Effect of Oxygen on Misonidazole Chemosensitization and Cytotoxicity in Vitro

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ABSTRACT

In order to assess the effect of oxygen on chemopotentiation by misonidazole (MISO), EMT-6/Ro tumor cells were exposed in vitro to combinations of CCNU and 1.0 mM MISO in culture medium equilibrated at various oxygen concentrations. The effect of oxygen on MISO cytotoxicity was similarly determined and compared with the relationship obtained for chemosensitization. MISO cytotoxicity and chemopotentiation were both oxygen sensitive, being maximal under anoxic conditions. Furthermore, the pattern of oxygen sensitivity was virtually identical for the two activities. These results suggest that a similar metabolic pathway, i.e., the oxygen-sensitive reduction of MISO to the nitroradical anion by cellular nitroreductases, is involved in the mechanism of both activities. The data further indicate that chemopotentiation can be expressed in cells treated at intermediate oxygen tensions. The implications of these findings with respect to the magnitude of chemopotentiation in vivo and the enhancement of normal tissue damage in animals treated with MISO and chemotherapy agents is discussed.

INTRODUCTION

Radiation sensitizers, particularly MISO, can potentiate the antitumor effectiveness of many conventional chemotherapeutic agents in vitro and in vivo (5, 15). Although the mechanism(s) or nature of the interaction(s) responsible for improved tumor cell killing is not yet completely defined, in vitro investigations have identified certain requisite conditions which must be satisfied before chemosensitization can be realized (5). Most notable among these is the requirement for exposing cells to MISO under hypoxic conditions prior to or concurrent with exposure to a chemotherapeutic agent. Several experimental studies strongly suggest that the induction of chemosensitization in vivo, although perhaps more complicated, is likewise dependent upon the presence of hypoxic tumor cells (23). However, the magnitude of chemopotentiation expressed in virtually all tumor systems analyzed greatly exceeds the enhancements predicted if sensitization was restricted to the radiobiologically hypoxic population. The diffusion of a hypoxia-derived mediator into oxic regions of the tumor is one mechanism proposed to rationalize this paradox. Equally possible is the probability that chemosensitization can be expressed to some extent in tumor cells at oxygen tensions intermediate between fully aerobic and radiobiologically hypoxic levels. If true, this might also account partially for the enhanced toxicity encountered in normal tissues (15).

This later possibility was evaluated in vitro by comparing the magnitude of potentiation achieved by combining MISO with CCNU for the treatment of EMT-6/Ro tumor cells at various oxygen tensions. Furthermore, the effect of oxygen on the cytotoxicity of MISO was determined and compared with the relationship determined for chemopotentiation.

MATERIALS AND METHODS

Cell Line and Drug Treatment. EMT-6/Ro cells (Rochester subline of EMT-6 mammary tumor cells) routinely maintained in Eagle's basal medium and 10% fetal calf serum were used in these studies. For each experiment, exponential cells were trypsinized from monolayer cultures and agitated in a gas-tight syringe at a concentration of 1 to 2 x 10^7 cells/ml for 15 min at 37°C prior to being injected into individual Type I treatment vials (24) containing 9.9 ml of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic-buffered Eagle's basal medium supplemented with 10% fetal calf serum. Cells were treated in suspension in these vials at a final concentration of 1 to 2 x 10^6 cells/ml. Prior to the addition of cells or CCNU, the medium in each vial was equilibrated with humidified gas mixtures certified to contain 21, 5, 1, 0.5, 0.1, 0.01 and 0.001% oxygen (Cg) in nitrogen. The length of the required equilibration period was dependent upon Cg and was determined for each gas mixture in independent experiments by monitoring the oxygen tension in the medium under experimental conditions.

Two different nitrosourea treatment protocols were used in these studies. In the first, complete CCNU dose-response curves were generated for cells treated for 4 hr in the presence of 1.0 mM MISO at each of the 7 different oxygen tensions, while in the second, cells were exposed for 4 hr to a fixed dose of CCNU in the presence or absence of 1.0 mM MISO.

CCNU was initially dissolved in absolute ethanol and diluted 100-fold upon addition to the treatment vials at the initiation of treatment. The final concentration of ethanol (1%) did not reduce control plating efficiencies. For MISO-treated groups, MISO was dissolved at the desired concentration directly in the culture medium prior to equilibration with the gas phase. Survival curves for EMT-6/Ro cells exposed for up to 5.0 mM MISO for up to 6 hr at the various oxygen concentrations were also determined.

