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ENHANCEMENT OF MELPHALAN (L-PAM) TOXICITY BY REDUCTIVE METABOLITES OF 1-METHYL-2-NITROIMIDAZOLE, A MODEL NITROIMIDAZOLE CHEMOSENSITIZING AGENT

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Abstract—Chemosensitization of bifunctional alkylators by misonidazole (MISO) and related nitroimidazoles *in vitro* has been shown to require hypoxic exposures. Presumably, reductive metabolism of the nitroimidazole under hypoxic conditions results in generation of a chemosensitizing intermediate(s) in a manner analogous to that described for the hypoxic toxicity of these compounds. In an attempt to identify these intermediates, we examined the ability of reductive metabolites of a model 2-nitroimidazole compound, 1-methyl-2-nitroimidazole (INO_2), to enhance the toxicity of melphalan (L-PAM) in HT-29 human colon cancer cells. INO_2 was a modest chemosensitizing agent, enhancing L-PAM only under hypoxic conditions. The 2-electron reduction product, 1-methyl-2-nitrosoimidazole (INO), was a potent chemosensitizer, enhancing L-PAM toxicity at micromolar concentrations under either aerobic or hypoxic conditions. In contrast, the 4- and 6-electron reduction products, 1-methyl-2-[hydroxylamino]imidazole and 1-methyl-2-aminoimidazole, respectively, failed to modify cell kill by L-PAM even at millimolar concentrations. These results suggest that nitrosoimidazoles may be the active chemosensitizing species generated upon the reductive metabolism of nitroimidazoles.

Misonidazole (MISO) and related 2-nitroimidazoles can enhance the cytotoxicity of several bifunctional alkylating-agents [most notably melphalan (L-PAM), cyclophosphamide and several nitrosoureas] *in vitro* and *in vivo*, an effect referred to as chemosensitization. The administration of 2-nitroimidazoles in combination with antineoplastic agents for the treatment of experimental rodent tumors results in preferential enhancement of anti-tumor effectiveness of the drug treatment. Dose enhancements for tumor response are typically reported to be in the range of 1.6 to 2.2 [1, 2], whereas enhancements of normal tissue toxicity rarely exceed 1.2 to 1.4 and are virtually always lower than those observed in tumors when the effects on both tumor and normal tissues are evaluated simultaneously. Because of the potential therapeutic advantage suggested by these compelling pre-clinical studies, the chemosensitization strategy has evolved to the stage of clinical evaluation. The potential of this treatment strategy was indeed demonstrated in a recently completed prospective, randomized Phase III clinical trial. Coleman *et al.* [3] observed a statistically significant improvement in response rate for lung cancer (non-small cell) patients treated with a melphalan–MISO combination when compared to the response rate of patients randomized to receive melphalan treatment alone. These results have prompted further clinical interest in nitroimidazoles as potential chemosensitizing agents.

In spite of impressive anti-tumor effects, the mechanism of chemosensitization by 2-nitroimidazoles has not been defined. However, it is well established that the mechanism is distinct from that involved in radiation sensitization by these agents. Furthermore, oxygen concentration has been shown to play a critical role in drug enhancement, chemosensitization being greatest under hypoxic conditions. The oxygen sensitivity of chemosensitization by MISO is very similar to that of MISO cytotoxicity [4, 5], a property which requires reduction of the parent compound to reactive intermediates under hypoxic conditions [6, 7] according to the reaction scheme depicted in Fig. 1 for a model 2-nitroimidazole, 1-methyl-2-nitroimidazole (INO_2), related in structure to MISO as shown.

In the presence of oxygen, the nitro radical anion (INO_2; 1-electron reduction product) is back-oxidized to the parent nitro compound resulting in a futile cycle without generation of active intermediates [8]. This back-oxidation reaction accounts for the lack of cell killing under aerobic conditions. However, as oxygen concentration is reduced, the reaction proceeds to the right with the sequential generation of the 2- (nitroso; INO), 4- (hydroxylamine; INHOH), and 6- (amine; INH_2) electron reduction products, one or more of which is hypothesized to be responsible for the cytotoxic [7] properties of the parent nitroimidazole.

