MOLECULAR PHARMACOLOGY, 22:175–181

The Effect of Misonidazole on the Cytotoxicity and DNA Cross-Linking Activity of an Activated Sulfidocyclophosphamide in Hypoxic Mouse Leukemia Cells

LORI M. RAMONAS, LEONARD C. ERICKSON,[1] AND MICHAEL E. MCMANUS[2]

Laboratories of Chemical Pharmacology and Molecular Pharmacology, and Laboratory of Carcinogen Metabolism, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20205

Received November 9, 1981; Accepted March 16, 1982

SUMMARY

The effect of misonidazole on the cytotoxicity of 4-*S*-(propionic acid)-sulfidocyclophosphamide (C-2) was assessed by measuring the colony-forming ability of mouse L1210 leukemia cells. C-2 under physiological conditions spontaneously hydrolyzes to 4-hydroxycyclophosphamide. Misonidazole alone at a concentration of 2.5 mM was only slightly toxic to hypoxic L1210 cells and allowed a greater than 90% survival following a 2-hr exposure. Combined treatment of C-2 and 2.5 mM misonidazole resulted in a cell kill that was greater than the additive toxicities of C-2 and misonidazole. The synergistic toxicity of the C-2 and misonidazole (2.5 mM) combination increased with increasing C-2 concentration, and at 0.01 survival the dose-modification ratio of C-2 alone versus the combination was approximately 1.5. Similarly, when the concentration of C-2 was held constant (10 μM) and the concentration of misonidazole varied from 2.5 to 25 mM, a cell kill greater than the additive toxicities of misonidazole and C-2 alone was observed. The kinetic patterns of formation and removal of DNA interstrand cross-links following a 2-hr treatment of 10 μM C-2 or 10 μM C-2 plus 2.5 mM misonidazole were similar. However, with the exception of the 0-hr time point, cells treated with the C-2 plus misonidazole combination showed consistently greater cross-linking of DNA than did cells treated with C-2 alone. The interstrand cross-link ratio closely correlated with the cytotoxic dose-modification ratio of the combination compared with C-2 alone.

INTRODUCTION

MISO[3] (1-[2-nitro-1-imidazolyl]-3-methoxy-2-propanol) has been used in clinical trials as a radiation sensitizer for hypoxic tumor cells for the past several years (1–3). However, neurotoxicity has been a major limiting factor in the use of this drug. The mechanism for sensitization is believed to involve an electron-transfer mechanism resulting in the interaction of MISO with a charged intermedite produced by direct energy absorption in a target molecule under anaerobic conditions (4). In addition to radiosensitization, MISO is also preferentially toxic toward hypoxic mammalian cells (5–7). In this case a different mechanism is proposed, whereby the nitro functional group of MISO is enzymatically reduced, resulting in the production of toxic metabolites (8). More recently, MISO has been shown to potentiate the cytotoxic action of certain chemotherapeutic agents in both *in vivo* and *in vitro* systems (9–13). Although the potentiation effects of MISO vary considerably depending on the tumor cell line or animal tumor system chosen for investigation, as well as the cytotoxic drug chosen for combined treatment with MISO, several different reports independently support the existence of this phenomenon. At present the mechanism of potentiation is not understood.

Because of the possible clinical significance and complications of drug potentiation by MISO, we decided to examine this effect at the cellular level in an attempt to gain some insight into how MISO influences the mode of action of certain antitumor drugs. Since cyclophosphamide remains one of the most widely used alkylating aents in clinical chemotherapy (14), C-2, an analogue of this compound, was chosen for combined treatment with MISO, under anaerobic conditions of mouse L1210 leukemia cells *in vitro*. The availability of 4-sulfidocyclophosphamides such as C-2, stable crystalline compounds that spontaneously hydrolyze to 4-hydroxycyclophosphamide under physiological conditions (15–17), makes *in vitro* investigations possible by circumventing the need to activate cyclophosphamide enzymatically to 4-hydroxycyclophosphamide. Insofar as 4-hydroxycyclophosphamide is believed to be the active transport form of cyclophosphamide responsible for antitumor activity *in*

[1] Laboratory of Molecular Pharmacology.

[2] Laboratory of Carcinogen Metabolism.

[3] The abbreviations used are: MISO, misonidazole; C-2, 4-*S*-(propionic acid)-sulfidocyclophosphamide.

0026-895X/82/040175-07$02.00/0

vivo (18), the sulfido compounds allow us to model the process of interaction of 4-hydroxycyclophosphamide and the tumor cell *in vitro*.

In this paper we present data that demonstrate the synergistic toxicity of misonidazole and C-2 on hypoxic mouse L1210 leukemia cells *in vitro*. Furthermore, our previous work on DNA damage produced by 4-sulfidocyclophosphamides on aerobic L1210 cells (19, 20) led us to examine the anaerobic effect of MISO on the DNA cross-linking capabilities of C-2. The experiments show that the combination of MISO and C-2 consistently increased levels of DNA damage in treated cells, and these increases correlated well with the increased cytotoxicity of the combination treatment.