At the conclusion of the exposure period, cells were removed from the vials, rinsed in fresh medium, counted, and plated for cell survival using a standard clonogenic assay. The plating efficiency of otherwise untreated EMT-6/Ro cells was not significantly reduced by a 4-hr hypoxic exposure.

Oxygen Level in Solution and Respiration Rate Measurements. Oxygen tensions in solution were measured using a Clarke-type O2-sensing microelectrode and amplification system manufactured by Yellow Springs Instrument Co. (Yellow Springs, OH). All O2 measurements were made in independent experiments under the same experimental conditions used in the actual experiments. Measurements were made in the absence or the presence of cells (1 to 2 x 10^6/ml). The former measurements were used to establish the length of the pretreatment gassing phase while the latter were performed to determine the actual equilibrium O2 tension (Cv) in medium containing respiring cells. This value can be significantly lower than the Cg used to purge the system (24). In the presence of respiring cells, it was only possible to actually determine Cv values for corresponding Cg values in excess of 0.5% (5000 ppm). This...
RESULTS

As shown in Chart 1A, the cytotoxicity of a 5.0 mM dose of MISO was dependent upon the length of exposure and the O₂ concentration of the medium. Although Chart 1A clearly shows the time dependency of MISO toxicity in the EMT-6/Ro cell line, the same data transposed to Chart 1B more clearly establish the influence of Cₐ on MISO activity. In Chart 1B, survival levels for exposure times from 2 to 6 hr are plotted as a function of Cₐ. Presentation of the data in this form reveals that the extent of cell kill achieved by exposure to 5.0 mM MISO increased markedly between Cₐ values of 0.05 and 0.01% (500 and 100 ppm, respectively). At Cₐ values above and below this range, the cell kill gradient is much less steep. These data suggest that a transitional oxygen concentration exists between approximately 500 and 100 ppm at which the cytotoxic efficiency of MISO becomes nearly maximal. To gain an estimate of this critical oxygen concentration the Kₐ(O₂)cytotoxicity value (i.e., the oxygen concentration at which the rate of cell inactivation by MISO is half-maximal) was determined. The method used to estimate the Kₐ(O₂)cytotoxicity value was the same as that described by Taylor and Rauth (19) and is graphically depicted in Chart 1A, inset. Briefly, from the terminal slopes of the MISO dose-response curves, the exposure time required to reduce survival by a factor of 10 (t₁₀) was determined for curves generated at the various Cₐ values. The relative t₁₀ values were then plotted as a function of Cₐ (ppm). From this plot, a Kₐ(O₂)cytotoxicity value of approximately 350 ppm was obtained.

A Kₐ(O₂)chemopotentiation value was calculated from complete dose-response curves for cells treated with CCNU and 1.0 mM MISO (Chart 2). However, since MISO primarily influenced the shoulder of the CCNU dose-response curves, it was not possible to make an estimate of the Kₐ(O₂)chemopotentiation by slope ratio analysis. Rather, a Kₐ(O₂)chemopotentiation value was determined by an alternate method based on a 2-fold reduction in the dose-effect factor (ratio of the dose of CCNU alone to the dose of CCNU in the presence of MISO required to reduce survival to 10⁻³). Under anoxic conditions and in the presence of 1.0 mM MISO, the dose of CCNU required to reduce cell survival 3 logs in 4 hr was 2.2 µg/ml. In contrast, 4.2 µg CCNU/ml was required to achieve a similar survival reduction under aerobic conditions without MISO, or aerobic conditions with or without MISO (dose-effect factor = 4.2/2.2 = 1.93). The combination treatment under anoxic conditions therefore resulted in a net CCNU dose reduction of ~2.0 µg/ml. From the data in Chart 2, it was calculated that a 2-fold reduction in chemosensitizing efficiency, corresponding to a 1-mg/ml dose reduction, would occur at a Cₐ value of approximately 420 ppm. This Kₐ(O₂)chemopotentiation value

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4 The relationship described by Taylor and Rauth states that

$$C_{a} = \frac{R_{g} R_{k}}{R_{k}}$$

where R = cellular oxygen consumption rate and is a function of time, oxygen concentration, and cell state, k is a geometry-dependent factor related to the efficiency at which oxygen passes into solution, and t is time. Readers should consult Ref. 19 for a more detailed discussion of this relationship.
and 0.01%. For comparison, the 4-hr MISO cytotoxicity data from Chart 18 are regraphael in Chart 3A. This particular data set was chosen for comparison with the data in Chart 3A because the exposure times and the magnitudes of the survival reductions (~20-fold) were equivalent for both. Comparison of the results in Chart 3A with other of the MISO cytotoxicity response curves (Chart 1B) would support a similar conclusion since they all show comparable breaks between 0.05 and 0.01% regardless of the ultimate magnitude of MISO cytotoxicity.

