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Mitomycin C : analysis of its cytotoxicity through studies using an analogue decarbamoyl mitomycin C

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Abstract

MITOMYCIN C : AN ANALYSIS OF ITS CYTOTOXICITY THROUGH STUDIES USING AN ANALOGUE DECARBAMOYL MITOMYCIN C

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Hypoxic regions in solid neoplasms have been associated with tumor recurrence and resistance to various forms of cancer treatment, including radiation therapy. Various strategies have been designed to target radioresistant cells, including the use of the bioreductive alkylating agent mitomycin C [MC], which has been shown to exert preferential cytotoxicity under hypoxic conditions in some cell lines. Analyses of the chemical mechanism of action of MC indicate that this drug can form cross-links with DNA, and it is currently thought that the bisadduct is the critical lesion responsible for the inhibition of DNA synthesis. Computer-generated models indicate that the MC adduct can fit snuggly into the minor groove of B-DNA, suggesting that it does not impose major distortion to the structure of the DNA molecule. Based on these observations, how can we explain the ability of MC to disrupt DNA synthesis as well as to exhibit preferential toxicity to hypoxic cells?

Numerous studies on MC have been performed in both chemical and biochemical systems. Several analogues of MC have also been the subject of research. In order to gain additional insight regarding the role of cross-linkage in the production of cytotoxicity, the analogue decarbamoyl mitomycin C [DMC] has been studied. DMC is structurally identical

to MC except for the replacement of the carbamoyl group at the C-10" position by a nonalkyating hydroxyl group. This structural alteration would be expected to result in functional changes which would prevent DMC from forming bisadducts with DNA. Chemical studies reveal that at least in chemical systems, DMC can produce only monoadducts. If indeed it is the MC-DNA cross-links which kill cells, it would be expected that DMC would produce less cytotoxicity than MC. Interestingly enough, results from tissue culture experiments suggest that DMC is at least as toxic as MC to the EMT6 mouse mammary tumor and to the wild type AA8 Chinese hamster ovary [CHO] cell lines.

To further investigate the cytotoxic nature of DMC, experiments were conducted using mutant CHO lines which have specific deficiencies in DNA repair. The UV4 and UV5 lines, which are hypersensitive to bulky monoadducts and crosslinks, were chosen to help delineate DMC's ability to produce cytotoxicity in these mutant phenotypes. The data revealed similar results for DMC and MC treatments, suggesting that in these biological systems DMC has a pattern of cytotoxicity and a potency that are similar to those of its parent compound mitomycin C. These observations strongly suggest that although crosslink formation by MC may partially account for its cytotoxicity, there may be another product, possibly a monoadduct that is generated by both DMC and MC, which also contributes to the antitumor activity exhibited by the two compounds.

MITOMYCIN C : AN ANALYSIS OF ITS CYTOTOXICITY THROUGH STUDIES USING AN ANALOGUE DECARBAMOYL MITOMYCIN C

A Thesis Submitted to the Yale University School of Medicine in Partial Fulfillment of the Requirements for the Degree of Doctor of Medicine

by

Susie Young Kim

May, 1994

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Introduction

The intimate relationship that is found in normal tissues between cells and their microenvironment is disrupted in solid neoplasms. Tumors are often characterized by abnormalities in blood flow, tissue oxygenation, nutrient supply, environmental pH, and bioenergetic status (Vaupel, 1989). A major focus of cancer research today relates to understanding the alterations which result from the malignant transformation of cells and the mechanisms by which neoplastic growth can be manipulated. Considerable research has been devoted to identifying characteristics that are unique to malignant cells which can then be exploited in selective antineoplastic therapy.

The purpose of this thesis is to examine in detail one mode of attacking solid neoplasms. The experiments that have been performed relate to investigation of a class of alkylating agents called mitomycins, an unusual group of antibiotics which are capable of mono- and bi-functional DNA alkylation. I was interested in analyzing the mechanism of action of the mitomycins at the molecular level. To carry out this research, an analogue of mitomycin C [MC], decarbamoyl mitomycin C [DMC], was studied extensively through tissue culture experiments under aerobic and hypoxic conditions. This compound was first examined using the EMT6 mouse mammary cell line. Currently the cytotoxicity of mitomycin C is attributed mainly to its ability to crosslink DNA and thereby inhibit DNA synthesis. Since DMC possesses only one of the two proposed alkyating sites in MC, DMC was expected to produce less cell kill; the results, however, do not support this hypothesis. In order to study how the toxicity of DMC might be related to the DNA lesions it creates, further investigation was performed using Chinese hamster ovary [CHO] cell lines having various deficiencies in DNA repair. A discussion of possible explanations for

the ability of DMC to exert cytotoxic effects will follow the presentation of the experimental data.

The remainder of the introduction will review topics pertinent to the research that will be presented. First, I will discuss hypoxia in solid tumors and its role in conferring radioresistance, the effects of hypoxia on radiotherapy, and the acidic environment that is characteristic of many tumor tissues. I will then review the concept of bioreductive agents and will discuss more specifically the mitomycins. The evolution of this class of drugs will be presented, as well as the current understanding of how these compounds function at the subcellular level. The analogue decarbamoyl mitomycin C will be described and discussed in further detail, since the focus of my research is centered around this molecule.

Hypoxia in Solid Tumors

With the advent of hybridoma technology and genetic engineering, a number of novel therapeutic agents have been developed. These include monoclonal antibodies conjugated with radionuclides, toxins, cytokines, enzymes, effector antibodies, immunotoxins, lymphocyte activated killer cells, and tumor infiltrating lymphocytes (Jain, 1989). Yet these modalities face the same problem that has already confronted the existing chemotherapeutic drugs, namely inadequate penetration of these agents to malignant cells. Interestingly, it has been observed that most forms of cancer therapy are affected in some way by the poor vasculature that has been found to be associated with solid tumors (Denekamp, 1993, 1991, 1990).

In order for an anti-cancer agent to reach neoplastic cells, it must first enter the tumor circulation. Gullino and Grantham hypothesized in 1962 that the tumor vasculature consists of vessels recruited from the pre-existing network of the host vasculature as well as vessels resulting from the angiogenic response to cancer cells (Gullino & Grantham, 1962). However, a key structural difference between normal and tumor vessels is that the latter are dilated, saccular, and tortuous, often containing tumor cells within the endothelial lining of the vessel wall (Jain, 1989; Jain, 1988). Also characteristic of the neoplastic process is that cells within these vessels proliferate at a slower rate than the rapidly dividing cancer cells. In addition, blood flow rates near the necrotic regions of tumors have been found to be low, whereas blood flow rates in the non-necrotic areas are variable and often substantially higher than those in surrounding normal tissues (Jain, 1988). Tumors have been shown to possess areas of high interstitial pressure, which has been correlated with a reduction in tumor blood flow and subsequent development of necrosis (Jain, 1989; Jain, 1987; Young *et al.*, 1950). Although the mechanisms responsible for intermittent blood flow in tumor tissue are not entirely clear, it is speculated that causative factors include vessel plugging by white blood cells, rouleaux formation of circulating tumor or red blood cells (Jain, 1989), collapse of vessels in areas of high tumor interstitial pressure (Sevick, 1989), and spontaneous vasomotion in incorporated host arterioles, affecting flow in downstream capillaries (Reinhold, 1977; Intaglietta, 1977). Consequently, the vascular network becomes less dense and is reduced to a degree that creates a general rarefaction of the vascular bed. As a result of these modifications, there is diminished oxygen transport capacity with progressive tumor growth, leading to regional hypoxia and anoxia, as well as glucose depletion, in the neoplastic tissue. These conditions have been strongly implicated as the cause for cytolysis, necrobiosis, and necrosis (Vaupel, 1977).

The presence of hypoxic cells in solid tumors was first demonstrated radiobiologically by Powers and Tolmach in 1963 using transplantable mouse lymphosarcomas (Powers & Tolmach, 1963). Further studies have demonstrated that the vast majority of cancer tissues possess hypoxic cells, frequently constituting 10-20% of the total tumor tissue population (Moulder & Rockwell, 1984). The imbalance between oxygen consumption and availability in solid tumors results from the abnormal vasculature and the absence of adequate perfusion. Heterogeneity of oxygenation within these tumors has been demonstrated by studies using oxygen microelectrodes, hemoglobin saturation measurements, cytochrome *c* reduction, and nuclear magnetic resonance spectroscopy (Moulder & Rockwell, 1990; Vaupel *et al.*, 1981). It has also been shown that the hypoxic cell fractions in many transplantable rodent tumor systems increase with tumor size (Moulder & Rockwell, 1984).

As a result of poor vascularization, solid tumors possess viable, oxygen-deficient cells of two different classes - chronically and transiently hypoxic cells (Rockwell, 1992; Vaupel & Thews, 1974; Thomlinson & Gray, 1955). Hypoxic cells are thought to be capable of proliferating and causing tumor recurrence, in part due to the protective nature of an oxygen-depleted environment against radiotherapy or chemotherapy. Thus the presence of anoxic cells in solid tumors is regarded as a major obstacle to the currently available treatment modalities for solid neoplasms.

Hypoxia and Its Effects on Radiotherapy

In 1923, Petri noted a relationship between radiosensitivity and the presence of oxygen from a study of the effects of radiation on vegetable seeds. About a decade later, Mottram reported that marginal cells of carcinomatous cell masses are more sensitive to γ–radiation than centrally located cells, and hypothesized that these peripherally located cells have a more abundant oxygen supply because of closer proximity to blood vessels, which renders them more radiosensitive (Mottram, 1936). Thomlinson and Gray later published some astonishing results which have made a great impact on studies of tumor biology. Their histological study of fresh specimens of human bronchial carcinoma revealed that avascular tumor areas with a radius of 200 μ or greater possessed necrotic centers which were surrounded by intact tumor cells. It appeared that these tumor cells could proliferate and grow actively only in close proximity to a supply of oxygen or nutrients from the stroma. It was concluded that oxygen concentration fell off rapidly with increasing distance from the blood capillaries which carried an abundant supply of oxygen. Cells situated in the region distant from the capillaries would be relatively resistant to radiation treatment due to their low oxygen tension and could thus provide a focus for subsequent tumor regrowth (Thomlinson & Gray, 1955).

Further evidence that hypoxic cells can be an important source for tumor regrowth has subsequently been obtained. Transplanted tissues from necrotic tumor regions have been shown to be capable of producing malignancies with a relatively high frequency (Goldacre & Sylven, 1962). In addition, it has been reported that fragments of the Walker 256 carcinosarcoma which were perfused for 56 hours with medium low in oxygen grew as well as transplants from well-oxygenated tumor tissue (Gullino, 1968).

These major discoveries triggered tremendous interest in oxygen as playing a critical role in radiotherapy. It is now known from *in vitro* studies that as the oxygen concentration increases, cells become progressively more sensitive to radiation, until in the presence of oxygen tensions of 20-40 torr, radiosensitivity plateaus, with cells becoming about three times as sensitive as under complete anoxia (Hall, 1988). Tumors contain cells at low oxygen concentrations, which are resistant to radiation while normal tissues are fully oxygenated, and fully radiosensitive. Research therefore has been focused on efforts to make tumor cells more amenable to the cytotoxic effects of radiation. Initially in the early 1960's, there was a search for compounds which mimic oxygen in sensitizing tissues to the effects of X-rays (Hall, 1988). Presently the problem of radioresistance is being approached from various angles, including the use of radiation sensitizers and protectors, hyperthermia, anti-angiogenesis, and the use of bioreductive agents (Horsman, 1993).

Environmental pH

In addition to inadequate oxygenation resulting from poor vascularization, inadequate nutrient supply has also been observed in solid tumors (Crabtree & Cramer, 1934; Mottram, 1936). It is thought that due to the high metabolic rate of these rapidly growing tumor cells, sufficient nutrient supply cannot be maintained. As Tannock and Steel had initially observed, cell death appears to be closely related to blood stasis, which may result from occlusion of vessels by thrombi, by mechanical pressure, or by a tendency for vessels which supply central tumor regions to be bypassed with formation of capillary shunts closer to the periphery (Tannock & Steel, 1969). Consequently there is a build-up of lactic acid and other acidic metabolites which causes lowering of the pH. This acidification of the microenvironment has been shown to inhibit cell proliferation, DNA

synthesis, and glycolysis (Cater & Phillips, 1954). Furthermore, lowering of the pH has been associated with a decrease in the radiosensitivity of mammalian cells, modulation of the cytotoxicity of certain anticancer agents, and enhancement of the cell killing effect of heat (Vaupel, 1989; Tannock & Rotin, 1989; Kennedy, 1987).

In rodent tumors, the pHs measured using microelectrodes vary from 5.80 to 7.52, and both intratumor and intertumor heterogeneity have been observed (Wike-Hooley *et al.*, 1984). Similar measurements made on the EMT6 tumors have demonstrated regional heterogeneity in pH, with values ranging from 5.8 to 7.6 (Rockwell *et al.*, 1991). Studies on human tissue have shown that while normal human tissue usually has pH values between 7.0 and 7.4, human tumors can exhibit pH values as low as 6.15 (Vaupel, 1989; Wike-Hooley *et al.*, 1984).