MATERIALS AND METHODS

Cell culture. Mouse L1210 leukemia cells were grown in spinner culture in Roswell Park Memorial Institute 1630 medium supplemented with 15% heat-inactivated (56° for 30 min) fetal calf serum, 1 mM L-glutamide, penicillin, and streptomycin. Cultures used in drug-treatment experiments were maintained in an exponential growth phase at densities between 0.3 to 1.8 × 10^6 cells/ml. The doubling time of the cells under these conditions was approximately 12 hr. One hour prior to drug treatment, cells were centrifuged (900 rpm for 5 min) and resuspended in fresh medium at a density of 1 × 10^6 cells/ml. The colony-forming ability of drug-treated cells in soft agar was determined according to the method of Chu and Fisher (21). The colony-forming ability of control cells was >75% in this system.

The DNA from L1210 cells utilized for alkaline elution experiments was labeled by growing the cells for at least 20 hr at 37° in either [^{14}C]thymidine, 0.02 μCi/ml (>56 mCi/mmole; New England Nuclear Corporation, Boston, Mass.), or [^{3}H]thymidine, 0.05 μCi/ml (20 Ci/mmole; New England Nuclear Corporation), and 10^{-6} M unlabeled thymidine.

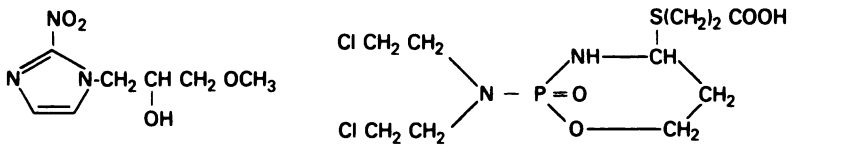
Anaerobic drug treatment. Misonidazole (Fig. 1) was supplied by the Developmental Therapeutics Program of the Division of Cancer Treatment, National Cancer Institute. The drug was dissolved in sterile phosphate-buffered saline (0.15 M NaCl/0.014 M KH_2PO_4/0.086 M K_2HPO_4), pH 7.4, immediately prior to the treatment of cell cultures.

The C-2 analogue (Fig. 1) was the generous gift of Drs. Helmut Ringsdorf and Wolfgang Klesse of the Institute of Organic Chemistry, Mainz University (Mainz, Federal Republic of Germany). In aqueous media, at 37°, this compound spontaneously hydrolyzes to 4-hydroxycyclophosphamide with a half-life for the hydrolysis of 8 min (22). Therefore, in order to avoid significant hydrolysis of C-2 to 4-hydroxycyclophosphamide during drug preparation, C-2 was dissolved in ice-cold sterile 0.1 M sodium phosphate buffer (pH 7.0) immediately prior to cell exposure.

All drug treatments, whether C-2 or MISO, or C-2 and MISO in combination, were carried out under anaerobic conditions for 2 hr at 37°. After treatment, drugs were removed by washing the cells three times by centrifugation, and the cells were resuspended in fresh medium. For the 0- hr time point, cells were washed with ice-cold medium. Following the wash, cells were either assayed for DNA cross-linking by alkaline elution, or for colony-forming ability in soft agar.

Hypoxia in L1210 cells was induced in the following manner. A small volume of L1210 cells (5–10 ml, 1 × 10^6 cells/ml) was placed in a sterile 25-ml Erlenmeyer flask equipped with a stir bar. At this point MISO, C-2, or the combination of MISO and C-2 was added to the cell suspension to achieve the desired final drug concentration. The flasks then were fitted with sterile rubber septum caps and placed over a magnetic stirrer in a 37° warm room. Since hyperthermia has been shown to increase the hypoxic toxocity of MISO (23), care was taken to maintain the temperature at 37°. Two sterile disposable needles, one 19-gauge, 1½ inches long and the other 20-gauge, 1 inch long, were inserted into the rubber septum of each flask. The larger-bore, longer needles were connected in parallel, via Tygon tubing, to a gas cylinder containing pure N_2, and the smaller-bore, shorter needles served as N_2 outlets. In this manner a small positive N_2 back pressure was maintained in each flask throughout the experiment. The L1210 cells were stirred and flushed with N_2 (400 ml/min) for the entire 2-hr drug treatment to ensure removal of air from the space above the cells and to provide a continuously exposed cell surface interface with the N_2 atmosphere in each flask. To prevent significant evaporation of the 1630 medium, the N_2 was humidified before flushing the treatment flasks. As a measure of the extent of hypoxia we determined the radiation dose required to reduce the surviving fraction of L1210 cells by two orders of magnitude. Under the above conditions the oxygen enhancement ratio of cells irradiated in air compared with N_2 pretreatment for 10 or 60 min was 2.6. At the conclusion of the drug treatment the cells were washed free of drug, as earlier described, under aerobic conditions.