Neither CCNU (20 μg/ml) nor MISO (5.0 mM), which produces a time and concentration dependent reduction of cellular respiration (1, 19) significantly altered the oxygen utilization rate of exponential EMT-6/Ro tumor cells (Table 1). Similarly, cellular respiration was not reduced significantly when the 2 agents were combined. Further evidence for this conclusion is provided by the MISO survival curves in Charts 1A, which do not bend upward at later exposure times as would be expected if cellular respiration rate declined with a resultant increase in C.(19).

DISCUSSION

In addition to its cytotoxic and radiation-sensitizing properties, MISO has been shown to enhance effectively the antitumor activity of several conventional chemotherapeutic alkylating agents in vivo and in vitro (5, 15). The ability to demonstrate this effect in vitro requires that cells be exposed to MISO for prolonged periods of time under hypoxic conditions, unless excessively large doses of MISO are utilized. A similar requirement for hypoxia for the manifestation of chemopotentiation by MISO in vivo has been more difficult to establish and therefore much more controversial. Wheeler et al. (23) reported recently that MISO failed to enhance the activity of 1,3-bis(2-chloroethyl)-1-nitrosourea against s.c. 9L rat brain tumors, which do not contain radiobiologically hypoxic cells. Similar treatment of hypoxic 9L cells in vitro resulted in significant chemopotentiation (16). These and other in vitro data suggest that hypoxic cells are required for chemosensitization by MISO. It is generally agreed, however, that the magnitude of chemopotentiation demonstrable in tumors exceeds what would be expected if the interaction between MISO and alkylating drugs was restricted to the radiobiologically hypoxic tumor cell population. Diffusion of a MISO metabolite from hypoxic cells into oxic regions of tumors has been proposed by some as a plausible explanation for this apparent paradox. A similar mechanism has been proposed to account for MISO cytotoxicity in excess of the tumor hypoxic fraction (4). However, to date attempts to identify and isolate such a reactive intermediate have met with little success. The hypothesis that chemosensitization might involve primarily oxygen-deficient tumor cells was also invoked to rationalize the observed selective effect of MISO chemopotentiation for tumors versus critical normal tissues, which lack radiobiologically hypoxic cells. While the magnitude of enhancement in most tumors indeed exceeds the concomitant increase in normal tissue toxicity, a modest potentiation of drug damage in dose-limiting tissues is nonetheless expressed in animals treated with MISO and alkylators (15). Consequently, a proposed mechanism for chemopotentiation must at once account for a large tumor effect in excess of that predicted on the basis of the hypoxic fraction, and a more limited effect in well-perfused normal tissues.

Both conditions would be satisfied if MISO chemopotentiation occurred to some extent in cells at oxygen tensions intermediate
between radiobiologically hypoxic and fully aerobic. Such a hypothesis might account for the increased toxicity observed in bone marrow and gut which would be expected to have a significant population of cells at venous oxygen tensions (~5%). Relative to normal tissues, tumors would be expected to have even wider oxygen-tension gradients and a larger proportion of cells at very low oxygen concentrations. Consequently, the potentiation of chemotherapeutic action by MISO would be larger in tumors. In order to test this hypothesis experimentally, the magnitude of chemopotentiation produced by combining 1.0 mM MISO with CCNU was determined for EMT-6/Ro tumor cells treated in vitro at 7 different oxygen concentrations. We were also interested in comparing the oxygen sensitivity of chemopotentiation with that for MISO cytotoxicity in an attempt to reveal any parallelisms between the mechanisms of the 2 activities. Therefore, MISO survival curves were likewise generated under various oxygen tensions.

As demonstrated in Chart 1A, MISO toxicity was both time and oxygen-concentration dependent. Assessment of the data as it is plotted in Chart 1B indicates that MISO toxicity increases abruptly at C_o values between ~0.05 and 0.01%, while at C_o above or below these limits, cell survival is much less dependent on changes in oxygen concentration. The K_m(O_2) cytotoxicity value for MISO cytotoxicity was calculated to be ~350 ppm (0.035%) (Chart 1A, inset) for the present experimental conditions. Our estimate of K_m(O_2) cytotoxicity is in good agreement with that reported by Taylor and Rauth (19) for Chinese hamster ovary cells similarly treated in vitro. The oxygen sensitivity of MISO cytotoxicity is attributable to a one-electron-transfer in the presence of oxygen, resulting in the regeneration of the parent compound (RNO_2) from the nitroradical anion (RNO_2^-) (2, 22) according to the reaction:

\[ \text{RNO}_2^- + \text{O}_2 \rightarrow \text{RNO}_2 + \text{O}_2^- \]

Since the reductive metabolism of MISO is considered to be responsible for its cytotoxic properties (14, 20), reversal of the initial reduction step would be expected to reduce cytotoxicity.

