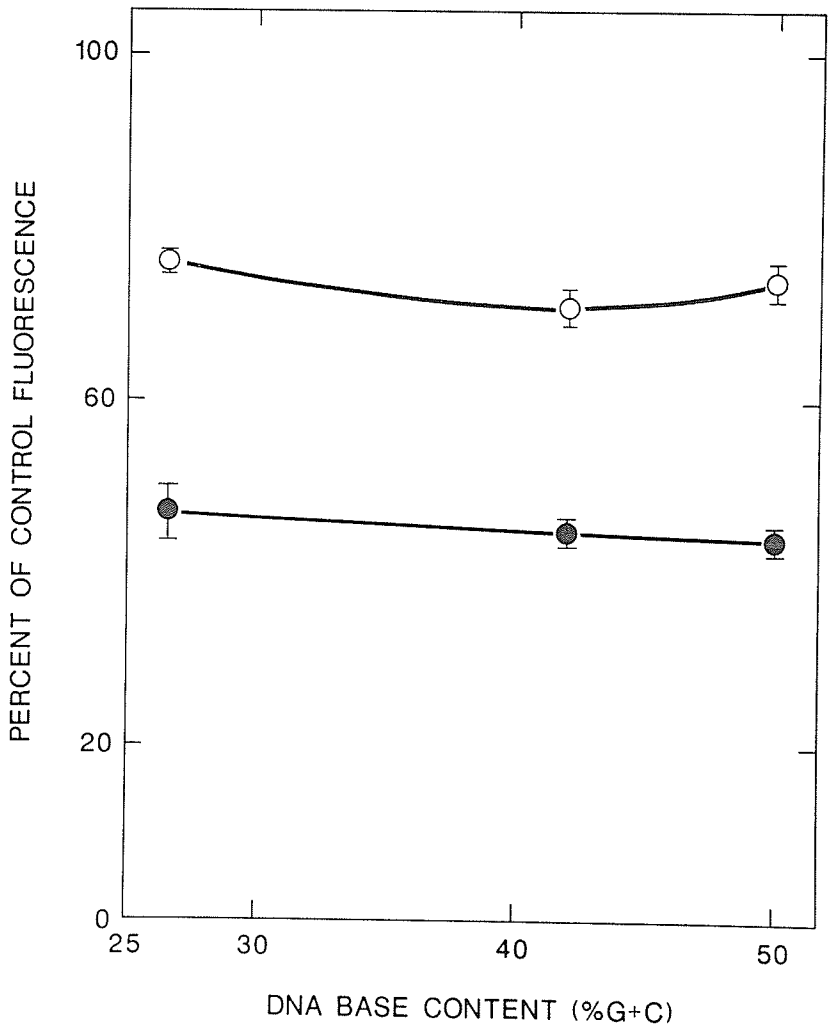


FIGURE 14: Effect of DNA base content on acridine orange displacement by CMA.

The ability of 125 nM (○) and 250 nM (●) CMA to displace acridine orange from *C. perfringens* (26.5 % G+C), calf thymus (42% G+C), or *E. coli* (50% G+C) DNA, was measured as described in "Materials and Methods" and expressed as a percentage of control fluorescence. *Points*, mean of 4-8 determinations; *bars*, standard error.

IV. DISCUSSION

CMA is a potent analog of the antitumor agent, ADR, and is of clinical importance because of its high therapeutic index, and activity against ADR-resistant tumors. It has been suggested that the enhanced antitumor activity of CMA could be related to its ability to produce DNA-DNA crosslinks. As only cyanomorpholinyl analogs of ADR possess this activity (70,154) and, as the cyanide group of CMA can act as a leaving group (100,153), alkylation may occur by cyanide displacement. However, other structural requirements for crosslinking, and the role of this activity in the cytotoxic action of CMA are still unclear. Since there has previously been no direct correlation between the crosslinking and the antitumor activities of CMA, this study has attempted to examine crosslinking in cells which show resistance to CMA. In addition, the role of the quinone group in the antitumor action of CMA has been examined by studying the cytocidal activity of the analog in quinone-resistant cells. It was previously observed that both the cytotoxic and crosslinking activities of ICMA, the 5-imino derivative of CMA, were reduced compared to CMA (70), suggesting that the quinone group may play a role in the actions of these analogs. The interaction of CMA, ICMA and CHL with DNA has therefore been examined in order to determine the role of the quinone group, and of intercalation, in the crosslinking activity of CMA.