Alkaline elution. The alkaline elution procedure that was used in these experiments and the factors governing DNA alkaline elution kinetics in general have been described in detail (24). L1210 cells used in this study were analyzed by the proteinase modification which minimizes



MISONIDAZOLE (MISO)

4-S- (PROPIONIC ACID)- SULFIDOCYCLOPHOSPHAMIDE (C-2)

FIG. 1. *Chemical structures of MISO and C-2*

the effect of DNA-protein cross-linking on the elution of DNA (25). In the modified assay, 5×10^5 drug-treated ^{14}C-labeled cells were mixed with 5×10^5 ^3H-labeled control cells, diluted with ice-cold 1630 medium, and irradiated at 0° with 300 rads of X-ray. This X-ray dose introduced a known frequency of DNA single-strand breaks. The cells were then diluted with 20 ml of ice-cold phosphate-buffered saline (0.15 M NaCl/0.014 M KH_2PO_4/0.086 M K_2HPO_4), pH 7.4, and layered onto a polyvinyl chloride filter (pore size 2 μm; diameter 25 mm; Type BSWP) (Millipore Corporation, Bedford, Mass.), using mild suction. The cells were immediately lysed with 5 ml of a solution containing 2% sodium lauryl sulfate, 0.1 M glycine, and 0.02 M EDTA (pH 10.0). The lysis solution was allowed to flow through by gravity, and, to increase deproteinization, 2 ml of lysis solution with proteinase K (0.5 mg/ml) were layered on the filter with the flow stopped. Thereafter, 40 ml of tetrapropyl ammonium hydroxide/0.02 M EDTA (pH 12.1), containing 0.1% sodium lauryl sulfate, were layered over the 2 ml of proteinase K solution. Both solutions were pumped through the filter, in the dark, at 0.035 ml/min. The effective proteinase digestion time was estimated to be approximately 1 hr. Eluted fractions were collected at 3-hr intervals and fractions and filters were processed as previously described (24). Radioactivity in each fraction was measured using a Packard 2450B liquid scintillation counter (^{14}C efficiency of 41% and ^3H efficiency of 16%).

Apparent DNA interstrand cross-link frequencies (in rad equivalents) induced by drug treatment were computed by using the formula (26)

$$(\sqrt{1 - r_0}/(1 - r) - 1) \times 300 \text{ rads}$$

where r is the fraction of [^{14}C]DNA retained on the filter relative to a constant fraction of [^3H]DNA retained for drug-treated cells and r_0 is this fraction for control cells irradiated with 300 rads.

RESULTS

Hypoxic cytotoxicity. The *in vitro* cytotoxicity of C-2, for various concentrations of the drug following a 2-hr exposure to hypoxic L1210 cells, is shown in Fig. 2. When a fixed dose of MISO (2.5 mM) was added to each C-2 concentration, a survival curve for C-2 and 2.5 mM MISO was generated. MISO at 2.5 mM allowed for greater than 90% survival of hypoxic L1210 cells (Fig. 3). This effect is shown by the *dashed line* in Fig. 2. However, C-2 and 2.5 mM MISO in combination resulted in a greater cell kill than would have been expected had the toxicities been additive. This synergistic toxicity of C-2 and 2.5 mM MISO conbination increased with the concentration of C-2, and ultimately reduced the survival of hypoxic L1210 cells by a dose-modification ratio of 1.5 at 0.01 survival. This ratio was found by comparing the dose of C-2 required to achieve the same cell kill as the C-2 and MISO combination.

Similarly, the complementary *in vitro* cytotoxicity of MISO, for concentrations ranging from 2.5 mM to 25 mM following a 2-hr exposure to hypoxic L1210 cells, is shown in Fig. 3. The survival curve for MISO shows a linear cell kill for doses of MISO up to 20 mM. The toxicity of MISO and C-2 in combination was measured by adding a fixed

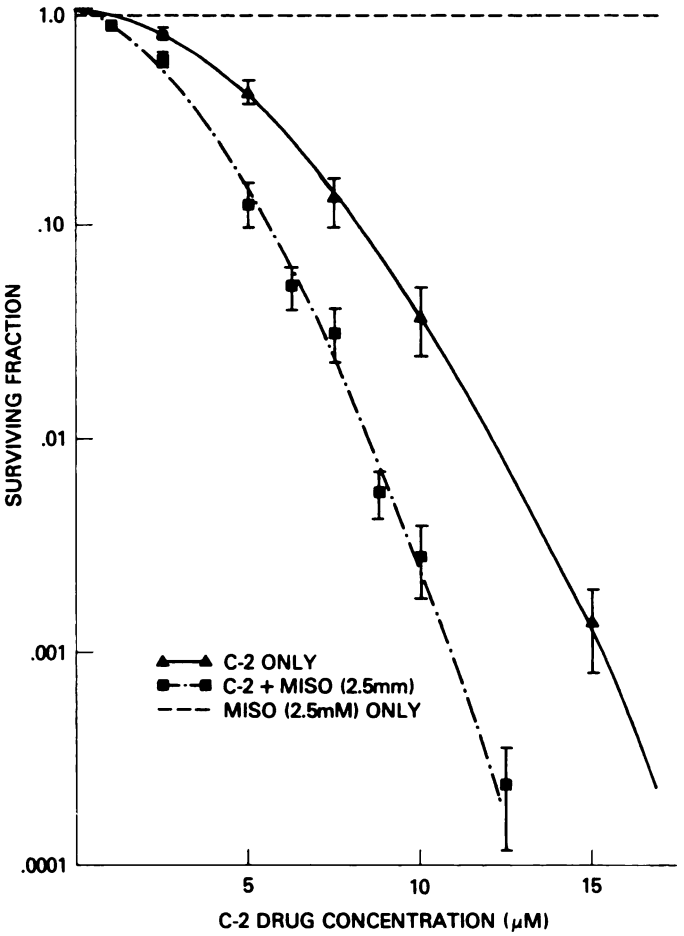


FIG. 2. *Survival of L1210 cells following 2-hr treatment with C-2 only, MISO only, and MISO + C-2*

