Enhancement by Hyperthermia of the \textit{in Vitro} Cytotoxicity of Mitomycin C toward Hypoxic Tumor Cells$^1$

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\textbf{ABSTRACT}

Mitomycin C and hyperthermia are both toxic to chronically hypoxic EMT6 tumor cells. Combinations of this drug and heat were tested \textit{in vitro} in normally aerated and chronically hypoxic EMT6 mouse mammary tumor cells to establish whether greater than additive cytotoxicity could be achieved by combined treatment. Cell survival was measured at four concentrations of mitomycin C (0.01, 0.1, 1.0, and 10 \(\mu\)M) at 37° or at elevated temperatures (41, 42, and 43°) for durations of 1, 2, 3, and 6 hr. At 42°, exposure to mitomycin C for 3 and 6 hr produced a 2- to 3-fold increase in hypoxic tumor cell kill at all drug concentrations over that expected for strict additivity. A 15-fold enhancement in the kill of hypoxic tumor cells was obtained at 1.0 and 10 \(\mu\)M mitomycin C at 43° for 6 hr of exposure. Under most conditions, additivity was observed for the antibiotic and heat in oxygenated cells, except at 43° with 0.01 and 0.1 \(\mu\)M mitomycin C following 3 and 6 hr of treatment, conditions under which a 5- to 10-fold potentiation of tumor cell kill was obtained. The rate of formation of reactive metabolites from mitomycin C under anaerobic conditions in EMT6 cell-free preparations was measured. A 30 to 50% increase in alkylating activity was observed at elevated temperatures, suggesting that the enhanced cytotoxicity of mitomycin C with heat toward hypoxic cells may, in part, be due to an increase in activation of the drug.

\textbf{INTRODUCTION}

Hyperthermia would appear to have therapeutic potential in the treatment of solid tumors, especially when used in combination with other treatment modalities, such as radiation therapy and chemotherapy (5, 17). Unlike X-ray irradiation which exerts its greatest lethality on well-oxygenated and rapidly proliferating tumor cells, heat appears to be at least as cytotoxic toward hypoxic and nonproliferating cells as it is toward exponentially growing cells (6, 21). The cytotoxic potential of hyperthermia toward hypoxic tumor cells would seem to be particularly important in solid tumor therapy, because hypoxic cells appear to be relatively resistant to the lethal actions of currently used modes of cancer therapy (5, 15). Malignant cells are preferentially destroyed by heat within the range of 41–43° whereas, at temperatures above 43°, this differential is lost (12, 19).

Mitomycin C, a naturally occurring bioreductive alkylating agent with clinical utility against several human tumors, has been shown to be selectively toxic toward chronically hypoxic tumor cells over a relatively wide range of concentrations \textit{in vitro} (13, 27) and has demonstrated potential as a hypoxic cell-selective chemotherapeutic agent \textit{in vivo} (24). We have demonstrated (13, 14) that, in the absence of oxygen, certain enzyme systems are capable of catalyzing the addition of electrons to suitable substrates leading to formation of a reduced species. Because hypoxia promotes conditions which are conducive to reductive reactions, mitomycin C is preferentially activated to an alkylating species by hypoxic cells, a phenomenon that results in enhanced cytotoxicity toward the hypoxic subpopulation of solid tumors (13, 16, 23, 26).

The present paper describes studies designed to examine the interactions of hyperthermia and mitomycin C on hypoxic tumor cells. The lethal effects obtained by simultaneous exposure of cultured EMT6 tumor cells to various concentrations of mitomycin C at a variety of temperatures and exposure times under oxygenated and hypoxic conditions were measured. Furthermore, because the activation of mitomycin C to alkylating species by tumor cell-free preparations can be estimated, the rates of formation of alkylating species from the antibiotic at various temperatures were also examined. The results indicate that synergistic kill of hypoxic EMT6 tumor cells occurs over a wide range of temperatures and concentrations of mitomycin C and that the enhanced kill of hypoxic cells by heat and the antibiotic may be, in part, the result of increased production of reactive species from the drug at elevated temperatures.