Bioreductive Alkylating Agents

In the 1930's, anaerobic cultures of microbes revealed that as cultures under either aerobic or anaerobic conditions grow and became more crowded, the redox potential of the microbial cultures decreases (Hewitt, 1936; Porter, 1964). By analogy, it has been hypothesized that hypoxic cells remote from the vasculature of a tumor mass might have a greater capacity for reductive reactions than their normal well-oxygenated counterparts (Kennedy, 1980), and therefore may be more sensitive to bioreductive alkylating agents (Sartorelli, 1988).

Two classes of agents are presently known to exhibit preferential cytotoxicity toward hypoxic cells through reductive activation: the quinone bioreductive alkylating

agents such as mitomycin C [MC], BMY-42355, and EO-9, and the nitroaromatic heterocyclic hypoxic cell sensitizers such as misonidazole and metronidazole. The action of these compounds is very dependent on the microenvironment, as evidenced by the prerequisite of reductive activation in order to produce their cytotoxic effects (Rockwell, 1992; Sartorelli, 1988; Kennedy *et al.*, 1980; Moore, 1977). In a reciprocal manner, the cell's ability to tolerate alterations in cell physiology produced by the chemical perturbation will dictate the outcome of the effects of these drugs.

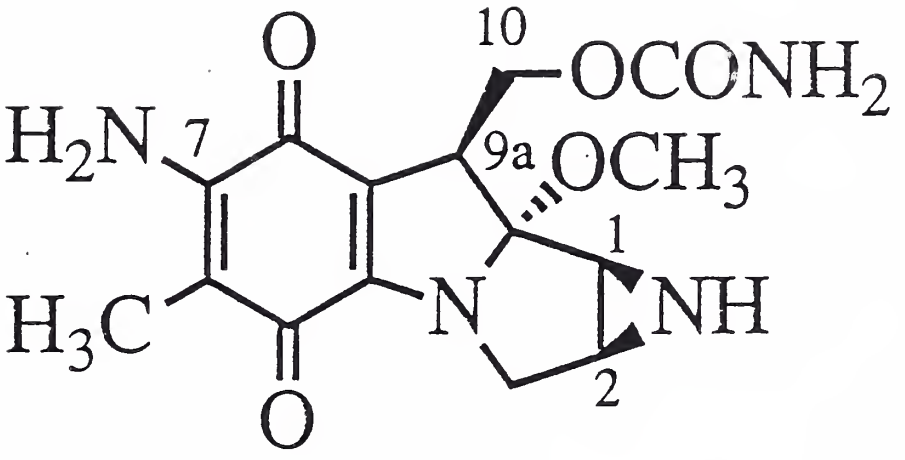
Mitomycin C : Chemical Strucure and Cytotoxic Activity

The mitomycins, which were first isolated from *Streptomyces caespitosus* in 1956 by Hata and co-workers (Hata *et al.*, 1956), interestingly possess antimicrobial (Kinoshita *et al.*, 1971) as well as antitumor activity (Crooke & Bradner, 1976). Several additional compounds which were subsequently isolated (Wakaki *et al.*, 1958; De Boer *et al.*, 1961) were found to be similar in structure and activity, and were therefore grouped and classified together as mitosanes. From this group of compounds, mitomycin C [MC] has been the most extensively studied. Its chemical structure was confirmed by structural analysis (Stevens *et al.*, 1964) and is illustrated in Figure 1. MC possesses three major functional groups: the quinone, the carbamate, and the aziridine ring (Teng *et al.*, 1969).

Early studies on the mechanism of its action as an antibiotic revealed that MC can inhibit DNA synthesis in bacterial cells (Sekiguchi & Takagi, 1959; Shiba *et al.*, 1959) and in mammalian cells (Sekiguchi & Takagi, 1960; Shatkin, 1962). Analysis using cesium chloride and cesium sulfate equilibrium density centrifugation enabled Iyer and Szybalski to hypothesize that inhibition of DNA synthesis by MC resulted from the cross-linking of complimentary strands of DNA (Iyer & Syzbalski, 1963). It has been demonstrated that MC is not active in its quinone form, but requires activation by enzymatic or chemical reduction (Iyer & Szybalski, 1963), or by mild acidic treatment (Tomasz, 1979). It is only after activation that MC can alkylate DNA to yield both mono- and bi-functional convalent adducts; because of this unique mechanism of action, MC has been referred to as the prototype bioreductive alkylating agent (Sartorelli, 1988).

FIGURE 1. The Chemical Structure of Mitomycin C

Mitomycin C possesses three major functional groups: the quinone, the carbamate, and the aziridine ring. The drug is not active in the quinone form, but must first undergo bioreductive activation. Analogues of mitomycin C which have alterations in one or more of these functional groups, such as decarbamoyl mitomycin C, have been analyzed in order to facilitate in elucidating the mechanism of action of this compound.


O
10
OCONH2
H2N
7
9a
OCH3
1
H3C
N
NH
2
O

Upon further analysis of its cytotoxic effects, it has also been demonstrated that MC creates single-strand breaks in bacterial DNA (Otsuji & Murayama, 1972). Investigation on mammalian DNA by alkaline elution, however, has not shown that single-strand or double-strand breaks are produced, which suggests that MC does not kill mammalian cells by generating oxygen free radicals, but instead acts primarily by alkylation when used within the pharmacological range (Dorr *et al.*, 1985; Pristos, 1986). Although these conclusions may appear contradictory, MC could actually exert its toxicity via two differect mechanisms (Kennedy *et al.*, 1981; Satorelli, 1988). First is its oxygen-dependent toxicity, which is proposed to occur by cyclic one-electron reduction of MC followed by oxidation by molecular oxygen to produce the original MC molecule and a superoxide radical (Bachur *et al.*, 1978, 1979). The second mechanism is thought to involve bioreductive activation of the MC molecule to a highly reactive bifunctional alkylating species (Iyer & Szybalski, 1963, 1964). Further investigation on the molecular damage produced by MC has been performed by analysis of mutations at the *hprt* locus of Chinese hamster V79 cells induced by MC (Davies, 1993); these experiments revealed that MC can produce total, partial, and point deletions on the *hprt* gene, suggesting that, at the molecular level, the alterations created by MC are possibly multilocus deletions.

The increased sensitivity to MC of hypoxic cells over their oxygenated counterparts has been attributed to the fact that oxygen decreases the formation of the reduced intermediate responsible for the production of what is presently considered to be the primary cytotoxic lesion, namely the cross-linking of DNA (Sartorelli, 1988). With the knowledge that some solid tumors contain hypoxic regions resistant to radiation, this concept of using this preferential cytotoxicity to hypoxic cells has since been applied by combining MC and radiotherapy (Rockwell, 1993, 1992). The ability of the mitomycin antibiotics to eradicate hypoxic cells in solid tumors has been noted in well-established implants of the EMT6 mammary sarcoma in BALB/c mice (Keyes *et al.*, 1985; Rockwell,

1983; Teicher *et al.*, 1981; Kennedy *et al.*, 1981; Kennedy *et al.*, 1980). Cell culture experiments on the effects of MC combined with radiation have produced data which indicate that the shape of the MC survival curve is not changed by irradiation, nor does the addition of MC alter the shoulder or slope of the radiation dose-response curve. Hence *in vitro* studies suggest that mitomycin C does not sensitize EMT6 cells to X-irradiation, and that these two modalities behave in an additive fashion (Rockwell *et al.*, 1985; Rockwell, 1982).

Studies on mitomycin C have been conducted under a number of conditions, including several chemical and biochemical systems, and cell cultures derived from normal and malignant tissues from humans and animals, as well as tumors and normal tissues of experimental rodents. Clinical trials on patients with various malignancies have also been conducted with MC and with another one of its analogues, porfiromycin. Although data generated from these various experiments provide useful information, interpretations must be made with caution, since each system possesses intrinsic differences which may influence the results.

Mitomycin C : Bioreductive Activation

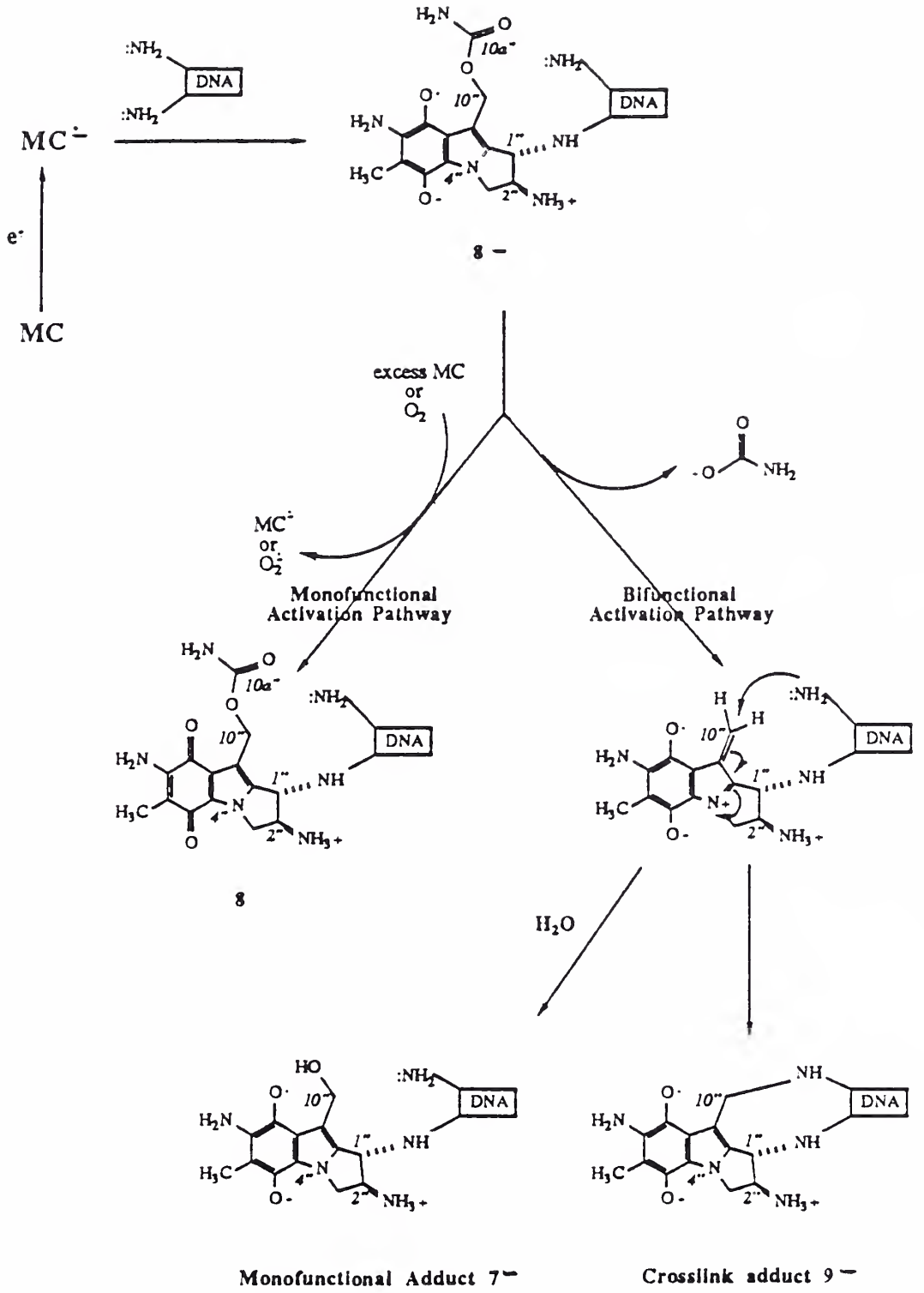
In order to produce cytotoxicity, mitomycin C must first undergo activation (Lin *et al.*, 1972; Schwartz *et al.*, 1963). The C-1 and C-10 positions of the compound (refer to Figure 1) are considered to be masked alkylating functions which become allylic, and hence activated, upon reduction of the quinone system (Tomasz *et al.*, 1988*a*). Previous studies have shown that enzymatic systems which activate these quinones and produce oxygen radicals include the NADPH-cytochrome *c* reductase, xanthine oxidase, and intact

mitochondria (Pristos & Sartorelli, 1986). Activation apparently occurs either via a one-electron reduction to produce a semiquinone or via a two-electron reduction to form a hydroquinone (Patrick *et al.*, 1964). In the mammalian system, the anaerobic metabolism of MC has been shown to be increased two-fold by the addition of cytochrome P-450 to the NADPH-cytochrome *c* reductase reaction mixture; it is thought that this action results from modulation of the activity of NADPH-cytochrome *c* reductase by the cytochrome P-450 (Sartorelli, 1988).