dose of C-2 (10 μM) to each MISO concentration. Although C-2 alone at 10 μM resulted in an approximate 2-log cell kill, we chose to work with this particular dose so that enough DNA interstrand cross-links would be formed by the alkylating agent to allow for accurate quantitation of cross-link frequency by the alkaline elution technique. Nevertheless, despite the substantial toxicity attributable to 10 μM C-2 alone, Fig. 3 illustrates that the combined treatment of MISO and 10 μM C-2 resulted in a cell kill that was again greater than the additive toxicities of MISO and C-2.

DNA interstrand cross-linking in hypoxic cells. Alkaline elution profiles of hypoxic L1210 cells exposed for 2 hr to 10 μM C-2, 2.5 mM MISO, or the combination of 10 μM C-2 and 2.5 mM MISO, and incubated at 37° with drug-free medium for 9, 12, or 15 hr under aerobic conditions are shown in Fig. 4. To test for DNA interstrand cross-linking, ice-cold ^{14}C-labeled cells received 300 rads of X-radiation immediately prior to elution to introduce a controlled number of random single-strand breaks. Other cells, either untreated controls or 2.5 mM MISO-treated, received no X-radiation and provide a measure of the production of single-strand breaks by MISO. As a result of interstrand cross-linking produced by C-2 drug treatment, there was increased retention of labeled DNA on the filter of C-2 treated cells resulting in an apparent reduction in the effect of the x-ray. ^3H-Labeled untreated

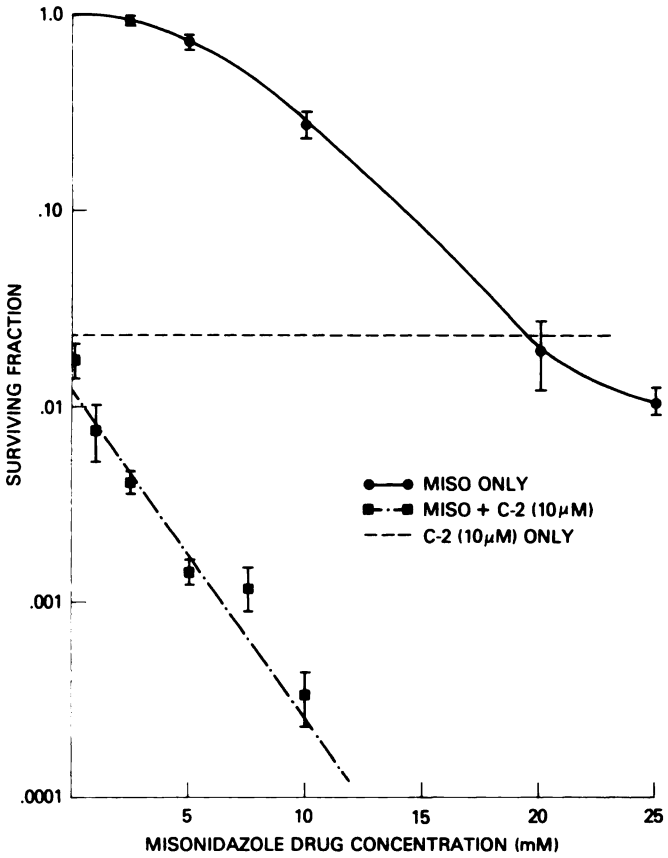


FIG. 3. *Survival of L1210 cells following 2-hr treatment with MISO only, C-2 only, and MISO + C2*

cells are mixed with ^{14}C-labeled cells prior to X-ray to act as an internal standard for the retention of DNA on the filter, and reduce quantitative variability in independent samples. In Fig. 4 an increased elution rate relative to controls in unirradiated samples would serve as a measure of drug-induced DNA single-strand breaks, and a decreased elution rate in irradiated samples is a measure of drug-induced DNA interstrand cross-links. In a previous study (19) we have shown that C-2 does not produce appreciable DNA strand breaks, and in Fig. 4 it can be seen that 2.5 mM MISO also produced few, if any, DNA single-strand breaks. It can be seen from the illustrated data that, with the exception of the 0-hr time point, the combination of 10 μM C-2 and 2.5 mM MISO resulted in a greater frequency of DNA cross-links than did 10 μM C-2. Although higher concentrations of MISO have been reported to cause DNA single-strand breaks in hypoxic cells (27), the observation that DNA of non-irradiated MISO-treated cells eluted like that from control cells at 0, 3, 6, 9, 12, and 15 hr following drug treatment (Fig. 4; 0, 3, and 6 hr not illustrated) indicates that 2.5 mM MISO caused negligible DNA strand breaks under our experimental conditions. This observation correlates well with our cytotoxicity data (Fig. 3) which demonstrated that 2.5 mM MISO was essentially nontoxic to hypoxic L1210 cells.