Hydrolyzed benzoquinone mustard is a model quinone antitumor agent which is thought to exert its antitumor action through the generation of free radicals. The hydrolyzed benzoquinone mustard-resistant cell line, L5178Y/HBM10, has been shown to have elevated levels of GSH, and

enhanced GST, DT-diaphorase, and catalase activity (12). As these systems can reduce oxygen-dependent free radical production, drug resistance in this cell line may be due to reduced free radical damage. Since there was no cross-resistance to CMA observed in the L5178Y/HBM10 cells (Fig.4 and Table 1), the ability of CMA to form free radicals may not be related to its antitumor action. This conclusion supports the findings of Peters *et al.* (101) who suggested that the redox activity of CMA was similar to that of ADR and not the reason for the enhanced potency. Thus, the quinone moiety of CMA may not play a direct role in the antitumor action of the drug.

The cytocidal activity of CMA was examined in two cell lines which were selected for resistance to alkylating agents. The L5178Y/AM6 cell line, which was 6-fold resistant to the model alkylating agent aniline mustard, showed only 2-fold cross-resistance to CMA (Fig.5 and Table 1). Similarly, the L5178Y/HN2 cell line which was 20-30-fold resistant to HN2 (55) and 2-3-fold cross-resistant to CHL, melphalan and MMC (52), showed 3-fold cross-resistance to CMA only at lower doses. The crosslinking activity of CMA was examined in the L5178Y/HN2 cell line, and it was found that there was no significant difference compared to the sensitive cells. These findings suggested that there was no correlation between crosslinking and the antitumor activity of CMA in these cells. However, since the level of cross-resistance was low, changes in crosslinking activity may not have been detected. As relatively small changes in the crosslinking activity of CMA can correlate with large changes in cytotoxicity (70), a 2-3-fold change in

drug sensitivity may not be enough to produce a change in crosslinking which is detectable by the elution assay. Perhaps a cell line which showed a higher level of cross-resistance would have provided a better indication of the role that DNA crosslinking plays in the cytotoxicity of CMA. The most direct way to approach this problem, would have been to attempt to develop a CMA-resistant cell line by continuously exposing cells to increasing concentrations of the drug. However, the scope of this project, and the limited availability of drug, prevented this from being done. An explanation for the low level of resistance to CMA in alkylator-resistant cells could be that the drug functions by a different mechanism than other alkylating agents, *eg.* different transport mechanisms, *etc.* It should be noted that CMA is an anthracycline, and even though it possesses alkylating activity, the drug may have a means of circumventing mechanisms of resistance to more simple alkylating drugs. If this were the case, one would not have expected to see alterations in crosslinking unless high levels of cross-resistance were observed.

This study has examined the structural characteristics of CMA in order to determine the requirements for cytocidal activity. As CMA was not cross-resistant to quinone-resistant cells, it is unlikely that the enhanced potency of the drug is related to its ability to produce oxidative DNA damage. Similarly, previous findings that CMA was not cross-resistant to ADR-resistant cells (71,125,134), suggested that the drug does not inhibit topoisomerase II at cytotoxic doses. While, CMA produced a low level of cross-resistance in alkylator-resistant cells,

the cytotoxic activity of the analog could not be correlated with crosslinking. Therefore, it is unclear whether the enhanced potency of CMA is a result of its alkylating ability.

This study examined the mechanism by which CMA produces crosslinking. Similar to previous findings with HT-29 human colon carcinoma cells (70), the potency of DNA crosslinking in L5178Y cells was in the order of CMA > ICMA > CHL (Fig.7). In isolated λ-phage DNA, CMA was 26-fold more active than ICMA, and 453-fold more active than CHL in forming DNA crosslinks (Fig.9). In contrast, CMA was only 6-fold more potent than CHL in its ability to alkylate the nucleophile, NBP (Fig.11). The alkylating activity of ICMA could not be determined because the visible absorbance of the analog interfered with the NBP assay. The discrepancy between the crosslinking and the alkylating activities of these agents, suggested that interaction of the drug with DNA potentiated the crosslinking activity of CMA and ICMA, but not that of CHL.

It is unclear from the literature whether 5-imino substituted anthracyclines have altered DNA intercalating activity. One report indicated that by using the change in Tm of DNA as a measure of intercalation, ICMA and the 5-imino derivative of MA showed reduced intercalating activity compared to their parent analogs (1). However, using the displacement of acridine orange as a measure of intercalation, there was no difference between the intercalating activities of ADR and 5-iminodaunorubicin (51). Thus, in the present study the intercalating activities of CMA, ICMA, and the non-

intercalating alkylating agent, CHL, were measured by both methods. It was observed that CMA and ICMA, but not CHL, were able to intercalate into DNA, and that CMA was 2-3-fold more active as an intercalator than its 5-imino analog as measured by the displacement of acridine orange and the change in Tm of calf thymus DNA (Table 2 and Fig.12). Additionally, the intercalating agent, ethidium bromide, inhibited the crosslinking activity of CMA (Fig.10), but did not inhibit crosslinking by CHL. These findings suggested that intercalation contributed to the formation of crosslinks by CMA, and that the decreased intercalating activity of ICMA compared to CMA, may account for its lesser crosslinking activity.