Figure 2 illustrates possible mechanisms by which MC undergoes activation. In 1966, Murakami (1966) postulated that one-electron reduction of the mitomycins to the semiquinones was important for DNA cross-linking. This creates a structural change in the nucleotide bases corresponding to their tautomeric isomers, particularly for cytosine and for guanine as compared to thymine and to adenine. It has been suggested that the semiquinone form is the active intermediate which cross-links the O^6 position of guanine and the N-4 of cytosine to the N-4 and O-5 of mitomycin. Other investigators have supported the existence of the semiquinone form with direct evidence by esr spectroscopy and by electron paramagnetic resonance spectrometry (Patrick *et al.*, 1964; Nagata & Matsuyama, 1969; Lown *et al.*, 1978; Bachur *et al.*, 1979; Kalyanaraman *et al.*, 1980). Furthermore, it has been proposed that following the single-electron reduction, reoxidation of MC may occur in the presence of oxygen to generate a superoxide radical, which may dismute to hydrogen peroxide and to other radicals known to be cytotoxic (Fridovich, 1972). There is also evidence supporting the two-electron reduction of the mitomycins (Workman & Stratford, 1993; Patrick *et al.*, 1964; Iyer & Szybalski, 1964). Studies from tissue culture experiments support the fact that the oxygen radicals may contribute to the aerobic cytotoxicities of some mitomycins (Pristos *et al.*, 1989) but do not explain the enhanced cytotoxicity of these drugs under hypoxic conditions (Rockwell *et al.*, 1982).

FIGURE 2. Possible Mechanisms for the Activation of Mitomycin C

Mitomycin C is initially reduced and alkylated to yield a monoadduct species **8**$^{\cdot}$. This intermediate subsequently enters one of two pathways. The monofunctional activation pathway is taken via electron transfer to unreacted MC, which occurs predominantly when the reduction rate is slow. The bifunctional pathway is taken via retro-Michael elimination of the C-10" carbamate to yield end products designated **7** and **9**. Chemical studies suggest that reduction kinetics, rather than redox potentials, as the critical factor in determining the pathway for the activation of mitomycin C. (Reprinted with permission, Tomasz *et al.*, 1988).


MC·⁻
e⁻
MC
DNA
8 –
excess MC
or
O2
MC·⁻
or
O2·⁻
Monofunctional
Activation Pathway
Bifunctional
Activation Pathway
8
H2O
Monofunctional Adduct 7 –
Crosslink adduct 9 –

Additional studies have suggested that the binding of MC to DNA is more dependent upon the semiquione form of the activated compound than upon the hydroquinone (Tomasz *et al.*, 1974). More recently, it has been observed that MC can also be activated solely by an acidic environment, an interesting observation in regard to the fact that lower pHs have been noted in many tumor tissues relative to normal tissue (Tomasz, 1979).

Mitomycin C : Mechanism of Action

The mechanism of action of MC has been investigated since the 1960's, when Iyer & Szybalski proposed both mono- and bi-functional alkylation by this agent. Tomasz and co-workers studied the adduct formation resulting from monofunctional and bifunctional activation in the chemical systems by treating DNA from *Micrococcus luteus* with MC and by analyzing the adduct distribution by high performance liquid chromatography (Tomasz *et al.*, 1988*a*,*b*). The proposed mechanism is diagrammed in Figure 3 and is outlined as follows: reduction of the quinone to the hydroquinone with elimination of the methoxy group, opening of the aziridine ring by an elimination process to yield a C-1 quinone methide, nucleophilic addition of DNA to the quinone methide to give a monoalkylated adduct, followed by intramolecular SN_2 displacement of the carbamate to form a second quinone methide at C-9, thereby yielding a cross-linked adduct (Moore & Czernik, 1981). After the sequential activation of the C-1 and C-10 positions of MC, covalent bond formation is thought to be preceded by a noncovalent association, presumably of the intercalative type, between the MC semiquinone and DNA. Alkylation of DNA by MC has been demonstrated to be exquisitely specific, with determination of the alkylation site to be solely at the N^2 position of guanine (Bizanek, 1993). According to these results, the ratio

FIGURE 3. The Mechanism of Action of Mitomycin C

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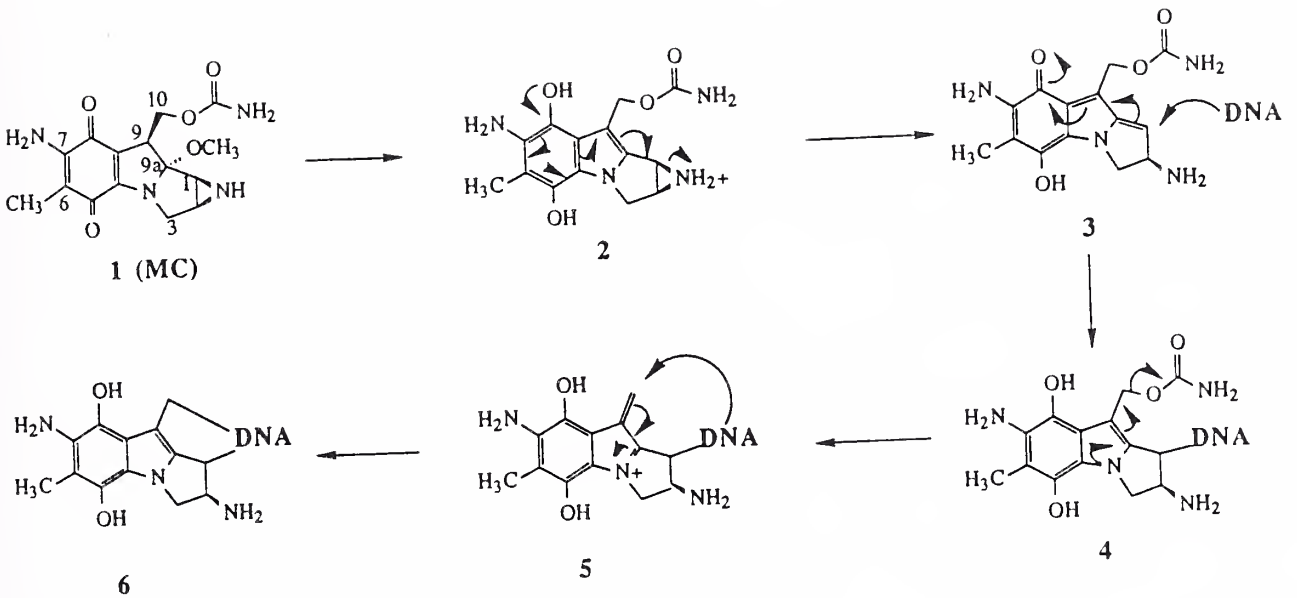

1 (MC)
2
3
4
5
6
DNA

of mono- to bi-functional adducts, previously determined to be in the order of 10:1 (Tomasz *et al.*, 1974), is largely dependent on the DNA base sequence. These investigators also report that the critical factor in determining this ratio appears to be reduction kinetics rather than redox potentials. Further analyses of MC binding to DNA have also suggested that the local DNA structure plays a critical role in determining the efficiency of cross-link formation (Basu *et al.*, 1993; Cera & Crothers, 1989; Teng *et al.*, 1989).

Iyer and Syzbalski showed that crosslinks between MC and DNA are formed and are stable during treatment with heat, alkali, formamide, and in a cesium chloride gradient (Iyer & Szybalski, 1964), but direct evidence for the existence of these complexes was not available for some time. There were several reasons for the difficulty in isolating the DNA-MC complexes, including the necessity for having to activate the compound *in vitro* and the fact that adduction of MC to DNA *in vitro* occurs at a rate of only one MC molecule per several hundred nucleotides (Weissbach & Lisio, 1965). However, the first direct proof of a DNA cross-link adduct with MC was reported in 1987, with isolation of two other adducts following immediately (Tomasz *et al.*, 1987; Tomasz *et al.*, 1988*a*,*b*). The structure of the bisadduct was determined by spectroscopic methods including proton magnetic resonance, differential Fourier transform infrared spectroscopy, and circular dichroism. Computer-generated models of the bisadduct incorporated into the center of the duplex synthetic B-DNA decamer d(CGTACGTACG)$_2$ indicated the stereochemical fit of this adduct to be in the minor groove with minimal distortion of the DNA structure. Analysis of factors influencing monofunctional versus bifunctional activation again suggested that the critical factor is reduction kinetics, rather than reduction thermodynamics. There is continued evidence suggesting that one-electron reduction is sufficient to activate both electrophilic MC centers, at the C-1 and C-10 positions.

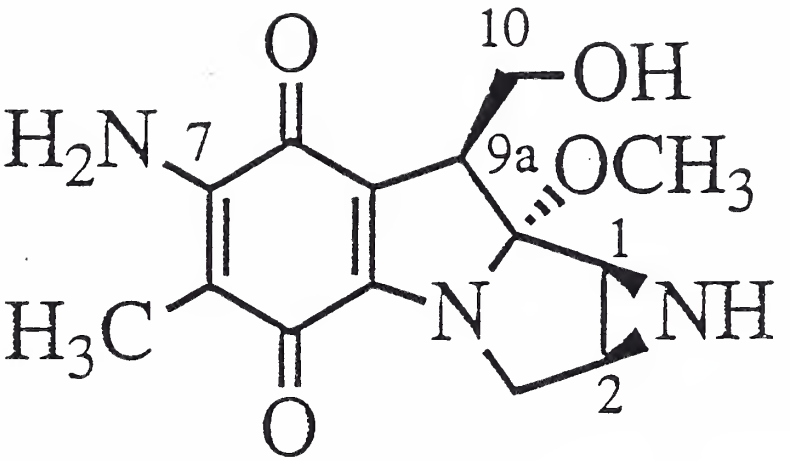
The products which result from mitomycin C -DNA interaction have been identified in a subcellular system as 2 monoadducts and 2 bisadducts; the bisadducts reflect both DNA-interstrand and intrastrand crosslinks (Bizanek *et al.*, 1992; Tomasz *et al.*, 1988*a*,*b*; Tomasz *et al.*, 1987; Tomasz *et al.*, 1986). Only recently have these entities been detected *in vivo*. A group in Japan has successfully synthesized [^{3}H]-labelled MC, which was used by Bizanek and colleagues on EMT6 mouse mammary tumor cells in the isolation and identification of MC-DNA adducts *in vivo* (Bizanek *et al.*, 1993). Analysis of these adducts indicated that the adducts found in living cells were identical to the ones which were previously isolated from chemical studies.

Decarbamoyl Mitomycin C

To further investigate the chemical mechanism of action of MC, analogues of MC with minor structural alterations have also been examined (Weiss *et al.*, 1968). One of these is decarbamoyl mitomycin C [DMC], which differs from its parent compound in that the C-10" carbamate is replaced by a hydroxyl group. DMC is synthesized from MC by treatment with NaOH (Kinoshita *et al.*, 1971). The structure of DMC is illustrated in Figure 4.

FIGURE 4. The Stucture of Decarbamoyl Mitomycin C

The analogue decarbamoyl mitomycin C is structurally identical to that of MC except that the carbamyl group at the C-10" position of MC is substituted by a hydroxyl group, a nonalkylating substituent. The structure of mitomycin C is diagrammed in Figure 1.


O
10
OH
H_2N
7
9a
OCH_3
1
H_3C
N
NH
2
O

Because of mounting interest in correlating the cytotoxicity of MC with its ability to form cross-links, its analogue DMC has been studied in various systems. Experiments performed on DNA from *E. coli* have suggested that DMC is not able to form cross-links (Otsuji & Murayama, 1972). The DNA lesions produced by DMC were also studied in Fanconi's anemia cells, as well as lymphocytes from Fanconi's anemia patients, and the results from alkaline sucrose profiles further support the monofunctional activity of DMC and its inability to form cross-links (Sasaki,1975; Fujiwara & Tatsumi, 1977).

Further investigation was performed using the sister-chromatid exchange (SCE) assay, a sensitive and quantitative measure of genetic alteration which is determined by an increase in frequency of exchange when cells are exposed to mutagens or to carcinogens. Carrano and co-workers treated Chinese hamster ovary cells with either MC or with DMC and found that DMC was actually a more potent inducer of SCE than MC (Carrano *et al.*, 1979). After drug treatment, DNA samples from the cells were denatured and bound to hydroxyapatite chromatography; in those samples that were treated with DMC, essentially all radioactivity was recovered, indicating that only single-strand adducts, rather than double-strand crosslinks, were produced.

The molecular reaction of DMC has also been analyzed through studies using DNA from *Micrococcus lutueus* and a chemical reduction system with sodium dithionite reduction. Once again DMC was observed to form strictly monoadducts from analysis of its products by high performance liquid chromatography (Tomasz *et al.*, 1988). Data from these experiments suggest that displacement of the C-10" leaving group is a critical step in the bifunctional pathway, and that DMC lacks the bifunctional potential. If indeed the cross-linking ability of MC accounts for most of its cytotoxicity, then DMC should be less cytotoxic than MC.

Materials and Methods

Drugs

Mitomycin C was provided without cost by Bristol Myers Squibb (Wallingford, CT). Lyophilized decarbamoyl mitomycin C was generously provided by Maria Tomasz (Department of Chemistry, Hunter College). The identity of DMC was reconfirmed by mass spectrophotometry at The Yale Comprehensive Cancer Center and the purity of the DMC sample was determined by TLC using a 5:1 mixture of acetone:chloroform solvent. The compounds were protected from light in order to prevent photodegradation. The drugs were dissolved in small volumes of sterile 70% ethanol to produce stock solutions of various drug concentrations which in the experiments were further diluted in culture medium.