The delayed formation and removal of DNA interstrand cross-links as a function of time following 2 hr of anaerobic treatment and subsequent removal of 10 μM C-2 or 10 μM C-2 plus 2.5 mM MISO in combination, for a single experiment (Experiment 7, Table 1), is shown in Fig. 5. For both C-2 and C-2 plus MISO in combination, cross-linking was highest approximately 6 hr following drug exposure, and cross-links were almost completely removed by 15 hr. However, with the exception of the 0-hr point, cells treated with C-2 plus MISO in combination show consistently higher cross-linking frequencies than those cells treated with C-2.

Table 1 summarizes data collected from multiple experiments examining DNA interstrand cross-link formation and removal as a function of time in hypoxic L1210 cells. Although the amount of cross-linking produced by a given drug treatment varied from experiment to experiment, we consistently found higher DNA cross-linking frequencies in those cells receiving the C-2 plus MISO than in C-2-treated cells. The only exception to this relationship appeared at the 0-hr time point which was obtained immediately after the cells were washed free of drug.

DISCUSSION

In this report, we have shown that MISO enhances the cytotoxicity of C-2 toward hypoxic L1210 cells in a dose-dependent manner. This enhancment resulted in a dose modification for C-2 at 0.01 survival due to the addition of MISO of approximately 1.5. The increased cytotoxicity of the combination compared with C-2 alone cannot be explained simply by the additive toxicities of C-2 and MISO alone. Furthermore, separate experiments showed that under similar experimental conditions, the surviving fraction of L1210 cells after a 2-hr exposure to 10 μM C-2 under either anaerobic (0.012 ± 0.003, mean ± SD; $n = 3$) or aerobic (0.017 ± 0.002, mean ± SD; $n = 3$) conditions was not significantly different. Our observation does not correlate with other reports indicating that cyclophosphamide selectively kills only well-oxygenated cells and spares hypoxic cells (28). This difference may be due to the chemical behavior of C-2, which spontaneously hydrolyzes to 4-hydroxycyclophosphamide in aqueous media, and thereby avoiding the crucial oxygen-dependent enzymatic activation to 4-hydroxycyclophosphamide that must occur with cyclophosphamide *in vivo* before it demonstrates antitumor activity.

By using the alkaline elution technique we also have shown that the presence of 2.5 mM MISO causes an increase in the frequency of DNA interstrand cross-links produced by 10 μM C-2 which parallels the increase in cytotoxicity seen with simultaneous C-2 and MISO treatment. This observation is in agreement with earlier studies from this laboratory which showed that sulfidocyclophosphamide-induced DNA interstrand cross-links are critical injuries capable of altering cell viability (19, 20).

Previously it was noted that 2.5 mM MISO alone did not cause quantifiable DNA damage, either as strand breaks or interstrand cross-links. Therefore, the enhanced interstrand cross-linking displayed by combined MISO and C-2 treatment cannot be explained as cross-linking enhancement due to additive DNA damage caused by the separate and independent actions of MISO and C-2 on cellular DNA. It is worthy to note, nevertheless, that if the concentration of MISO is raised 4-fold, a