Considering the similarities in oxygen sensitivities for hypoxic cytotoxicity and chemosensitization of

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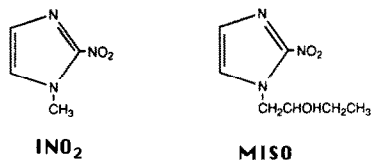

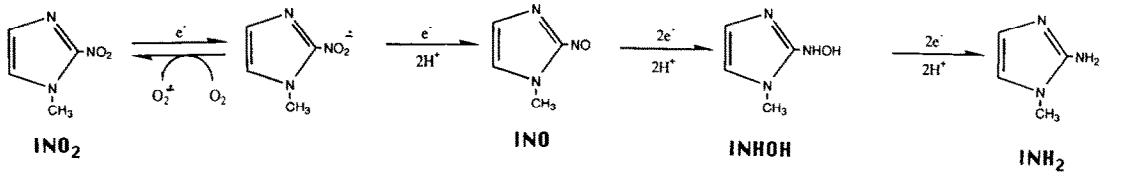

Fig. 1. Structure and reduction scheme of the model 2-nitroimidazole, 1-methyl-2-nitroimidazole (INO_2). The structure of misonidazole (MISO) is included for comparison.

MISO, we hypothesized that the generation of chemosensitizing equivalents likewise requires enzyme-catalyzed nitroreduction of the parent nitroimidazole at reduced oxygen concentrations, and that one or more of the reductive metabolites is responsible for the expression of chemosensitization [4]. In an attempt to identify reductive products which may mediate the chemosensitizing properties of 2-nitroimidazoles, we have examined the ability of the reductive metabolites of a model 2-nitroimidazole (INO_2) to enhance the toxicity of L-PAM in HT-29 colon cancer cells.

METHODS

Materials. The metabolites of INO_2 were synthesized according to techniques published previously [9] employing 2-hydroxylamino-1-methylimidazole hydrochloride generated by electrochemical reduction of 1-methyl-2-nitroimidazole. For all experiments concentrated stock solutions of INO and INHOH were prepared in ice-cold distilled water. INO_2 and INH_2 were dissolved directly in culture medium.

L-Buthionine sulfoximine (BSO) and L-PAM were purchased from the Sigma Chemical Co. BSO was dissolved directly in culture medium; L-PAM was dissolved as described below.

Cell line and drug treatments. The human colon cancer cell line, HT-29, was used for these studies. Cells were maintained in exponential growth in α-Minimum Essential Medium (α-MEM) supplemented with 10% fetal bovine serum and gentamycin (50 μg/mL). Cultures were incubated at 37° in an atmosphere of 97% air/3% CO_2 and transferred at weekly intervals. All cultures were determined to be Mycoplasma free.

The technique used to treat cells under aerobic or hypoxic conditions has been described in detail previously [10]. All cell treatments were performed in Dulbecco's modified Eagle's medium (DMEM) lacking ascorbate to avoid rapid reduction of INO and INHOH [11]. For aerobic exposures, exponentially growing HT-29 cells were suspended in 10 mL of DMEM at a concentration of 1–2 × 10^5 cells/mL and transferred to glass treatment vials. For hypoxic exposures (INO_2 and INO only) the cells were injected into 10 mL of medium in treatment vials only after the medium had been gassed for 3 hr with a 97% N_2/3% CO_2 gas mixture. Prior to being injected into the treatment vials at the conclusion of the 3-hr gassing phase, the cells were incubated for 10 min at a concentration of 1–2 × 10^7/mL in a Hamilton gas-tight syringe at 37° to deplete oxygen by consumption.

To deplete cellular levels of GSH, HT-29 cells were incubated in the presence of 1.0 μM BSO for 20–24 hr prior to L-PAM treatment. This pretreatment reduced GSH levels to 75 ± 4% of control HT-29 cells but did not reduce plating efficiency relative to untreated controls.