Cells

In order to study the structure and properties of solid tumors, several model systems have been proposed and utilized for experimental oncology. The simplest of these are exponentially growing monolayer cultures of tumor cells. It has been known for some time that density-inhibited cultured cells (plateau phase) and populations of solid tumor cells *in vivo* share many properties. More specifically, both groups contain large fractions of out-of-cycle but potentially clonogenic cells which constitute a major obstacle to the successful treatment of many solid tumors (Hahn *et al.*, 1968; Hahn & Little, 1972; Ray *et al.*, 1973). Noncycling cells include those that cannot produce new cells and are therefore

considered to be clonogenically non-viable, as well as clonogenically viable cells, which can proliferate if stimulated by an environmental change. Quiescent cell populations are thought to occur in adequately perfused regions within tumors (Tannock, 1978), and have been shown to be less radiosensitive than their aerobic counterparts (Kallman *et al.*, 1980). Because exponentially growing and plateau phase cultures have similar responses (Rockwell, 1983), only exponentially growing cultures were used in these studies.

The development of the colony formation assay in 1955 has allowed for the measurement of the survival of cells treated in vitro with radiation, drugs, and other agents (Puck & Marcus, 1955). An ingenious system developed for experimental oncology is the *in vivo-in vitro* tumor system, which is based upon transplanted tumor cell lines that have been adapted to or selected for growth in cell culture. Unique to the *in vivo-in vitro* model is that it allows detailed examination of the effects of therapeutic agents on tumor cell populations *in vivo* and *in vitro* by colony formation assay with a quantitative precision that is not possible with older animal tumor models (Rockwell, 1977). This system therefore allows *in vitro* assaying of clonogenic cells suspended from tumors that are treated *in vivo* .

The EMT6 *in vivo-in vitro* tumor system was developed by Dr. Rockwell using a mouse mammary tumor originally developed from mamary tumor KHJJ (Rockwell, 1977, 1973). This system has been utilized in the past to study mitomycin C and its N-methyl derivative, porfiromycin [POR]. In the studies which are presented in this thesis, the first set of experiments analyzing MC and DMC, utilized this system.

Further examination of the mitomycins was then performed on another set of cell lines derived from Chinese hamster ovary [CHO] cells. The wide use of CHO cells is mainly atttributed to the fact that these cells grow well and have a high plating efficiency (Hahn *et al.*, 1968; Stewart *et al.*, 1968; Hickson & Harris, 1988). To gain a better

understanding of the relationship between DNA damage and DNA repair, cell survival and mutation, a series of DNA repair-deficient mutants of Chinese hamster ovary cell lines has been developed and studied. The general approach in these studies has been to mutagenize the parent population with radiation or with an alkylating agent, to screen for isolates hypersensitive to a particular DNA-damaging agent, and to examine the hypersensitive sublines (Hickson & Harris, 1988). Hoy, Thompson, and other colleagues have identified DNA repair-deficient mutants from five genetic complementation groups isolated from Chinese hamster cells (Hoy *et al.*, 1984; Hoy *et al.*, 1985). From their studies they characterized and isolated two mutant strains with deficiencies in removal of mono- and bi-functional adducts; the UV4 mutant line has the lowest efficiency in removing cross-links, where the UV5 subline has intermediate efficiency. Testing of 22 different alkylating agents on these two mutant lines have suggested that sensitivity is determined by cross-links, and not by the monoadducts that are produced (Hoy *et al.*, 1985).

EMT6 mouse mammary tumor cells (subline EMT6/Rw)

The first set of experiments with mitomycin C [MC] and with decarbamoyl mitomycin C [DMC] was performed using EMT6 mouse mammary tumor cells (subline EMT6/Rw). Stock cultures of this line were seeded at a concentration of 1 x 10^6 cells and grown as monolayers in T-25 flasks (25 cm^2 plastic tissue culture flasks, Falcon). This line was propagated in 10 ml of Waymouth's medium (Hazelton) which was supplemented with 15% fetal bovine serum (Gibco) and antibiotics (fungizone, gentamycin, penicillin, and streptomycin, all from Gibco). Cultures were grown in a 37°C incubator with a humidified atmosphere of 95% air/5% CO_2. With a doubling time of 12-14 hrs (Rockwell, 1977), these monolayers were subcultured every 3 - 4 days.

Chinese hamster ovary cell lines AA8, UV4, and UV5

The next set of tissue culture experiments was on the AA8, UV4, and UV5 lines of Chinese hamster ovary [CHO] cells, which were obtained from Dr. Larry Thompson at Lawrence Livermore National Laboratory. Stock cultures of these lines were seeded at a concentration of 1 x 10^4 cells and grown as monolayers in T-25 flasks (25 cm^2 plastic tissue culture flasks, Falcon). These lines were propagated in 10 ml of alpha-Minimum Essential Medium (α-MEM, Gibco) which was supplemented with 10% fetal bovine serum and antibiotics (fungizone, gentamycin, penicillin, and streptomycin, all from Gibco). Stock cultures were subcultured every 7 days and were grown in a 37°C incubator in a humidified atmosphere of 95% air/5% CO_2. The doubling time of these monolayers was 20 hours.

Cell Culture Studies

In setting up cells for the experiments which compared the sensitivity of fully aerobic and fully hypoxic cells *in vitro* to the drugs, the monolayers were washed with 5 ml of 0.05% trypsin followed by trypsinization with 5 ml of the trypsin for about 10 minutes. The proteolytic action of the enzyme was then neutralized with 5 ml of serum-supplemented medium, Waymouth for the EMT6 cells and α-MEM for the CHO cell lines. Cells were gently pipetted into single-cell suspensions and were counted using a Coutler Counter Model ZBI (Rockwell, 1977). Cultures were set up by plating 2 x 10^5 cells into glass milk dilution bottles; these flasks also contained 10 ml medium which had been pre-equilibrated for at least one hour in a 37°C humidified incubator to attain optimal pH and temperature for cell growth. Once the cells were seeded, they were allowed to grow for 3-

4 days in a humidified atmosphere of 95% air/5% CO_2 at 37°C. On the day of drug treatment, the medium was replaced with 5 ml of fresh medium (Waymouth for the EMT6 cells and α-MEM for the CHO cell lines). At this point, the flasks were divided into 2 groups, one exposed to the drug in an aerobic environment and the other treated under hypoxic conditions.

The hypoxic apparatus was set up such that a gas mixture of 95%N_2/5%CO_2 (containing < 10 ppm O_2) was warmed and humidified by bubbling through a Corning humidifier containing degassed H_2O. To eliminate all oxygen in the hypoxic apparatus, the system was flushed with the N_2/CO_2 gas mixture for one hour before treatment of the cultures.

When the cultures were to be exposed to the hypoxic environment, the caps on the flasks were removed and the glass bottles were stoppered with tight-fitting rubber sleeves. One 13-gauge and one 18 gauge needle were inserted through the septum of each rubber stopper. The humidified mixture of 95%N_2/5%CO_2 was made to flow through the flasks by attaching the plastic tubing of the hypoxic system to the 13 gauge needle as previously described (Kennedy, 1980; Rockwell, 1988). In this system, outflow from the gassed flasks was through 18-gauge needles connected to rubber tubing whose ends were submerged in degassed H_2O. This allowed visual monitoring of gas flow by continuous bubbling of H_2O, and it also prevented backflow of air into the culture flasks. Previous studies have shown that this method produces severe hypoxia after 1 hour (Kennedy, 1980; Rockwell, 1988). Cultures for the experiments presented in this thesis were gassed with N_2/CO_2 for 2 hours prior to drug treatment.

The cell cultures were treated with graded doses of drug for 1 hr. Cells which were exposed to the aerobic environment were treated with the drug at 37°C. To treat the

cultures in hypoxia, the drugs at various concentrations were injected through the septums of the rubber stoppers while maintaining an anaerobic environment; this procedure did not introduce sufficient oxygen to alter the radiosensitivity of the hypoxic cells and did not alter the pH of the culture medium (Rockwell, 1988).

At the end of the drug treatment, the medium which contained the drug was removed, and the cells were washed with 5 ml of 0.05% trypsin and trypsinized with 5 ml of the trypsin for about 10 minutes. Following neutralization with 5 ml of medium, the single cell suspension was then counted on the Coulter Counter Model ZBI (Rockwell, 1977). Three to four dilutions were made and plated at low densities in quadruplicate in 60 mm^2 plastic tissue culture dishes (Costar). These dishes contained 5 ml medium which had been pre-equilibrated in a 37°C incubator overnight. After plating, the culture dishes were returned to the 37°C incubator for colony formation assay to assess cell viability.

Cell Survival Studies

The EMT6 cell culture experiments were incubated for 14 days and the CHO cell lines for 7 days at 37°C to allow viable cells to grow into macroscopic colonies. On day 14 and day 7 for the EMT6 and CHO cell line experiments, respectively, the growth medium was removed and the cultures were washed with 0.9% saline solution. The colonies were fixed and stained for 20 minutes with a solution of crystal violet in formyl methanol. Only colonies containing more than 40 cells were counted; the number of colonies per plate was calculated from the average of the colony counts of the set of four dishes that had been seeded in quadruplicate. Surviving fractions for treated cultures were calculated using plating efficiencies of untreated controls assayed on the same day. Vehicle-treated controls,

subjected to all of the experimental conditions and treated with maximal concentrations of sterile 70% ethanol, were also included in each experiment and were used to assess and to correct for any cytotoxic effects from the experimental manipulations.

Results

The effects of mitomycin C [MC] and decarbamoyl mitomycin C [DMC] were examined under hypoxic and aerobic conditions using the EMT6 cell line, and subsequently the Chinese hamster ovary cell lines AA8, UV4, and UV5. Survival curves were generated in order to: (1) compare the cytotoxicity of DMC to the various cell lines, (2) evaluate the relative cytotoxicity of DMC to aerobic and hypoxic cells, and (3) compare the cytotoxicity of DMC to that of its parent compound MC. All of the studies using DMC were performed under similar conditions, with one hour drug treatments at varying drug concentrations. Although the major focus of this thesis is on the analysis of the effects of DMC on cell lines deficient in DNA cross-linkage repair, data from these studies can also be used to compare the cytotoxicity of DMC with the effects of the other mitomycins. Previous studies on the MC toxicity in some CHO cell lines have analyzed MC at a given drug concentration with different times of drug exposure, and the results are reprinted in this thesis with permission (Hughes *et al.*, 1991). Although the experimental design differs slightly from the studies conducted for this thesis, comparisons can be made with previous results given the fact that antecedent studies have demonstrated that the most important parameter in determining the overall cytotoxicity of MC is the concentration times the duration of drug exposure (Hughes *et al.*, 1991; Marshall & Rauth, 1988).

Effects of MC and DMC on EMT6 Cells

The results from exposure of EMT6 cells to DMC under aerobic and hypoxic conditions at the time of drug exposure are graphed in Figure 5. Figure 6 compares the response of the EMT6 cells to MC and to DMC under aerobic and hypoxic conditions at the

FIGURE 5. Survival of EMT6 Cells After Treatment With DMC Under Aerobic and Hypoxic Conditions. The EMT6 cell line was exposed to one hour drug treatment with DMC at various concentrations. Each point represents the mean of three to five independent determinations +/- SEMs (standard errors of the mean). Key: closed circles represent aerobic conditions, and open circles represent hypoxic conditions.