The formation and removal of the crosslinks produced by CMA and CHL were examined in L5178Y cells (Fig.8). A previous report had indicated that both the induction of crosslinks by melphalan and HN2, and the rate of their removal were important for the cytotoxicity of these agents (65). In the present study, it was found that both the onset and rate of removal of CMA crosslinks were more rapid than those of CHL. Similar findings have also been reported by another group (121), who suggested that the reduction of CMA crosslinking over time may reflect DNA fragmentation as a result of cell death. Although there is evidence that CMA can fragment DNA soon after exposure (151), there was little or no cell death in the present study, as measured by trypan blue extrusion, until greater than 50% of the crosslinks had been removed. Regardless of whether these observations represent DNA fragmentation or repair of crosslinking, it is apparent that the

crosslinks produced by CMA differed from those produced by CHL in the speed of their formation and the ease of their removal. The faster onset of crosslinking by CMA, compared to CHL, may be related to the intercalative ability of the drug.

It was previously reported that the reducing agent, dithiothreitol, produced no change in the crosslinking activity of either CMA or ICMA in isolated DNA (70). However, in the present study spectrophotometric analysis suggested that this result was due to inadequate reduction of the quinone group. In contrast, the stronger reducing agent, sodium borohydride, reduced the quinone group of CMA, and produced a 55% and a 41% decrease in the crosslinking activity of CMA and ICMA, respectively (Fig.9). As well, sodium borohydride produced a corresponding decrease in the intercalating activity of CMA and ICMA (Fig.12), but had little or no effect on the alkylating activity of CMA (Fig.11). These results confirmed the contribution of intercalation to the crosslinking activity of CMA, and suggested that the quinone ring plays an important, but indirect, role in regulating the activity of the drug by influencing its ability to intercalate. Previous studies (99,107), have shown that the anthracycline chromophore, which contains the quinone group, was responsible for the intercalative nature of these agents. During the intercalation process, the quinone and dihydroquinone rings of the chromophore were shown to hydrogen-bond with adjacent base pairs of DNA. Thus, reduction of the quinone group, or a 5-imino substitution, may interfere with the ability of the chromophore to form such bonds, and

thus decrease intercalation.

The anthracyclines do not appear to have a preference for DNA base content for intercalation (42,102); however, many alkylating agents preferentially bind to G-C bases of DNA (66). In this study, we examined both the crosslinking and the intercalating activities of CMA with DNAs of varying G-C content. Increasing G-C content resulted in increased crosslinking by CMA (Fig.13), whereas, it had no effect on the ability of the drug to intercalate into DNA (Fig.14). These findings indicated that the crosslinking of CMA to G-C bases was due to preferential alkylation of these bases rather than to altered intercalation.

In summary, although a low level of cross-resistance was observed to CMA in alkylator-resistant cell lines, the antitumor activity of the drug could not be correlated with crosslinking activity. The role of the quinone ring of CMA in free radical-mediated cytotoxicity was examined, and it was found that this activity may not be important to the enhanced potency of the drug. CMA crosslinking was potentiated by the ability of the drug to intercalate into DNA, and the quinone ring played an important, but indirect role, in the crosslinking activity by modulating the ability of the drug to intercalate. Similar to other alkylating agents, CMA was observed to preferentially crosslink G-C rich DNA, as a result of preferred alkylation to these bases; however, the rate of formation and removal of CMA crosslinks differed markedly from that of other alkylating agents.

Future studies

Despite evidence suggesting that DNA crosslinking is responsible for the enhanced potency of CMA, a direct correlation between crosslinking and cytotoxicity has yet to be demonstrated. This study attempted to make a connection between these two activities; however, the unavailabilty of a suitable model of CMA resistance made this impossible. The development of a specific CMA-resistant cell line may provide a solution to this problem if a high enough level of resistance can be achieved to measure significant alterations in crosslinking activity. This project would depend upon the availability of an adequate supply of CMA since at this time quantities of this agent are limited.

The observation that crosslinks produced by CMA appear to be rapidly removed should be examined in greater detail. It is not clear whether the removal of crosslinks was due to DNA repair processes within the cell, or to removal of DNA adducts as a result of drug decomposition. If CMA crosslinks are being rapidly repaired, it would suggest that this activity may not have a role in the cytotoxic action of the drug.

CMA is of obvious clinical importance because of its high potency and its high therapeutic index. Since this drug is effective against ADR-resistant cells, it may provide a means of effectivly treating patients which have become resistant to anthracyclines. Similarly, CMA may be a useful in treating patients resistant to alkylating agents, since the drug maintains its anthracycline stucture. However, even if

this agent is not clinically useful, CMA is the first anticancer agent to be developed which contains both strong intercalating and alkylating activities, and therefore may provide a model for the development of other drugs of this kind.

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