L-PAM was dissolved in acid ethanol, further diluted in absolute ethanol, and finally diluted 100-fold by injection into each treatment vial at the initiation of drug exposure. In all cases, cells were exposed to L-PAM and INO_2 or reductive metabolites concurrently. For INO and INHOH exposures, various concentrations of the compounds in distilled water were diluted 100-fold by injection into appropriate treatment vials. INO_2 and INHOH were dissolved directly in culture medium. At the conclusion of the exposure interval the cell suspensions were centrifuged, washed in drug-free medium, and prepared for survival assay.

Cell survival assay. Survival was determined using a standard plating efficiency assay. Colony formation in treated and control groups was enumerated 12–14 days after plating, and survival was calculated as the ratio of plating efficiency (PE) of treated groups to that for controls (PE = 50–70%).

RESULTS

The chemosensitizing potency of the model 2-nitroimidazole, INO_2, was determined by exposing HT-29 cells to various concentrations of the sensitizer

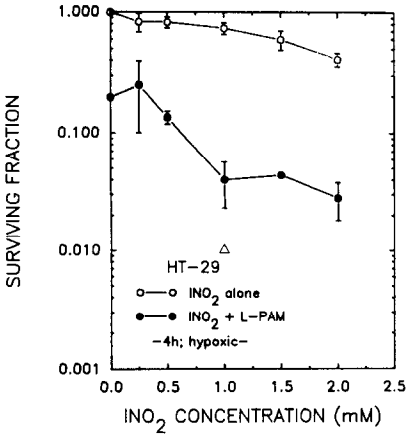

Fig. 2. Effect of INO_2 on L-PAM toxicity. HT-29 cells were incubated with various concentrations of INO_2 alone (○) or in combination with L-PAM (7.5 μM) for 4 hr under hypoxic conditions (●). For reference, the effect of 1.0 mM MISO on L-PAM toxicity is also shown (△). Survival data for the combination were corrected for INO_2 toxicity. Values are means ± SD of 3–4 determinations, with the exception of 1.5 mM which is the average of two determinations.

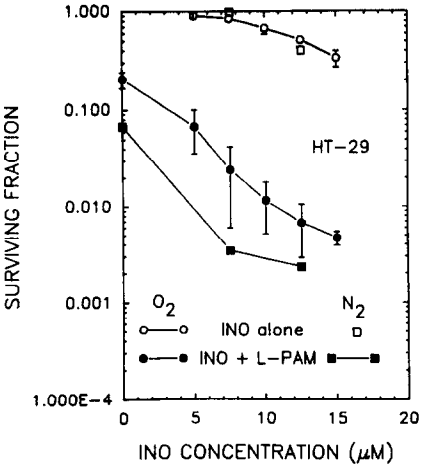

Fig. 3. Enhancement of L-PAM toxicity by INO under aerobic or hypoxic conditions. HT-29 cells were incubated with various concentrations of INO alone or in combination with L-PAM (15 μM) for 1 hr under aerobic and hypoxic conditions. Survival data for the combination were corrected for INO toxicity. Values are means ± SD of 4–6 determinations. (E-4 = 0.0001.)

and a fixed concentration of L-PAM (7 μM) for 4 hr under hypoxic conditions. Previous experiments with MISO [4, 10] have established that these exposure conditions are adequate for the expression of chemosensitization. As shown in Fig. 2, hypoxic exposure to minimally cytotoxic concentrations of INO_2 enhanced L-PAM toxicity in a concentration-dependent fashion, although less effectively than MISO (Fig. 2; open triangle). No sensitization was observed under aerobic exposure conditions (data not shown). By virtue of its ability to enhance L-PAM toxicity in these experiments, INO_2 was considered to be an effective model chemosensitizing nitroimidazole which could be utilized in experiments designed to evaluate the chemosensitizing potency of individual reductive metabolites.