EMT6 CELLS TREATED WITH DMC

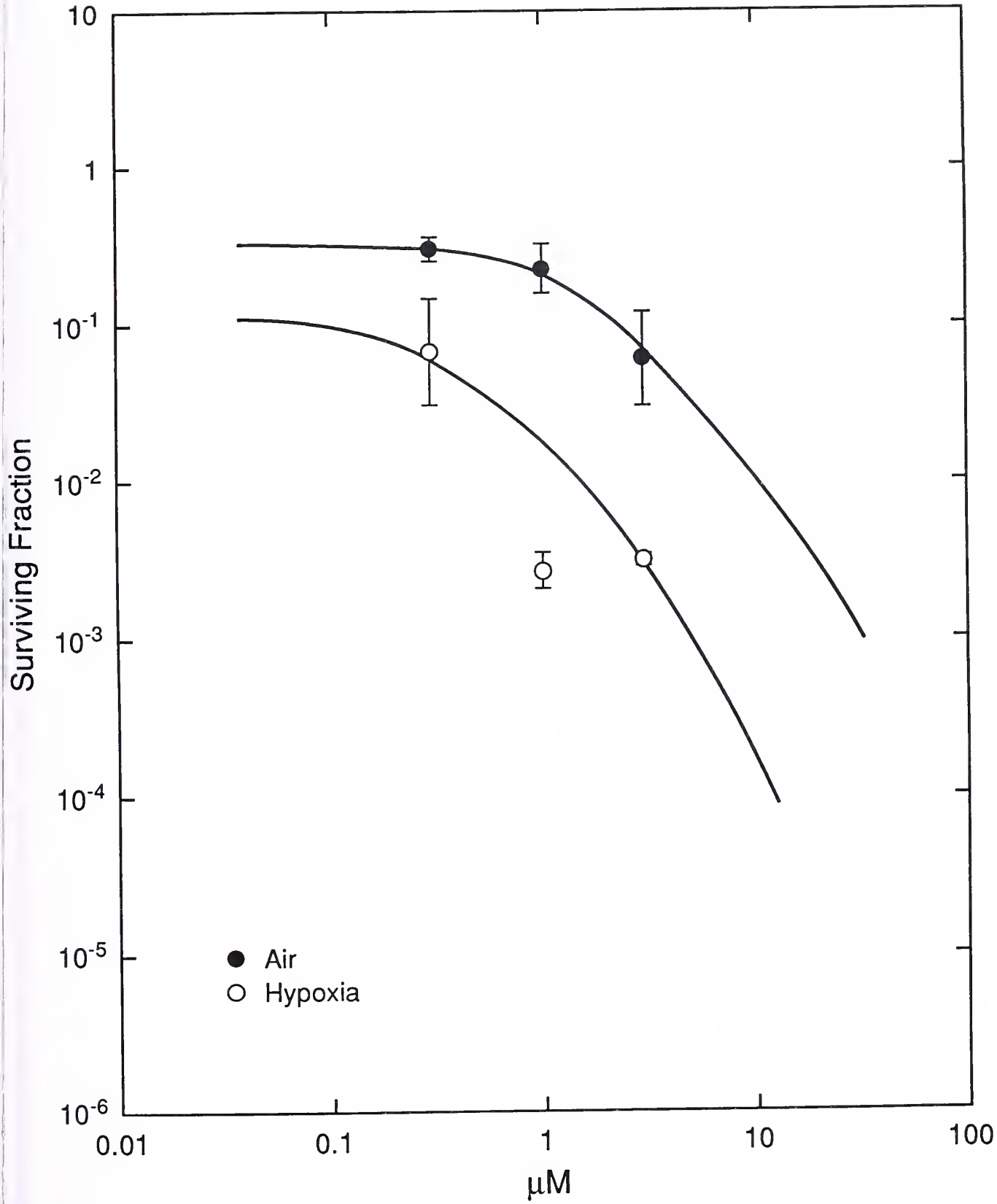
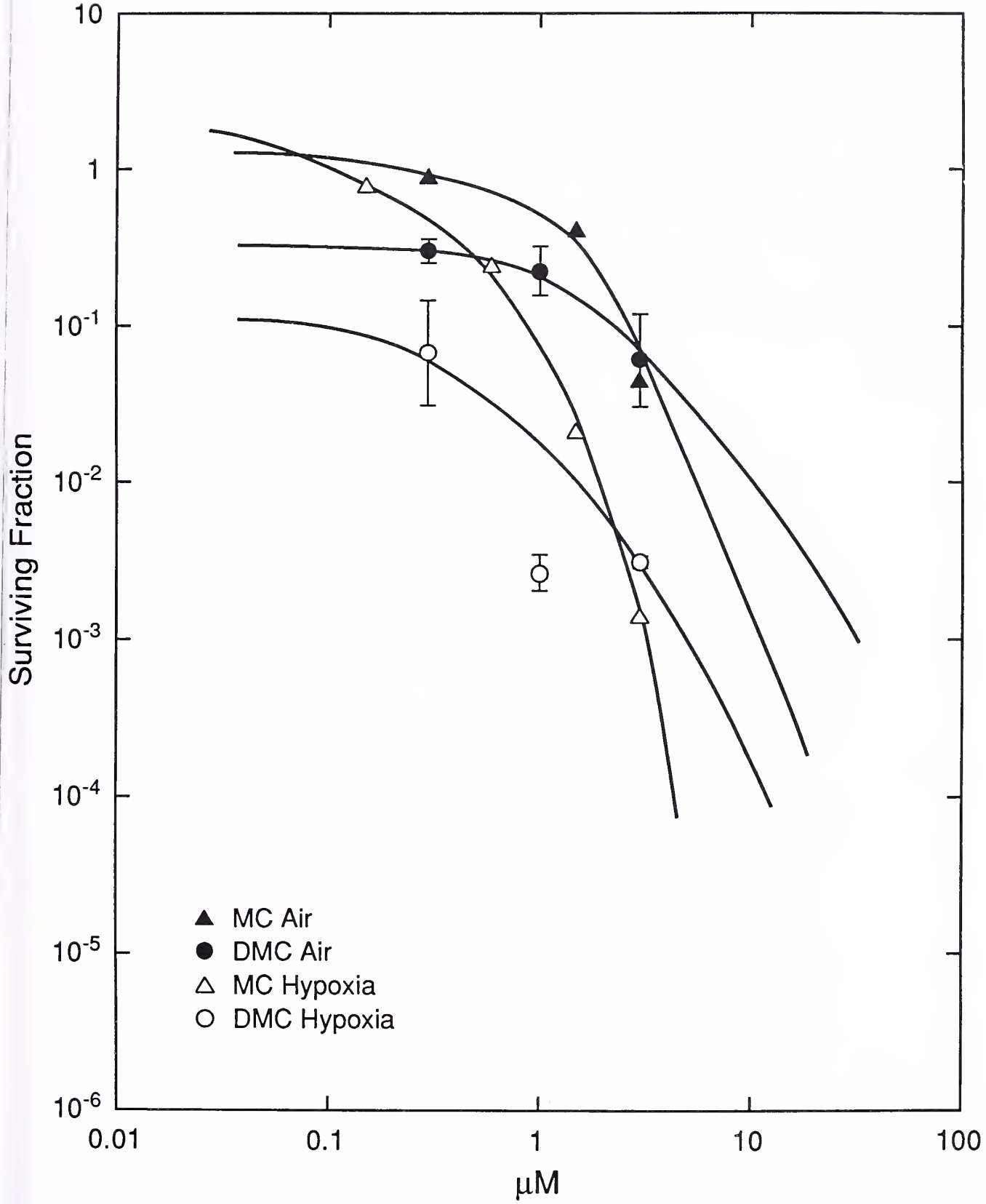


FIGURE 6. Survival of EMT6 Cells After Treatment With MC and DMC Under Aerobic and Hypoxic Conditions. Each point represents the mean from two to five independent determinations. SEMs are shown for points based on three or more determinations. Key: closed symbols represent aerobic conditions and open symbols represent hypoxic conditions. The triangles repesent MC treatment and the circles represent DMC treatment.

EMT6 CELLS TREATED WITH MC AND DMC



time of drug exposure. Data obtained from treatment of this cell line with MC in these experiments are similar to those reported in earlier studies (Kennedy *et al.*, 1980, Keyes *et al.*, 1984, 1985; Rockwell, 1983, 1986). The toxicity of DMC is surprisingly similar to that of MC, as suggested by the similarity in the survival curves. Furthermore, both compounds appear to confer preferential cytotoxicity towards hypoxic cells.

The Effects of MC and DMC on the CHO Cell Lines

Because the identity of the toxic lesion(s) could potentially vary with the cell line and the cellular environment (Keyes *et al.*, 1984, 1985 *a,b*; Lown *et al.*, 1976; Tomasz, 1979; Underberg & Lingeman, 1983), MC and DMC were also tested on another cell line, the Chinese Hamster Ovary [CHO] cell line AA8. This particular CHO cell line was chosen due to the availability of several mutant sub-strains which possess specific defects in DNA repair (Hoy 1984; Hoy *et al.*, 1985). The AA8 cell line was therefore an important system to study, because comparisons between AA8 and the mutant strains would facilitate in elucidating the cytotoxic lesions that are produced by DMC and MC. Figure 7 shows the results from treatment of the wild type AA8 CHO line with DMC under aerobic and hypoxic conditions. In comparing the data from the AA8 line with those from the EMT6 cells (see Figure 6), the cytotoxic effects of DMC appear to be similar on both cell lines. The effects of MC on the AA8 cell line are graphed in Figure 8 with permission. Comparing the graphs in Figures 7 and 8 suggest that MC and DMC have similar effects on the AA8 cell line.

FIGURE 7. Survival of AA8 Cells After Treatment With DMC Under Aerobic and Hypoxic Conditions. The AA8 cell line was exposed to one hour drug treatment with DMC at various concentrations. Each point represents the mean from two to five independent determinations +/- SEMs. Key: closed circles represent aerobic conditions, and open circles represent hypoxic conditions.

AA8 CELLS TREATED WITH DMC

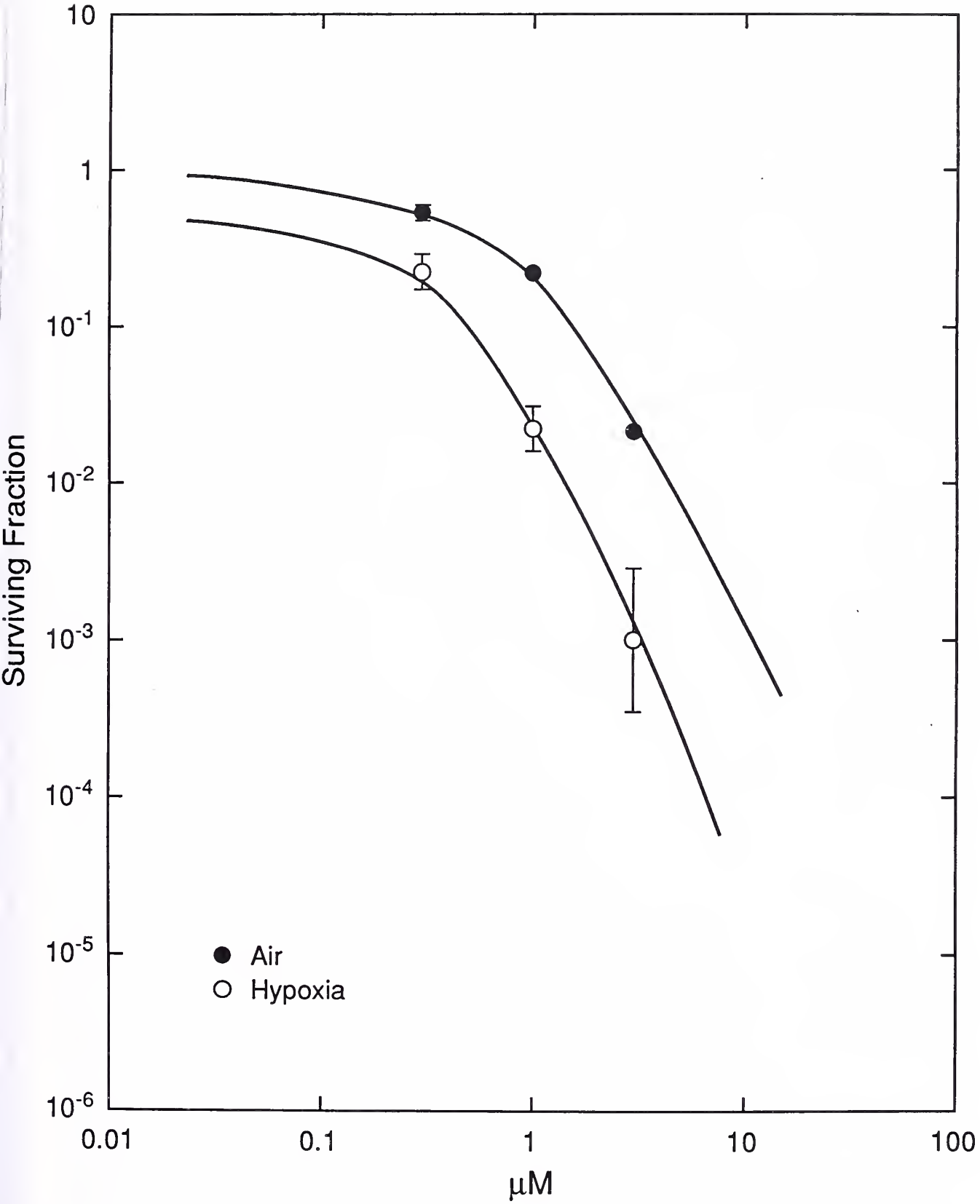
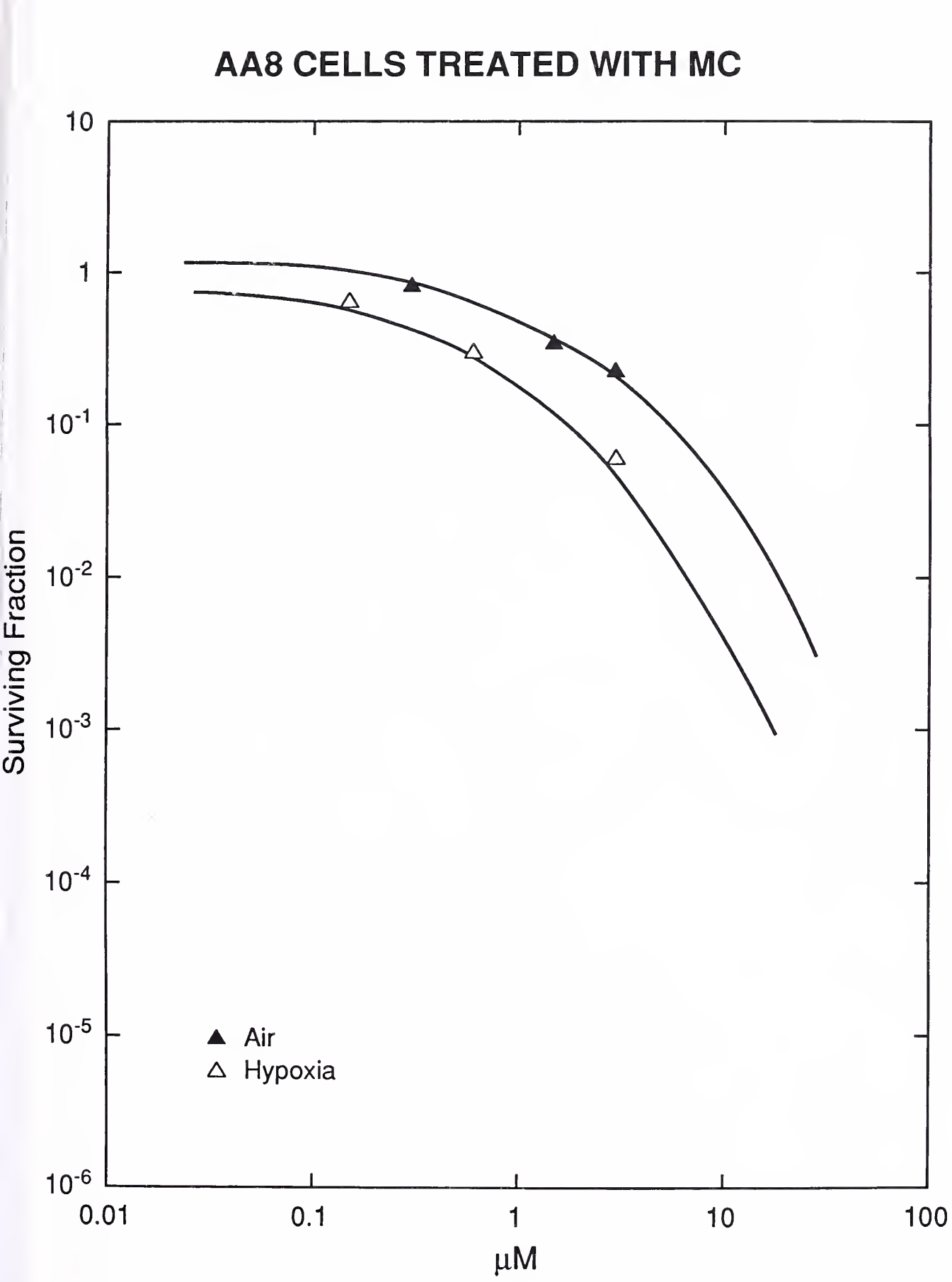