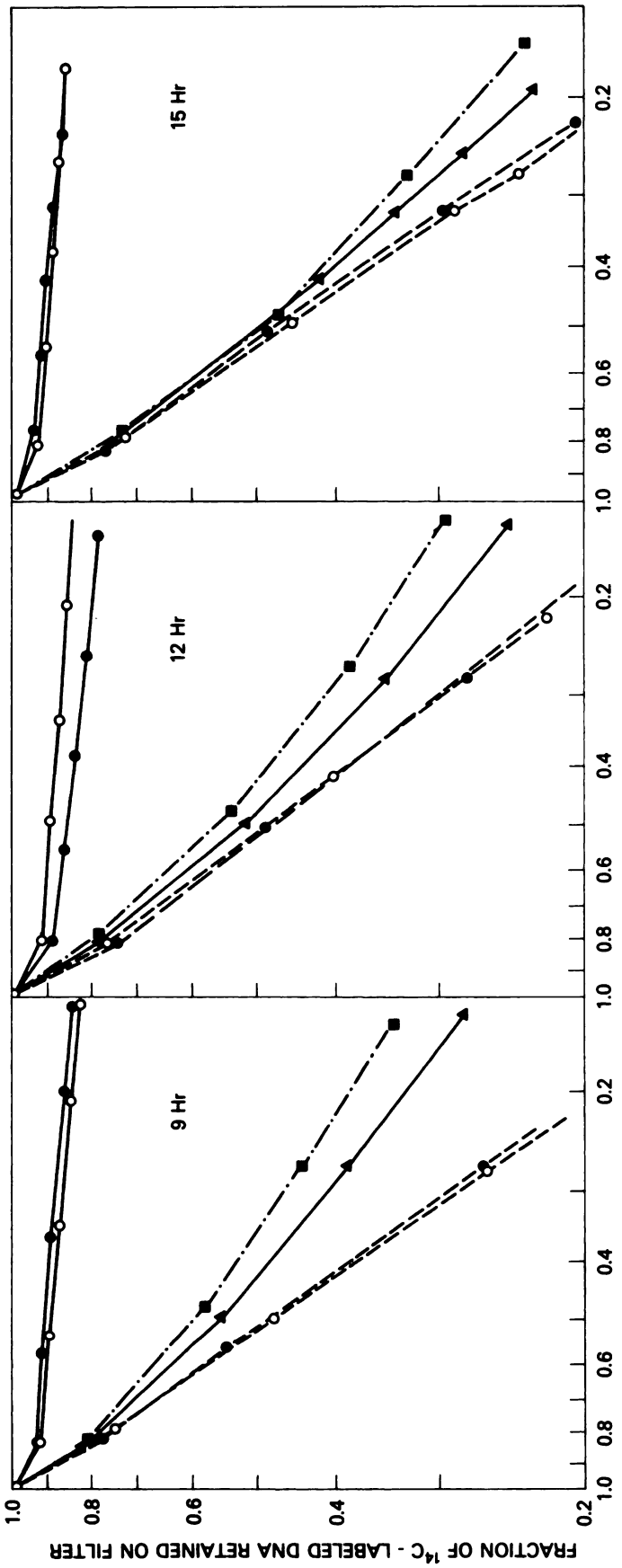


FIG. 4. *Alkaline elution profiles of L1210 cells 9, 12, and 15 hr after 2-hr treatment with 10 μM C-2, 2.5 mM MISO, and the combination* Treatment conditions were as follows: ○—○, control cells, no drug, no X-ray; ●—●, 2.5 mM MISO for 2 hr, no X-ray; ■—■, 2.5 mM MISO plus 10 μM C-2, irradiated with 300 rads X-ray; ▲—▲, 10 μM C-2, irradiated with 300 rads X-ray; ●- - -●, 2.5 mM MISO, irradiated with 300 rads X-ray; ○- - -○, control cells, irradiated with 300 rads X-ray.

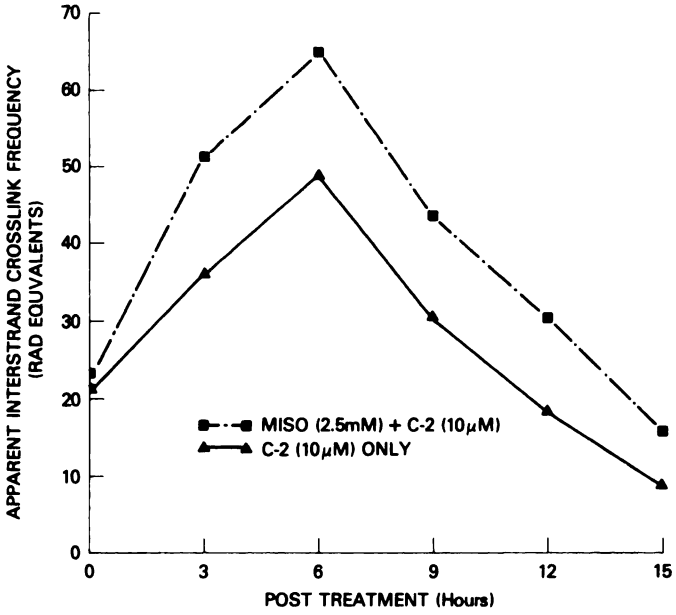


FIG. 5. *Apparent DNA interstrand cross-linking in L1210 cells exposed to 10 μM C-2 only (▲) and 2.5 mM MISO + 10 μM C-2 in combination (■)*

2-hr exposure of 10 mM MISO to hypoxic L1210 cells will produce detectable amounts of DNA strand breaks which are completely removed within 4 hr following drug removal.

Although the cross-linking data consistently indicated statistically significant (*t*-test for paired samples, Table 1) higher cross-linking levels for C-2 and MISO in combination, Table 1 clearly shows that the magnitude of cross-linking varied substantially from one experiment to another. The reason for the large variability in cross-linking frequencies among separate experiments remains unclear. However, because hypoxia severely retards cell cycling, multiple factors such as drug penetration, drug inactivation, and DNA repair may be uniquely influenced in each group of cells that is used to generate an individual experiment (12).

With respect to the potentiation of cytotoxic drugs, it is possible for MISO to act at the cellular level by changing drug penetration into the cell and/or drug action at the intracellular target site. Close examination of the data in Table 1 reveals that C-2 and MISO together induced more DNA cross-links than C-2 alone at all time points investigated with the exception of the 0-hr point. If MISO significantly promoted C-2 or 4-hydroxycyclophosphamide transport across the L1210 cell membrane, higher cross-linking levels would be expected immediately following drug removal as well. Since this is not the case, a more plausible explanation is that MISO has affected the action of C-2 at the DNA target. Bifunctional alkylating agents, such as C-2, have been reported to show a time lag of several hours before maximal cross-linking is observed (29). This lag is thought to represent the slow second step in a two-step reaction whereby the drug-DNA monoadducts initially formed are converted to interstrand cross-links. If MISO impeded C-2 monoad-