As it has been suggested [8] that hypoxic exposure conditions are primarily required in order to avoid reoxygenation of the nitro radical anion to the inactive parent, thereby allowing for the generation of active chemosensitizing intermediates (Fig. 1), the sensitizing effectiveness of the three reductive metabolites of INO_2, namely INO, INHOH and INH_2, was examined under aerobic conditions. Because of the very short half-lives ($T_{1/2}$) of INO and INHOH (a few minutes [9] and less than 1 min [12] respectively), the exposure interval was reduced to 1 hr in experiments using these compounds and the concentration of L-PAM was correspondingly increased to 15 μM in order to achieve iso-effective treatments.

INO proved to be a very potent chemosensitizer which dramatically increased L-PAM cell kill at extremely low doses (Fig. 3). The magnitude of enhancement achieved in cells exposed to INO and L-PAM under hypoxic conditions was comparable to that observed in aerobic experiments. The concentration–response curve for the hypoxic

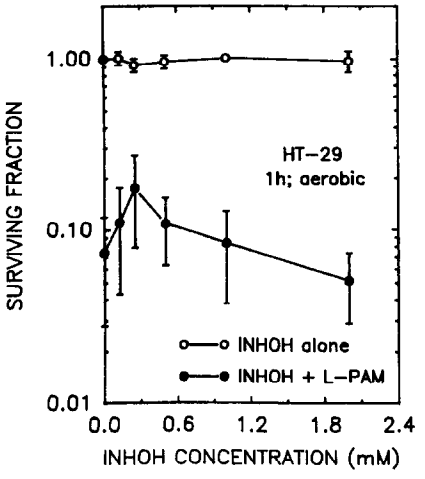

Fig. 4. Effect of INHOH on L-PAM toxicity. HT-29 cells were treated with INHOH as described in the legend of Fig. 2. Values are means ± SD of 4 determinations.

treatment paralleled the aerobic concentration response but was shifted to lower survival levels as a result of increased L-PAM toxicity produced under hypoxic conditions. This enhanced activity of L-PAM under hypoxic conditions is a consistent finding in our experience. Toxicity resulting from exposure to INO alone was comparable to that previously reported and was not dependent upon oxygen concentration. In contrast, neither INHOH (Fig. 4) nor INH_2 (Fig. 5) was cytotoxic, and both failed to augment L-PAM toxicity, even at concentrations exceeding those used in the chemosensitizing experiments with the parent compound, INO_2. It should be noted that experiments with INHOH were conducted several months after those with the other

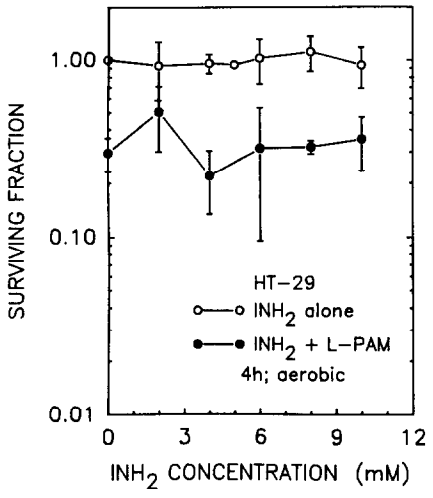

Fig. 5. Effect of INH_2 on L-PAM toxicity. HT-29 cells were treated with INH_2 as described in the legend of Fig. 2. Values are averages ± range of 2 determinations.

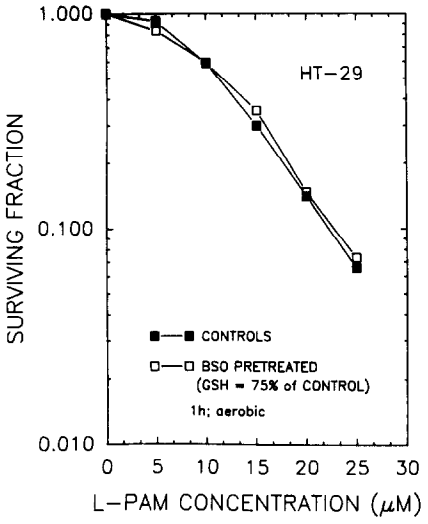

Fig. 6. Survival of control and BSO-treated HT-29 cells following L-PAM exposure. The GSH content of HT-29 cells was reduced to 75% of control levels by incubation with 1.0 μM BSO for 20 hr prior to treatment with various concentrations of L-PAM for 1 hr (control GSH levels = 12.5 ± 2.5 fmol/cell). BSO pretreatment did not alter the response of the cells to L-PAM. Results are from a single determination.

compounds. At that time (and consistently thereafter) the HT-29 cells were slightly more sensitive to L-PAM and, consequently, lower survival levels are evident in Fig. 4.