\textbf{MATERIALS AND METHODS}

\textbf{Chemicals.} Mitomycin C was the gift of Dr. Maxwell Gordon of Bristol Laboratories, Syracuse, N. Y. NADP$^+$, glucose 6-phosphate, and glucose-6-phosphate dehydrogenase were obtained from Sigma Chemical Co., St. Louis, Mo. All other reagents were obtained from standard chemical sources unless otherwise indicated.

\textbf{Tumor Cells.} Experiments were performed using EMT6 mouse mammary tumor cells \textit{in vitro} in Waymouth’s medium supplemented with 15% newborn calf serum (Grand Island Biological Co., Grand Island, N. Y.). Cytotoxicity studies were performed using exponentially growing monolayer cultures of EMT6 cells. The techniques used for propagating the cells and measuring cell survival by colony formation have been described in detail previously (22–24, 28).

\textbf{Hypoxic Conditions.} Cells were made hypoxic as described elsewhere by preincubation for 4 hr with a humidified atmos-
phere of 95% N₂-5% CO₂ prior to the addition of mitomycin C and incubation at elevated temperature (13, 24, 27). Normally aerated cultures were incubated in a humidified atmosphere of 95% air-5% CO₂. The pH of the incubation medium was stable at 8.15 ± 0.05 under the various atmospheric conditions, drug concentrations, and temperatures used. Incubation of the cells was carried out at 37, 41, 42, or 43° for 1, 2, 3, or 6 hr at mitomycin C concentrations of 10, 1.0, 0.1, and 0.01 µM. Each combination was tested in at least 3 separate experiments. For the colony-forming assay, each flask was plated at 3 dilutions in replicate dishes and incubated at 37° under 95% air-5% CO₂; colonies grew to readily countable sizes in 8 to 10 days. No difference existed between the survival of untreated cells or vehicle-treated cells maintained under the aerobic or hypoxic conditions used; the plating efficiencies for these control situations were 65 to 80%.

**Hyperthermia.** EMT6 cell monolayers in sealed 25-sq cm plastic culture flasks were maintained within ±0.1° of the desired temperature by totally immersing the flasks in a circulating water bath. The temperature was monitored with a National Bureau of Standards thermometer (0.1° gradations) and a thermocouple digital thermometer (Model BAT-8C; C. Bailey Instruments Co., Saddlebrook, N. J.). Mitomycin C was added to the cells at the time of initiation of hyperthermia and was removed by washing at the conclusion of the heat treatment; at this time, the cells were cloned.

**Assessment of Alkylating Metabolites.** The rates of formation of alkylating metabolites were assessed at 37, 41, 42, and 43° as described previously (13). Briefly, sonicated EMT6 cells were incubated under a nitrogen atmosphere in the presence of an NADPH-generating system, mitomycin C and 4-(p-nitrobenzyl)pyridine. After a 20-min incubation, the reaction was stopped by the addition of acetone and 1 M NaOH. The reaction mixture was then extracted with ethyl acetate, and the absorbance of the alkylated 4-(p-nitrobenzyl)pyridine complex was determined at 540 nm (31). Cell sonicate protein was determined by the method of Lowry et al. (18).

**Calculation of Additive and Synergistic Effects.** Results of the combined treatment of EMT6 cell monolayers with mitomycin C and hyperthermia were termed additive or synergistic according to the method and definitions of Valeriote and Lin (30). To estimate an additive effect, it was assumed that each agent acted independently of the other; consequently, the product of the individual surviving fractions was used.