TABLE 1

DNA interstrand cross-link formation[a] and removal following 2-hr treatment with C-2 only or C-2 + MISO

Drug	Experiment	Time after drug removal[b]					
		0 Hr	3 Hr	6 Hr	9 Hr	12 Hr	15 Hr
C-2 only	1	20	35	34	—	—	—
C-2 + MISO		20	41	59	—	—	—
C-2 only	2	26	35	35	—	—	—
C-2 + MISO		27	34	47	—	—	—
C-2 only	3	33	61	45	—	—	—
C-2 + MISO		29	68	50	—	—	—
C-2 only	4	—	—	—	21	35	31
C-2 + MISO		—	—	—	42	38	38
C-2 only	5	—	—	—	30	36	29
C-2 + MISO		—	—	—	38	53	40
C-2 only	6	—	—	—	37	28	10
C-2 + MISO		—	—	—	53	37	30
C-2 only	7	20	36	49	30	19	9
C-2 + MISO		23	52	66	44	31	15
$\bar{X}$[c] (C-2 only)		24.8	41.8	40.8	29.5	29.5	19.8
$\bar{Y}$ (C-2 + MISO)		24.8	51.5	55.5	44.3	39.8	30.8
SED		1.47	2.25	4.21	2.69	2.93	3.19
p Value		1.00[d]	0.023	0.039	0.012	0.039	0.041

[a] Rad equivalents.
[b] Incubation under aerobic conditions at 37°.
[c] *t*-Test for paired determinations at each time point.
[d] Not significant.

duct removal, the over-all effect would be exactly that which we observe in Table 1, namely an apparent increase in C-2-induced DNA cross-links at later times due to the larger population of C-2 monoadducts available for conversion to cross-links. Such a phenomenon has been postulated as the mechanism responsible for manifesting nitrosourea sensitivity and resistance in normal and transformed human cell lines (30). Glutathione depletion may also be involved in the potentiation of the cytotoxic action of C-2 by MISO, since under anaerobic conditions glutathione reduces the covalent binding of MISO to microsomal protein (8).

The present study clearly demonstrates that MISO potentiates the toxicity of C-2 as measured by the colony-forming ability of L1210 cells. In addition, this increase in cytotoxicity was paralleled by an increased frequency of DNA interstrand cross-links. The latter observation may help to explain the mechanism of potentiation of MISO on the cytotoxic action of certain chemotherapeutic agents (9–13). Whether this potentiating effect of MISO holds any promise as an innovative treatment modality is unclear, since it is often associated with an increase in host toxicity so that no real gain in the therapeutic index of the chemotherapeutic agent is achieved (9–13).

ACKNOWLEDGMENTS

The authors wish to thank Susan Hurst-Calderone and Ernestine Gregory for assistance with cell cultures, Edward Walsh for performing the data computations, and Drs. Daniel Zaharko and Kurt Kohn for valuable discussions of the manuscript.