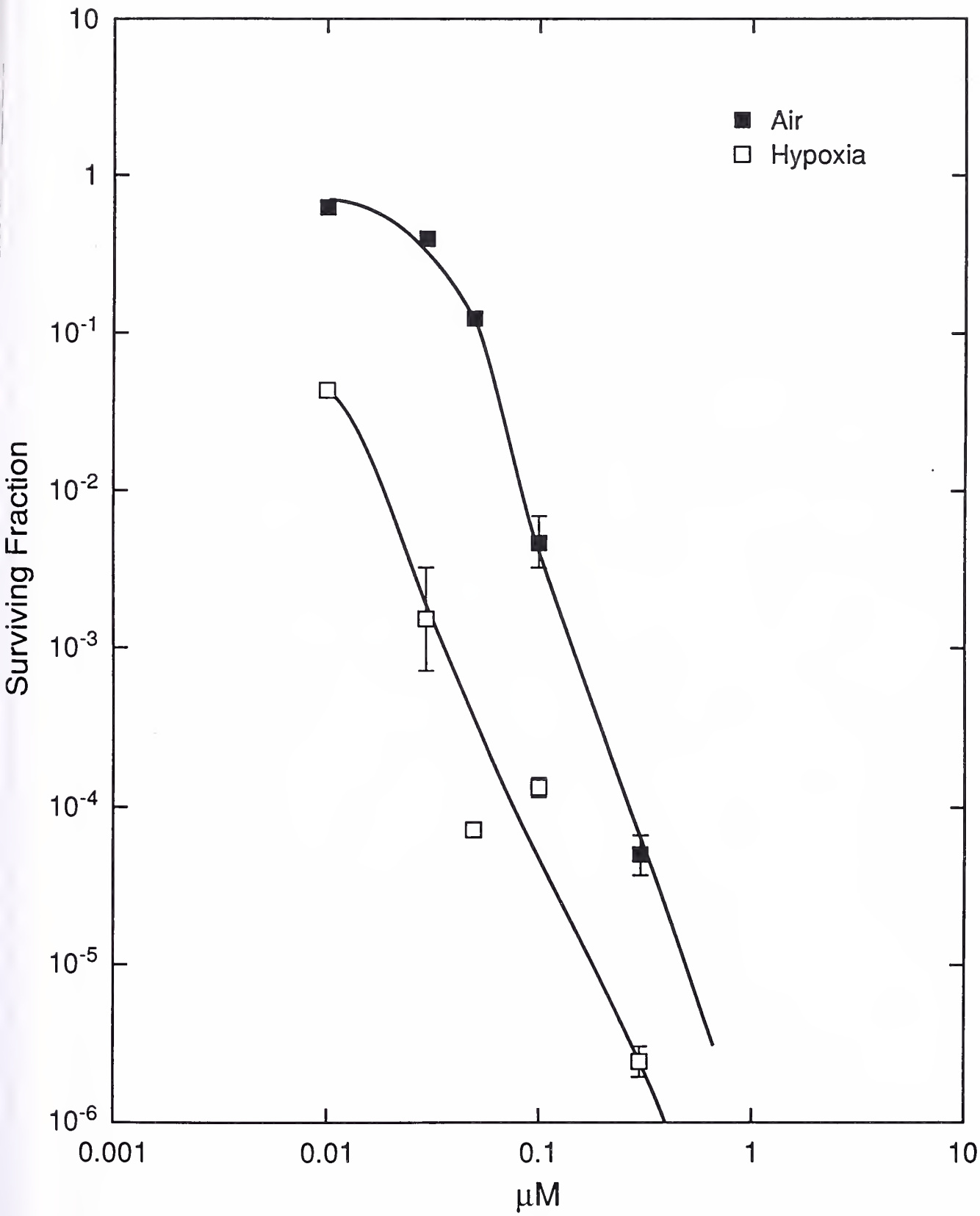
FIGURE 8. Survival of AA8 Cells After Treatment With MC Under Aerobic and Hypoxic Conditions. The AA8 cell line was exposed to one hour drug treatment with MC at various concentration. Each point represents the mean of three to five independent determinations +/- SEMs. Key: filled triangles represent aerobic conditions, and unfilled triangles represent hypoxic conditions.


AA8 CELLS TREATED WITH MC
Surviving Fraction
10
1
10-1
10-2
10-3
10-4
10-5
10-6
0.01
0.1
1
10
100
μM
Air
Hypoxia

The DNA repair-deficient sub-strains UV4 and UV5, derived from the AA8 line, were used to examine more closely the cross-linking ability of the mitomycins. Results from treating the UV4 mutant line with DMC are shown in Figure 9, and results from treatment with MC are presented in Figure 12. Data from treatment with MC have been reprinted with permission from Dr. Christine Hughes (Hughes *et al.*, 1991). Previous studies indicate that the UV4 strain is very sensitive to MC under aerobic and hypoxic conditions. The current results suggest that DMC exerts cytotoxicity similar to that of MC on the UV4 cells. However, the sensitivity of these cells to DMC is more pronounced under hypoxic conditions. It should be noted that the concentrations of both MC and DMC used to treat the UV4 line were diluted 10-fold lower than the concentrations to which the wild type cells were exposed.

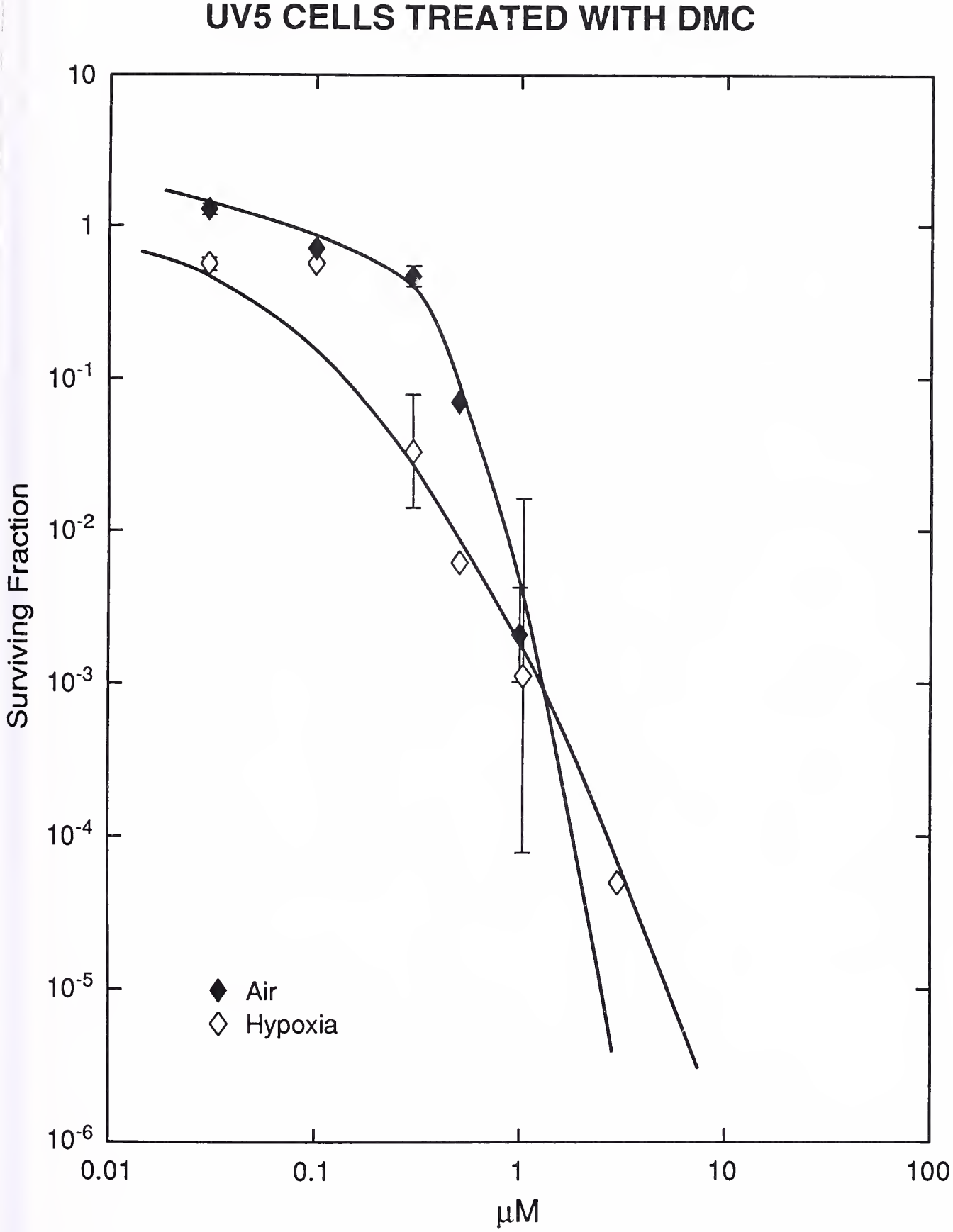
FIGURE 9. Survival of UV4 Cells After Treatment With DMC Under Aerobic and Hypoxic Conditions. The UV4 cell line was exposed to one hour drug treatment with DMC at various concentrations. Each point represents the mean of two to four independent determinations. SEMs are shown for points based on three or more determinations. Key: filled squares represent aerobic conditions and unfilled squares represent hypoxic conditions.

UV4 CELLS TREATED WITH DMC



DMC was tested on the other DNA repair-deficient mutant line, UV5, and the results are graphed in Figure 10. Data from treatment of this mutant strain with MC are reprinted in Figure 12 for comparison (Hughes *et al.*, 1991). Previous studies with MC on the UV5 line indicate intermediate sensitivity to the effects of this alkylating agent, and futhermore no real differential toxicity between drug exposure under aerobic or hypoxic conditions has been observed. Interesingly enough, DMC also appears to exert intermediate cytotoxic effects on this particular mutant strain, with no obvious preferential hypoxic cell kill. The magnitude of the hypoxic/oxic differential for both MC and DMC thus appears to be much lower in the UV5 line than in the AA8 and UV4 lines.