Exposure to INO has been shown [9, 11] to result in dose-dependent decreases in intracellular glutathione (GSH) and, since GSH depletion can sensitize cells to L-PAM exposure, we attempted to determine whether the level of GSH depletion accompanying exposure to the concentrations of INO used in the present experiments could account for the increased toxicity observed in the chemosensitizing experiments. The GSH content of HT-29 cells was reduced to 75% of controls by 20 hr of incubation with 1 μM buthionine sulfoximine (BSO), a specific inhibitor of GSH synthesis, prior to exposure to L-PAM. Although comparable to the level of depletion induced by exposure to 20 μM INO, an effective chemosensitizing concentration, the sensitivity of the BSO pretreated HT-29 cells to L-PAM was not significantly different from that of controls (Fig. 6).

DISCUSSION

Previous studies conducted in our laboratory demonstrated that the magnitude of chemosensitization by MISO was inversely proportional to oxygen concentration [4]. Furthermore, these studies revealed that the k_mO_2 (the oxygen concentration at which an effect is half-maximal) value for chemosensitization was quite similar to that reported for hypoxic cell killing by MISO [13], suggesting possible similarities in mechanism for the two activities. Similar findings were also reported by Roizin-Towle *et al.* [5]. By analogy to the generation of toxic metabolites of MISO [7] under hypoxic conditions, we hypothesized that generation of chemosensitizing equivalents likewise requires nitroreduction of the parent nitroimidazole according to the reduction scheme shown in Fig. 1.

According to this hypothesis, hypoxic conditions are primarily required to avoid the back-oxidation of the nitro radical anion (1-electron reduction product) to the inactive parent nitro compound, thus generating the active chemosensitizing intermediate(s). Consistent with this prediction and typical for chemosensitization experiments, the parent nitroimidazole, INO_2, only enhanced L-PAM effectiveness under hypoxic conditions in these experiments. In contrast, INO produced equal enhancements of L-PAM toxicity under aerobic and hypoxic conditions at concentrations 2 orders of magnitude lower than those required for the parent nitroimidazole. Combined with the data for INO_2, this oxygen-indifferent sensitization provides direct evidence that reduction of the parent nitro compound is required for activity and further suggests that the nitroso intermediate or one of its more reduced forms is indeed capable of mediating an interaction with bifunctional alkylating agents, like L-PAM, to enhance cell kill.

Unambiguous assignment of chemosensitizing activity to a specific reduction product is difficult at this time because of the unknown fate of the various compounds once administered to cells in culture. For example, INO may react directly with intracellular targets or be further reduced to INHOH and ultimately INH_2. Another confounding factor, the back-oxidation of INHOH to INO under aerobic conditions, has also been reported [14]. Assessment of the role of INHOH is further complicated by its extremely short $T_{1/2}$ in cell culture conditions [12]. Nevertheless, the evidence presented in this report, namely the extremely low concentrations of INO required to observe enhanced cell kill under aerobic or hypoxic conditions and the lack of sensitizing activity observed with relatively high concentrations

of the exogenously added downstream reduction products, INHOH and INH_2, implicates INO as the active metabolite. Since INO was capable of enhancing L-PAM toxicity, we compared the frequencies of DNA–DNA interstrand cross-links in HT-29 cells treated with L-PAM alone or with the L-PAM/INO combination by alkaline elution. While preliminary, these studies have revealed an increase in cross-link formation in the cells treated with the L-PAM and INO combination (Mulcahy RT, unpublished observations). As enhanced cross-link formation is considered to be a hallmark of chemosensitization by MISO, its detection in INO/L-PAM-treated cells lends further support to an active role of INO in chemosensitization.