**RESULTS**

The effects of mitomycin C on the survival of aerobic and hypoxic EMT6 tumor cells at 37° were measured, and the results obtained are shown in Chart 1 (top). EMT6 cells were exposed to mitomycin C at concentrations from 0.01 to 10 µM for 1, 2, 3, or 6 hr in the presence or absence of oxygen. Mitomycin C was markedly more cytotoxic to chronically hypoxic cells than to their normally oxygenated counterparts at all drug concentrations and durations of exposure tested. After

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**Chart 1.** Time course for the survival of EMT6 tumor cells exposed to various concentrations of mitomycin C under aerobic or hypoxic conditions at 37, 41, 42, and 43°. • 0.01 µM mitomycin C; □, 0.1 µM mitomycin C; ○, 1.0 µM mitomycin C; O, 10 µM mitomycin C. Each point represents the average of 3 separate experiments.
the initial hr of exposure to the drug, the survival of cells at all concentrations of mitomycin C continued to decrease but at a decreasing rate. After 6 hr of exposure to the antibiotic, 6 to 9 times more kill of hypoxic cells than aerobic cells was produced by mitomycin C at the various concentrations used.

The effects of mitomycin C on the survival of aerobic and hypoxic EMT6 tumor cells at elevated temperatures are also shown in Chart 1 (bottom). At 41°, the cytotoxicity of mitomycin C toward oxygenated cells was comparable to that observed at 37°. In addition, under conditions of hypoxia, very little increase in cell kill over that obtained at 37° occurred at 0.01 and 0.1 µM mitomycin C at 41°. When the ratios of aerobic to hypoxic survivals were compared, those at 0.01 and 0.1 µM mitomycin C were similar at both temperatures. At higher drug concentrations (1.0 and 10 µM), however, an increase in the ratio of aerobic to hypoxic cell survival was apparent, reaching 30 times at 1.0 µM mitomycin C and 13 times at 10 µM mitomycin C after 6 hr of exposure to the antibiotic at 41°.

At 42° and 43°, substantial increases were observed in the cytotoxicity of mitomycin C toward chronically hypoxic EMT6 cells as compared to that toward their normally aerated counterparts. At 42°, the ratios of the survival of oxygenated cells to hypoxic cells were 78 at 10 µM, 18 at 1.0 µM, 12 at 0.1 µM, and 5 at 0.01 µM mitomycin C after 6 hr. Exposure to the antibiotic for 6 hr at 43° produced 130 times more kill of hypoxic cells than of oxygenated cells at 10 µM mitomycin C and 70 times greater kill of hypoxic cells at 1.0 µM mitomycin C. At a level of 0.1 µM mitomycin C, 18 times more kill of hypoxic tumor cells than of their oxygenated counterparts occurred after 6 hr at 43°. At the lowest drug concentration tested, 0.01 µM mitomycin C, the ratio of aerobic to hypoxic survival was only 4 at 43° after 6 hr of exposure to the drug; this decrease in ratio was due primarily to an enhancement of oxygenated cell kill under these conditions.

Using the methodology described by Valeriote and Lin (30), calculations were made of the expected surviving fractions for the combination treatments under conditions of additive activity for all mixtures of drug concentration, temperature, and duration of treatment from survival data obtained from exposure to mitomycin C at 37° and heat-only controls. By comparing the expected cell survival for additive treatment conditions with experimentally observed survivals, it is possible to quantify the degree of interaction between mitomycin C and hyperthermia. Table 1 lists the ratios of the expected surviving fraction for conditions of treatment additivity to the experimentally observed surviving fractions for oxygenated and hypoxic EMT6 cells under each of the experimental conditions. A ratio of 1.0 indicates an additive effect, while ratios greater than 1.0 indicate a potentiation of tumor cell kill by the combined treatment modalities (4). A marked enhancement of the kill of hypoxic cells occurred at 43° at all drug concentrations, especially after 6 hr of exposure to 10 or 1.0 µM mitomycin C, where 15 times or 1.5 logs greater toxicity toward hypoxic cells by the combination treatment occurred than was expected from an additive effect. A large increase in the degree of enhanced cell kill was observed with hypoxic cells exposed to the antibiotic for 6 hr as the temperature was increased from 42 to 43°. Primarily additive effects occurred under most treatment conditions in cells maintained under oxygenated conditions. However, at the lowest concentrations of mitomycin C, 0.01 and 0.1 µM, and at 43°, a substantial enhancement in the degree of cell kill was observed, reaching a maximum of about 10-fold after 6 hr of exposure to 0.01 µM mitomycin C.