FIGURE 10. Survival of UV5 Cells After Treatment With DMC Under Aerobic and Hypoxic Conditions. The UV5 cell line was exposed to one hour drug treatment with DMC at various concentrations. Each point represent the mean of two to five independent determinations. SEMs are shown for points based on three or more determinations. Key: filled diamonds represent aerobic conditions, and unfilled diamonds represent hypoxic conditions.


UV5 CELLS TREATED WITH DMC
Surviving Fraction
10
1
10-1
10-2
10-3
10-4
10-5
10-6
0.01
0.1
1
10
100
μM
Air
Hypoxia

To facilitate the comparison of the effects of decarbamoyl mitomycin C on the mutant strains relative to its cytotoxicity to the AA8 cell line, the combined results from treatment with DMC on the AA8, UV4, and UV5 cells are graphically depicted in Figure 11. Similarly, the effects of MC on these same lines are illustrated in Figure 12.

FIGURE 11. Survival of AA8, UV4, and UV5 Cells After Treatment With DMC Under Aerobic and Hypoxic Conditions. Graphs from figures 7, 9, and 10 are plotted together in order to facilitate in comparing the effects of DMC on the wild type AA8 cell line to its effects on the mutant lines UV4 and UV5. Key: open symbols represent aerobic conditions, and closed symbols represent hypoxic conditions.

AA8, UV4 AND UV5 CELLS TREATED WITH DMC

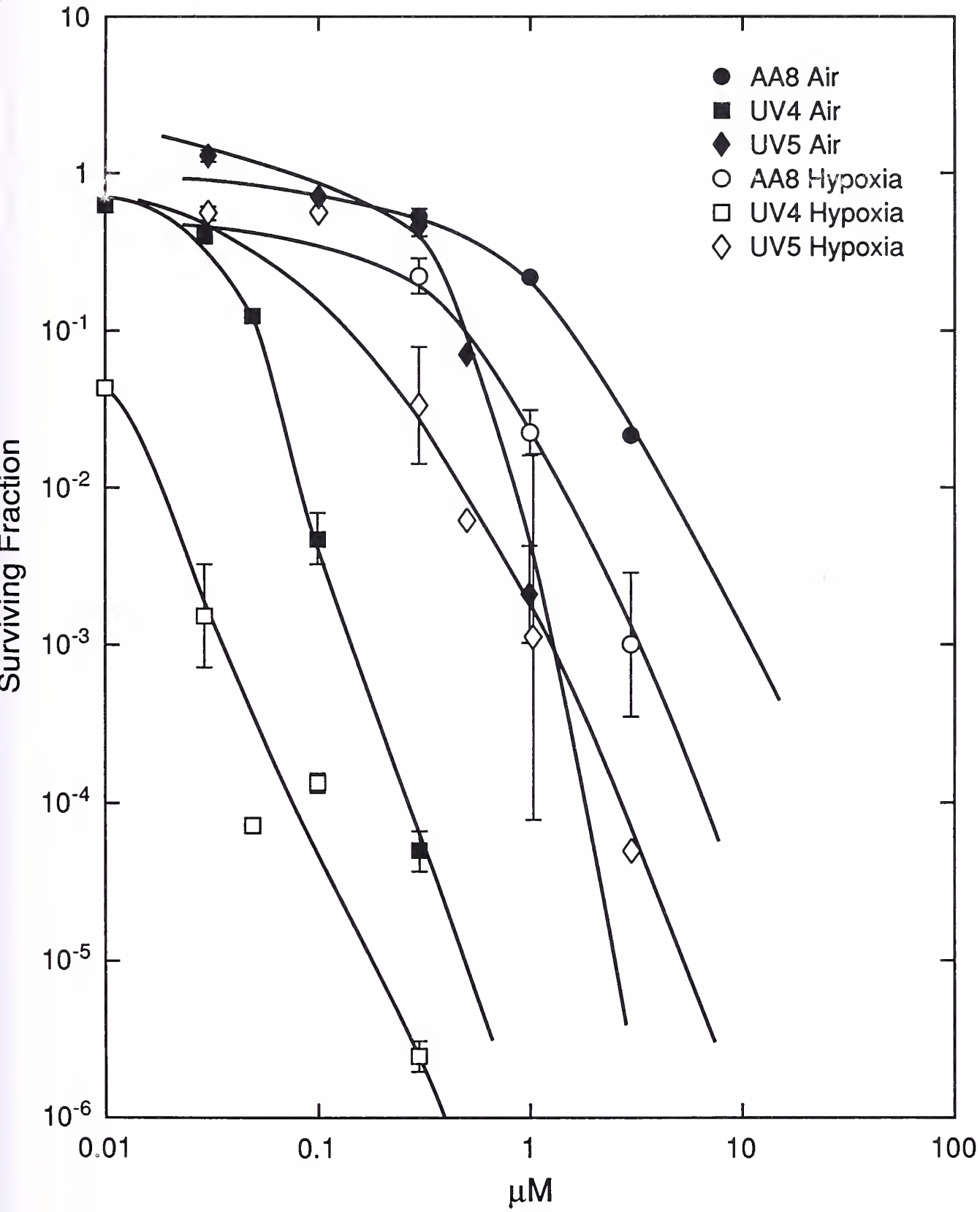
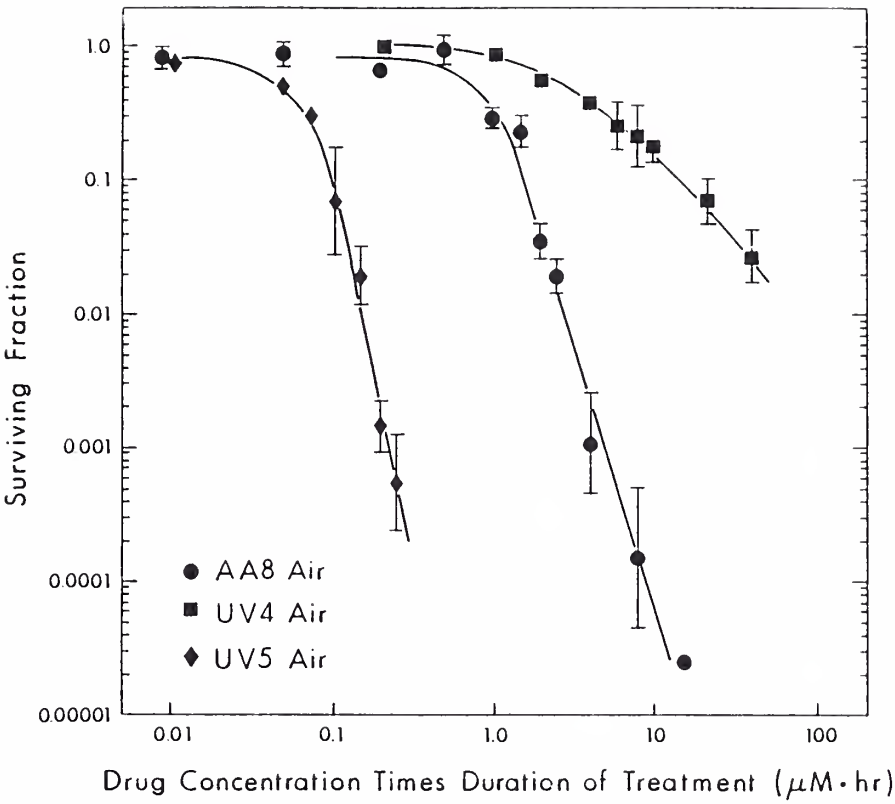
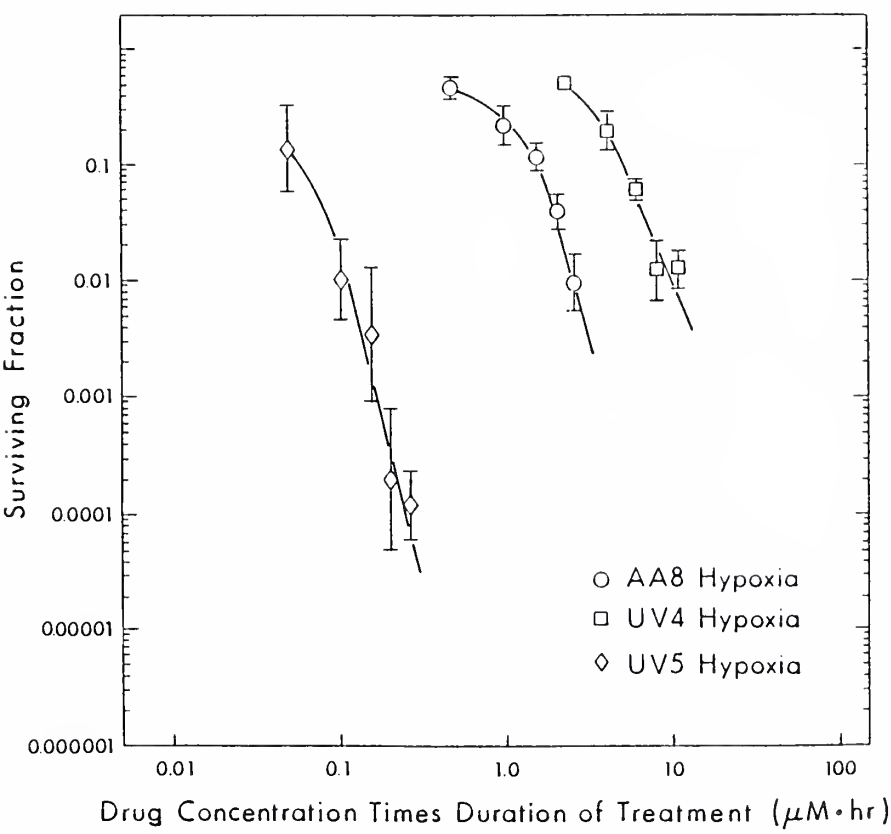


FIGURE 12. Survival of AA8, UV4, and UV5 Cells After Treatment With MC Under Aerobic and Hypoxic Conditions. The effects of MC on the CHO line AA8 and on its substrains UV4 and UV5 are represented in this graph. Key: open symbols represent aerobic conditions, and filled symbols represent hypoxic conditions. The data on the UV4 and UV5 lines are reprinted from Hughes *et al*, 1991.

AA8, UV4 AND UV5 CELLS TREATED
WITH MC UNDER AEROBIC CONDITIONS



AA8, UV4 AND UV5 CELLS TREATED
WITH MC UNDER HYPOXIC CONDITIONS



Chemical Analysis of the Compound DMC

DMC was chemically synthesized from mitomycin C, and the purity of the sample was analyzed by mass spectrophotometry and by thin-layer chromatography [TLC]. Using the electrospray method, the mass spectrophotometry readout indicated a peak at 262 consistent with the molecular weight of DMC. There were two other peaks of much higher molecular weight; these were too high to correspond to mitomycin C, but their identity remains unknown. One possibility is the dimerization of DMC. The major concern was possible contamination with MC, which would have produced a peak at 360; however, the results demonstrate that the sample was free of MC. The author wishes to thank Mr. Walter McMurray for running the sample through the mass spectrophotometer.

Further analysis on the purity of the compound was performed by thin-layer chromotography. Using the solvent system containing 5:1 acteone:chloroform, the average R_f ratios obtained were : 6.0 for DMC, 5.5 for DMC:MC mixture, and 5.0 for MC. There was no evidence for MC contamination in the DMC sample. The author wishes to thank Dr. Juliang Zhu for helping set up the TLC system.

Discussion

Analysis of the effects of decarbamoyl mitomycin C on several cell lines have yielded intriguing results which serve as a reminder of the complex nature not only of the chemistry of the mitomycins but also of the cellular response to such perturbations. Results from these experiments, particularly those with the DNA repair-deficient CHO strains, provide additional insight into the action of the mitomycins. This section of the thesis will integrate data from the DMC experiments with the current understanding of the antineoplastic effects of the mitomycins.

Decarbamoyl mitomycin C is structurally identical to mitomycin C except for its lack of an alkylating group at the C-10" position. A strong correlation between interstrand crosslinks and cytotoxicity has been observed in dose-response studies with MC on various cell lines (Keyes *et al.*, 1991; Dorr, 1985; Kohn, 1981; Kinoshita *et al.*, 1971). The cytotoxic effects of mitomycin C have been attributed to its ability to crosslink DNA; hence the analogue DMC, previously shown to be incapable of forming bisadducts in chemical systems, was predicted to be less cytotoxic. Results from these experiments performed on four cell lines, EMT6, AA8, UV4 and UV5, suggest otherwise.

Initial experiments with DMC have been performed on the wild type EMT6 and AA8 strains. Comparison of survival curves for DMC with those for MC suggest that DMC possesses activity similar to its parent compound, and demonstrate that DMC is preferentially cytotoxic to hypoxic cells. Because these results came as a surprise, there was concern for the possibility that DMC might be contaminated with MC. The DMC sample utilized in the experiments was therefore verified by mass spectrophotometry and by TLC methods to be free of MC. The outcome of these experiments raises several questions. First, with the prediction that DMC is less potent than MC, how is the

decarbamoyl analogue able to kill cells with similar efficacy as its parent compound? Also, could it be that lesions other than bisadducts, i.e. monoadducts, play a significant role in the production of cellular toxicity? Finally, is it possible that DMC produces crosslinks *in vivo* which have yet to be identified?