Chemopotentiation by MISO was likewise oxygen sensitive, being maximal for cells in anoxic conditions (Chart 2) with a K_m(O_2) chemopotentiating value of ~420 ppm. The good agreement between K_m(O_2) cytotoxicity and K_m(O_2) chemopotentiating suggests that oxygen-sensitive reduction of MISO by cellular nitroreductase may be a requisite step for the induction of chemopotentiation as well as for MISO cytotoxicity. A similar conclusion is suggested by investigations demonstrating that cellular changes associated with the reductive metabolism of MISO, such as glutathione depletion (6, 21), are important factors in chemopotentiation. However, the present experiments provide even more direct evidence for an association between MISO reduction and the ability of MISO to potentiate chemotherapeutic efficacy. It should be noted that while the K_m(O_2) estimates obtained in the current studies are similar to those reported previously (19), the C_o values for K_m(O_2) reported here should be viewed with some reservation, since both are below the lower limit of sensitivity (0.1%, 1000 ppm) of our Clarke-type oxygen electrode. However, in all cases, the rapid change in cytotoxicity and chemopotentiation were observed at oxygen concentrations be-
low this limit of detectability (Charts 18 and 3B). Therefore, $K_m(O_2)$ values less than 1000 ppm seem reasonable estimates for our experimental conditions. Nevertheless, our $K_m(O_2)$ estimates should be considered accurate only to within a factor of 2 or 3.

The purpose of these studies was to compare the oxygen sensitivity of MISO toxicity and chemopotentiation. Such a comparison does not require quantitative assessment of low $C_w$ values and can be made qualitatively providing equilibrium conditions in the cytotoxicity and chemosensitization experiments are reproducible. Since in all cases the treatment protocol was held constant and since CCNU and/or MISO concentrations equal to or exceeding those used for treatment did not significantly modify the $O_2$ consumption rate of EMT-6/Ro cells (Table 1), this condition was satisfied. Therefore, the conclusion that the oxygen sensitivity for MISO cytotoxicity and chemopotentiation are similar is valid, regardless of the uncertainties associated with the assignment $C_w$ values below ~1000 ppm.

The chemosensitization data shown in Chart 2 also confirm the hypothesis that moderate enhancements are produced in cells treated with CCNU and MISO at intermediate oxygen concentrations. To further characterize this potentiation, a series of experiments were performed evaluating the effectiveness of a fixed dose of CCNU either alone or in combination with 1.0 mM MISO at various oxygen concentrations. The results of these experiments (Chart 3A) reveal 2 important properties of MISO chemosensitization in this cell line: (a) the shape of the response curve as a function of oxygen concentration is similar to that for MISO cytotoxicity (Charts 1B and 3B); and (b) enhancement of CCNU toxicity was consistently produced at measured $C_w$ values as high as 2 to 3%. Interestingly, this is very near the reported venous oxygen-tension (P0v, 40 mm Hg; ~5%) for many normal tissues in vivo (10). In fact, it can be calculated from this example that the magnitude of the enhancement observed when MISO and CCNU are combined at $C_w$ equal to 2 to 3% could correspond to a dose modification factor of 1.1 to 1.2. Larger enhancements could be expected if normal cells exist at $O_2$ tensions below venous levels, as has been suggested by microelectrode measurements (3, 10) and radiobiological experiments (8, 9, 12). While the actual value obtained in normal tissues may ultimately depend on tissue-dependent $O_2$ tensions and nitroreductase activities, the estimate made from the current data is at least compatible with the dose modifications already reported for many dose-limiting tissues. In addition, some of the enhanced damage observed in normal tissue may also be attributable to altered pharmacokinetics associated with the combination of high doses of MISO and chemotherapeutic agents (7, 11, 17).

It can be concluded therefore that (a) chemopotentiation by MISO in vitro is oxygen sensitive, occurring to a variable extent in cells maintained between fully aerobic and anoxic oxygen concentrations, and (b) the $K_m(O_2)$ values for MISO cytotoxicity and chemopotentiation are very similar, strongly suggesting that MISO reduction is integral to both activities. Extension of these observations and conclusions to the more complex in vivo situation would go a long way toward explaining (a) the larger-than-anticipated magnitude of chemosensitization based on hypoxic fraction considerations, (b) a larger enhancement in tumors than in normal tissues, and (c) some enhancement of normal tissue toxicity.

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