Since mitomycin C is preferentially activated to an alkylating species under hypoxic conditions (13, 25, 26), enhanced drug cytotoxicity in combination with heat could be due to an increase in the rate of formation of alkylating metabolites. To test this possibility, the capacity of sonicates of EMT6 cells to produce reactive metabolites from mitomycin C was measured by trapping these reactive species with 4-(p-nitrobenzyl)pyridine. The results, shown in Chart 2, demonstrate that the rates of formation of reactive species were increased by 30 to 50% in EMT6 cell-free preparations incubated at 41–43°, when compared to rates in 37° control preparations.

<table>
<thead>
<tr>
<th>Concentration (µM)</th>
<th>Temperature (°C)</th>
<th>Aerobic cells</th>
<th>Hypoxic cells</th>
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<tbody>
<tr>
<td></td>
<td>1 hr</td>
<td>2 hr</td>
<td>3 hr</td>
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<tr>
<td>0.01</td>
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<td>0.9</td>
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<tr>
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DISCUSSION

Three factors are known to be of major importance in cellular response to hyperthermia. These are pH, nutritional status, and oxygenation; variations in all of these factors are likely to occur in solid tumors. Cells at low pH and under conditions of nutritional deprivation are especially sensitive to heat (5, 7, 8, 20). Clearly, these conditions may occur in combination with hypoxia in solid tumors. Synergism following treatment with mixtures of hyperthermia and chemotherapeutic agents, as demonstrated by a decrease in cell survival greater than that expected from addition of the treatment effects, has been produced by several agents (8). The results clearly demonstrate that mitomycin C produces enhanced selective toxicity toward hypoxic tumor cells at elevated temperatures. The survival of heated hypoxic EMT6 cells markedly decreased as the drug concentration and temperature increased, while the kill of well-oxygenated EMT6 cells was only slightly enhanced under most treatment conditions.

It is probable that more than one mechanism for the synergistic interaction between mitomycin C and heat may be operating. Changes in membrane structure under thermal stress may increase cellular permeability to drugs, as seen with Adriamycin (9, 11). Hyperthermia may also inhibit cellular repair processes (9, 10, 29). Furthermore, an enhancement of drug cytotoxicity by heat may result from an increased rate of activation of the drug from an inactive precursor or increased accessibility or sensitivity of a metabolic target. Since metabolic reduction requires anaerobiosis, the data presented in this report suggest that the potentiation of hypoxic cell killing by mitomycin C at elevated temperatures may be due, at least in part, to an increased rate of formation of the active quinone methide alkylating species of the drug in hypoxic tumor cells.

Although scant information is available on blood and tissue levels of mitomycin C in humans, the concentration range of drug examined in this study, 10 to 0.01 μM, is clearly inclusive of levels achievable in humans (3). Total-body hyperthermia can be conducted safely at 42°C for durations of up to 4 hr (2). For local heating, temperatures of 43°C and higher have been utilized clinically. Although no substantial enhancement of cell kill was demonstrated in the in vitro test system used in the present study at 41°C, significant effects were observed at 42°C, and very marked enhancement of mitomycin C cytotoxicity was found at 43°C, especially in chronically hypoxic tumor cells. These findings corroborate and extend the findings of Barlogie et al. (1), who demonstrated an enhancement of mitomycin C cytotoxicity by heat in LoVo cells (a human colon cancer cell line) under oxygenated conditions at drug concentrations near 10 μM at 41 and 42°C in both exponentially growing and plateau-phase cultures.

In summary, the combination of mitomycin C and heat has been demonstrated to produce a potentiation of cytotoxicity against an established tumor cell line in vitro. The further testing of this combination in animal systems should provide useful information on the potential clinical usefulness of these 2 agents.

REFERENCES

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