To examine the possible relationship between monoalkylation and cytotoxicity, the cell lines UV4 and UV5, which possess deficiencies in DNA crosslink repair, were exposed to DMC. The mutant CHO line UV4 lacks the ability to remove DNA crosslinks and bulky DNA monoadducts, whereas the UV5 strain is not capable of removing bulky monoadducts, but still has an intact DNA-DNA crosslink repair mechanism (Hoy,1984, 1985). In the studies reported here, the results with the UV4 cells suggest that this mutant strain is very sensitive to both MC and DMC. It should be noted that the concentrations of the drugs used to treat this cell line were much lower than those used to treat the wild-type AA8 strain. While MC appears to exert similar effects on the aerobic and hypoxic UV4 cells, sensitivity to DMC is more pronounced under hypoxic condtions. If the assumption holds that DMC is not capable of forming crosslinks in DNA, the implication is that a monoadduct of DMC is responsible for the cytotoxicity of this compound, and that more of these lesions are generated under hypoxic conditions.

The UV5 cells exhibited intermediate sensitivity towards both MC and DMC. Because the UV5 strain is capable of repairing crosslinks, but not removal of large monoadducts, the data reported here support the theory that monoadducts play an important role in cell killing. Its intermediate response can be interpreted as partial recovery by its ability to repair crosslinks. Nonetheless, the fact that both the UV4 and UV5 lines, two substrains which have the inability to remove bulky monoadducts, have demonstrated increased sensitivity to DMC when compared to the wild-type strain AA8, strongly

suggests that another lesion, most likely a monoadduct, contributes to the lethal activity of the mitomycins.

Furthermore, in these four cell lines treatments with either MC or DMC have resulted in similar outcomes, providing additional support for the hypothesis that the crosslinks themselves may not solely account for the cytotoxic nature of the mitomycins. In fact, after Iyer and Szybalski hypothesized that the mitomycins were bifunctionally masked alkylating agents, there was speculation as to whether or not the aziridine ring and the carbamoyl groups were essential to account for their biological activity. Kinoshita and colleagues have analyzed and compared the 10-acyloxy derivatives of the decarbamoylmitosane and decarbamoylmitosene to the mitosane and mitosene containing the carbamoyl group (Kinoshita *et al.*, 1981). Their data indicate that these compounds exhibit substantial antibacterial activity, suggesting that the carbamoyl group at the C-10" position of mitomycin C is not of significance, at least with respect to the antibacterial activity of the compound.

Two other research groups have reported results which further question the importance of the cross-linking action of the mitomycins. First, a critical experiment by Carrano and co-workers used Chinese hamster ovary cells to compare the ability of MC and DMC to induce sister-chromatid exchanges [SCEs], a sensitive and quantitative assay used to measure genetic alteration. DMC was actually found to be more effective than MC in inducing SCEs, leading to the proposal that the DNA-interstrand crosslink is not the major lesion responsible for inducing SCEs in CHO cells but may in fact confer lethality beyond that from monofunctional alkylation (Carrano *et al.*, 1979). Dorr and his colleagues have also investigated the relationship between interstrand crosslinks and cytotoxicity in dose-response studies using MC. They raise the possibility that MC's impressive ability to produce cross-links could potentially prevent the detection of DNA-

strand breaks which are produced simultaneously by mechanisms involving quinone-generated oxygen free radicals, but which are not identifiable by current methods (Dorr *et al.*, 1985).

Due to the short-lived existence of the active form of MC, the DNA-drug complex has been difficult to isolate. As a consequence, identification and isolation of the lesions produced has been done primarily through manipulation of chemical systems. In their earlier studies on the bifunctional alkylating ability of mitomycin C, Tomasz and co-workers found that the initial monofunctional step of DNA binding consistently occurs at the C-1 position of MC (Tomasz *et al.*, 1988). The bifunctional activation of MC has been proposed to subsequently occur at the C-10 position, and the product distribution between monofunctional and bifunctional adduct formation is thought to depend on the rate of product formation rather than on the redox potentials. Although preferential crosslinking by MC in hypoxia would have been an attractive model to account for the differential toxicity in aerobic and anaerobic environments, current studies in cellular systems reveal that fully bifunctional activation of MC can also occur under aerobic conditions (Tomaz *et al.*, 1987; Chowdary & Tomasz, 1987). This further supports the possibility that, although MC is capable of crosslinking DNA, other entities may also produce toxic effects.

Several adducts have successfully been isolated from additional chemical studies. One monoadduct, interestingly, has been reported to be common in the pathways by which both MC and DMC appear to exert their cytotoxic effects (Tomasz *et al.*, 1988*a,b*). Furthermore, both DMC and MC have been shown to exhibit the same 5'-CG specificity in DNA interaction (Kumar *et al.*, 1992; Tomasz *et al.*, 1988*b*). Crosslinks generally have been considered to be more lethal than the monofunctional adducts, because crosslinkage results in an irreversible block to DNA replication. However, it has also been hypothesized that perhaps after the monoalkylation step, the specific orientation of the monoadduct,

namely with the C-10" position extending in the 5'-direction, may be critical in inhibiting DNA synthesis (Kumar *et al.*, 1992; Small *et al.*, 1976; Mercado & Tomasz, 1972; Kinoshita *et al.*, 1971). In their studies using synthetic oligonucleotides reacted with mitomycin C under chemical conditions which restrict MC to monofunctional alkylating activity, Kumar and colleagues have shown that there is site selectivity in the monoalkylation of guanine, particularly in the 5'-GCG sequence. It is currently thought that this specificity is related to thermodynamic stability. Molecular modeling has supported the notion of specificity in the orientation of the compound, and from these observations one can hypothesize that perhaps the initial alkylation of MC, which appears to be identical to the action of DMC, plays a more significant role in damaging the structural integrity of DNA than was previously thought.

Tomasz and colleagues have recently been working with site-specifically modified oligonucleotides which were synthesized to contain either of the two identified MC-DNA monoadducts. Interestingly, their data suggest that the monoadducts of MC are strong enough blocks of replication to be potentially lethal lesions *in vivo* (Basu *et al.*, 1993). Molecular modeling studies indicate that a MC monoadduct fits snuggly into the minor groove of the duplex B-DNA, but it has also been postulated that the unique noncovalent, hydrogen-bonding capacities of the mitomycin-DNA interaction creates a thermodynamically stable, distorted structure at the replication fork, resulting in a polymerase block and hence termination of DNA synthesis. More specifically, the two polar functional groups of the bound MC, i.e. the 2"-NH_3 and 10"-carbamate, have been identified as the main interactive elements. Experiments on DMC reveal that its 10"-OH group possesses similar properties. It is now known that both MC and DMC produce a common monoadduct, and it is tempting to speculate that this product could in fact be responsible for the cytotoxicity of the mitomycins.

Recently, the availability of [6-CH_3-^{3}H]-mitomycin C has made it possible to follow the processing of MC in cellular systems. This is considered to be the most direct method for detecting and identifying DNA adducts formed in intact cells. The lesions that have been isolated, both mono- and bis-adducts, were identified to be identical to the ones found in the chemical systems (Bizanek *et al.*, 1993). When radiolabelled DMC becomes available, this will undoubtedly aid in determining whether the lesions produced by DMC in cellular systems correspond to those found in chemical systems. In addition, more extensive comparisons can be made between the lesions produced by MC and DMC *in vivo*.

It is also necessary to evaluate the biological response triggered by these structural modifications. One consideration is the possibility that DMC may form crosslinks in biological systems which have not been producible under chemical conditions. Due to the complex nature of the cellular environment, it is reasonable to postulate that certain cellular components, i.e. enzymes and cofactors, could facilitate the formation of crosslinks which have yet to be found with chemical reaction systems (Bizanek *et al.*, 1993; Rockwell, 1986). Vos and Hanawalt have developed a system, referred to as the RAGE technique, that can been used to detect DNA crosslinks in cellular systems. This technique has been utilized to study psoralen, a furocoumarin which is known to intercalate DNA and to form covalent adducts with pyrimidine bases when exposed to near-ultraviolet light (Vos & Hanawalt, 1988; Misra & Vos, 1993). After exposure of the DHFR gene in cultured human cells with 4'-hydroxymethyl-4,5',8-trimethylpsoralen [HMT], the DNA is rapidly denatured and electrophorectically separated on agarose gel. Southern hybridization is then performed in order to quantify the level of cross-linking in the DNA samples. This method is presently being modified for use in investigating the possibility of crosslink formation by DMC in EMT6 cells (P. Glazer, personal communication). The results of this study will,

hopefully, provide further insight into the mechanisms by which the mitomycins produce their cytotoxic actions.

Regions within tumors have transient or persistent deprivation in perfusion, resulting in the development of hypoxia, acidity, and nutritional deficits (Rockwell, 1992; Thomlinson & Gray, 1955). It has been well established that solid tumors in experimental animal tumors contain hypoxic cells which limit their curability (Kennedy *et al.*, 1980). Recent advancements in laboratory techniques combined with the ever increasing fund of knowledge have resulted in providing more direct evidence for hypoxia in solid tumors, particularly in human neoplasms (Rockwell, 1992). Unlike chemotherapy, which can confront resistance and tumor recurrence from the cytogenetic alterations produced after drug treatment, radiotherapy does not appear to be dramatically affected by genetic changes (Rockwell, 1992; Deacon *et al.*, 1984). The cellular environment, however, does markedly alter neoplastic response to radiation, with hypoxia inducing resistance to this treatment modality (Rockwell, 1992; Vaupel, 1989).

Due to the existence of radio-resistant cell populations in solid tumors, there has been substantial interest by laboratory and clinical investigators in developing agents which circumvent the protective effect of hypoxia. Oxygen, even at low concentrations, has been demonstrated to act as a radiosensitizer (Gray *et al.*, 1953). The half-maximum for radiosensitization occurs at about 3 torr, and the curve plateaus at oxygen tensions (pO_2) of approximately 20-40 torr, which is the oxygen tension found in venous blood. Because normal tissue is usually well aerated, most normal tissues are fully, or almost completely, sensitive to radiation. With the rationale to enhance the effects of radiotherapy on hypoxic cells in solid tumors while minimizing its effects on normal tissue, the use of hypoxia-directed drugs such as mitomycin C have been studied both in the laboratory as well as the clinical research settings.

Mitomycin C was first used as an adjunct to radiotherapy in head and neck cancer treatment at the Yale Comprehensive Cancer Center (Weissberg *et al.*, 1989). In their first randomized clinical trial of 120 patients with biopsy-proven squamous cell carcinoma of the head and neck, the 5-year acturarial local recurrence-free survival was significantly better in the radiation therapy plus mitomycin C group as compared to the group treated with radiation alone (87% and 66%, respectively, $p < 0.02$). Hence the use of this alkylating agent appears to improve local tumor control in patients with tumors of the oral cavity, oropharynx, larynx, hypopharynx, and nasopharynx, thereby producing therapeutic gain.

More recently, clinical trials in Toronto using mitomycin C as an adjunct to 5-fluorouracil plus radiotherapy in the treatment of carcinomas of the cervix and the anal canal have demonstrated improvement in local control with MC, as compared to treatment without mitomycin in the regimen (Rockwell, 1992; Thomas *et al.*, 1990). Other clinical applications of mitomycin C are also underway.

Conclusion

Mitomycin C is an interesting compound in many respects. It is capable of exhibiting antimicrobial as well as antitumor activity. Activation is required before it can produce lethal effects. Furthermore it possesses the ability to cross-link DNA in addition to forming monoadducts. The precise mechanism of its ability to produce cytotoxicity, however, remains to be solved. Studies on its analogue decarbamoyl mitomycin C, reported here, seem to indicate that the primary cytotoxic lesion may in fact be a monoadduct.

The mitomycins have been regarded as a challenging structure-activity problem. In cellular systems, factors such as oxygenation, pH, and cell proliferation patterns need to be taken into account (Rockwell, 1983), as well as the state of differentiation of cells, growth fraction, and intracellular communication (Sutherland *et al.*, 1970). Other factors that need to be taken into account include the rate of transport, the intracellular location of activating enzymes, the rate of drug inactivation, intracellular pH, and the cellular capacity to repair drug-induced damage (Sartorelli, 1988). The issue of physiologic, metabolic, and environmental alterations which accompany hypoxia in solid neoplasms also needs to be addressed (Kennedy *et al.*, 1980).

Thus studying the cytotoxic effects of the mitomycins requires acknowledging the complexity in the interaction between mitomycin C and cells. New avenues have been considered which in the future may facilitate the elucidation of the mechanism of action of this drug. Studies using the RAGE technique to separate chemically cross-linked and un-cross-linked fragments of DNA are presently under way. In the future radiolabelled decarbamoyl mitomycin C could also provide useful information in understanding the mechanism of action of the mitomycins.

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