The suggestion that the nitroso compound is involved in chemosensitization is also consistent with accumulating data implicating these intermediates in several other biological effects of nitroimidazoles. In comparing the bactericidal activity of 4- and 5-nitroimidazoles with their corresponding nitroso-imidazoles, Ehlhardt *et al.* [15] recently found the latter compounds to be much more active than the parent nitro compounds under both aerobic and anaerobic conditions, although they could not conclusively demonstrate that they themselves were the biologically active species. Similarly, Noss *et al.* [9] and our own laboratory [11] recently published data compatible with the hypothesis that the nitroso-intermediate may also be responsible for the toxicity and GSH depletion resulting from hypoxic exposure of mammalian cells to nitroimidazoles. Incubation with INO was extremely toxic to aerobic and hypoxic cultures and resulted in concentration-dependent depletion of intracellular GSH. INO produced significant DNA damage in treated cells (Mulcahy RT, unpublished results), produced single-strand breaks in plasmid DNA in the presence of GSH* (Mulcahy RT, unpublished results) and was capable of forming a stable conjugate with GSH in chemical systems (McClelland RA, unpublished results). Ehlhardt and Goldman [16] recently reported that 1-methyl-4-phenyl-5-nitrosoimidazole binds to DNA, and that the binding process is enhanced by 2–3 orders of magnitude in the presence of physiological concentrations of GSH.

In an excellent series of studies, Varghese and Whitmore [7, 17–20] provided chemical and biological evidence to suggest that the hydroxylamine derivative of MISO is the intermediate responsible for toxicity, macromolecular binding, and thiol depletion in cultured cells incubated with MISO under hypoxic conditions. This compound interacted with glutathione (GSH) to form a stable product and formed specific covalent adducts in cellular DNA. However, the toxicity of the hydroxylamine was not determined directly. Other attempts to demonstrate toxicity and/or thiol depletion in mammalian cells following exposure to hydroxylamine derivatives in culture have not met with success [9]. Our failure to induce either cell killing or chemosensitization with INHOH is consistent with this latter experience. Whether this apparent inactivity is real or is an artifact associated with the physical properties (i.e. short $T_{1/2}$) of the compound when added exogenously to cells in culture is currently being investigated using radiolabeled compounds. In contrast, aminoimidazole metabolites have been shown almost universally to be inactive as GSH depleting agents ([7]; Mulcahy RT, unpublished results) and cytotoxins [9], so their lack of chemosensitizing activity in these experiments was not surprising.

The depletion of intracellular GSH resulting from treatment with INO could conceivably account for the enhancement of L-PAM toxicity observed in these experiments. Cells can be sensitized to L-PAM exposures by reducing intracellular GSH concentrations to low levels [21–23]. However, preincubation with a specific inhibitor of GSH synthesis, BSO, while reducing GSH concentrations to levels comparable to those produced by the highest doses of INO (i.e. 20 μM) used in our chemosensitization experiments, failed to alter the response of HT-29 cells to subsequent L-PAM exposure. Although GSH depletion by these two different classes of compounds should not necessarily be considered entirely analogous, our data suggest that GSH depletions of the magnitude produced by chemosensitizing doses of INO (i.e. $< 25\%$) are not sufficient to account for the enhancements observed.

In summary, our results strongly suggest that the nitroso-reduction products are active intermediates in the expression of chemosensitization by 2-nitroimidazoles. However, in spite of strong data showing that INO can induce enhanced toxicity, our current data do not directly rule out the possibility that other metabolites, combination of metabolites, or reaction products are also involved. The most conservative interpretation of the data suggests that the nitroso-intermediate is the active species or is converted to the ultimate intermediate more efficiently than any of the other compounds tested. Additional experiments, already in progress, are required to further define the pathways involved in the expression of chemosensitization.

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