REFERENCES

1. Thomlinson, R. H., S. Dische, A. J. Gray, and L. M. Errington. Clinical testing of the radiosensitizer Ro-07-0582. III. Response of tumors. *Clin. Radiol.* **27**:167–174 (1976).
2. Dische, S., M. I. Saunders, M. E. Lee, G. E. Adams, and I. R. Flockhart. Clinical testing of the radiosensitizer Ro-07-0582: experience with multiple doses. *Br. J. Cancer* **35**:567–579 (1977).
3. Gray, A. J., S. Dische, G. E. Adams, I. R. Flockhart, and J. L. Foster. Clinical testing of the radiosensitizer Ro-07-0582. I. Dose, tolerance, serum and tumor concentrations. *Clin. Radiol.* **27**:151–157 (1976).
4. Adams, G. E., and M. S. Cooke. Electron-affinic sensitization. I. A structural basis for chemical radiosensitizers in bacteria. *Int. J. Radiol. Biol. Relat. Stud. Phys. Chem. Med.* **15**:457–471 (1969).
5. Moore, B. A., B. Palcic, and L. D. Skarsgard. Radiosensitizing and toxic effects of the 2-nitro-imidazole Ro-07-0582 in hypoxic mammalian cells. *Radiat. Res.* **67**:459–473 (1976).
6. Whitmore, G. F., S. Gulyas, and A. J. Varghese. Sensitizing and toxicity properties of misonidazole and its derivatives. *Br. J. Cancer (Suppl III)* **37**:115–119 (1978).
7. Wong, T. W., G. F. Whitmore, and S. Gulyas. Studies on the toxicity and radiosensitizing ability of misonidazole under conditions of prolonged incubation. *Radiat. Res.* **75**:541–555 (1978).
8. McManus, M. E., M. A. Lang, K. Stuart, and J. Strong. Activation of misonidazole by rat liver microsomes and purified NADPH-cytochrome C reductase. *Biochem. Pharmacol.* **31**:547–552 (1982).
9. Tannock, I. F. *In vivo* interaction of anti-cancer drugs with misonidazole or metronidazole:methotrexate, 5-fluorouracil and adriamycin. *Br. J. Cancer* **42**:861–870 (1980).
10. Tannock, I. F. *In vivo* interaction of anti-cancer drugs with misonidazole or metranidazole:cyclophosphamide and BCNU. *Br. J. Cancer* **42**:871–880 (1980).
11. Stratford, I. J., G. E. Adams, M. R. Horsman, S. Kandarija, S. Rajaratnam, E. Smith, and C. Williamson. The interaction of misonidazole with radiation, chemotherapeutic agents, or heat. *Cancer Clin. Trials* **3**:231–236 (1980).
12. Roizin-Towle, L., and E. J. Hall. Studies with bleomycin and misonidazole on aerated and hypoxic cells. *Br. J. Cancer* **37**:254–260 (1978).
13. Twentyman, P. R. The response of EMTG tumor spheroids to combined treatment with misonidazole and either nitrogen mustard, adriamycin, or BCNU. *Cancer Clin. Trials* **3**:253–256 (1980).
14. Hill, D. L. *A Review of Cyclophosphamide.* Charles C Thomas, Chicago, 3–7 (1975).
15. Hirano, T., W. Klesse, J. H. Heuringer, G. Lambert, and H. Ringsdorf. Synthesis of activated cyclophosphamide derivatives bearing functional groups. *Tetrahedron Lett.* **10**:883–886 (1979).
16. Peter, G., and H. J. Hohorst. Synthesis and preliminary antitumor evaluation of 4-(SR)-sulfidocyclophosphamides. *Cancer Chem. Pharmacol.* **3**:181–188 (1979).
17. Peter, G., T. Wagner, and H. J. Hohorst. Studies on 4-hydroperoxycyclophosphamide (NSC-181815): a simple preparation method and its application for the synthesis of a new class of "activated" sulfur-containing cyclophosphamide (NSC-26271) derivatives. *Cancer Treat. Rep.* **60**:429–435 (1976).
18. Brock, N. Comparative pharmacologic study *in vitro* and *in vivo* with cyclophosphamide (NSC-26271), cyclophosphamide metabolites, and plain nitrogen mustard compounds. *Cancer Treat. Rep.* **60**:301–308 (1976).
19. Erickson, L. D., L. M. Ramonas, D. S. Zaharko, and K. W. Kohn. Cytotoxicity and DNA crosslinking activity of 4-sulfido-cyclophosphamides in mouse leukemia cells *in vitro*. *Cancer Res.* **40**:4216–4220 (1980).
20. Ramonas, L. M., L. C. Erickson, W. Klesse, K. W. Kohn, and D. S. Zaharko. Differential cytotoxicity and DNA cross-linking produced by polymeric and monomeric activated analogues of cyclophosphamide in mouse L1210 leukemia cells. *Mol. Pharmacol.* **19**:331–336 (1981).
21. Chu, M. Y., and G. A. Fisher. Incorporation of cytosine-^{3}H-arabinoside and its effect on the murine leukemia cells (L5178Y). *Biochem. Pharmacol.* **17**:753–767 (1968).
22. Hirano, T., H. Ringsdorf, and D. S. Zaharko. Anti-tumor activity of monomeric and polymeric cyclophosphamide derivatives compared with *in vitro* hydrolysis. *Cancer Res.* **40**:2263–2267 (1980).
23. Stratford, I. J., and G. E. Adams. Effect of hyperthermia on differential cytotoxicity of a hypoxic cell radiosensitizer, Ro-07-0582, on mammalian cells *in vitro*. *Br. J. Cancer* **35**:307–313 (1977).
24. Kohn, K. W., L. C. Erickson, R. A. G. Ewig, and C. A. Friedman. Fractionation of DNA from mammalian cells by alkaline elution. *Biochemistry* **15**:4629–4637 (1976).
25. Ewig, R. A. G., and K. W. Kohn. DNA damage and repair in mouse leukemia L1210 cells treated with nitrogen mustard, 1,3-bis-(2-chloroethyl)-1-nitrosourea, and other nitrosoureas. *Cancer Res.* **37**:2114–2122 (1977).
26. Ewig, R. A. G., and K. W. Kohn. DNA-protein cross-linking and DNA interstrand cross-linking by haloethylnitrosoureas in L1210 cells. *Cancer Res.* **38**:3197–3203 (1978).
27. Palcic, B., and L. D. Skarsgard. Cytotoxicity of misonidazole and DNA damage in hypoxic mammalian cells. *Br. J. Cancer* **37**:54–59 (1978).
28. Dixon, B., J. V. Moore, and H. Speakman. Radiogiological hypoxia of a transplanted rat tumour and the effect of treatment with cyclophosphamide. *Eur. J. Cancer* **14**:1383–1387 (1978).
29. Ross, W. E., R. A. G. Ewig, and K. W. Kohn. Differences between melphalan and nitrogen mustard in the formation and removal of DNA crosslinks. *Cancer Res.* **38**:1502–1506 (1978).
30. Erickson, L. C., M. O. Bradley, J. M. Ducore, R. A. G. Ewig, and K. W. Kohn. DNA crosslinking and cytotoxicity in normal and transformed human cells treated with antitumor nitrosoureas. *Proc. Natl. Acad. Sci. U. S. A.* **77**:467–471 (1980).

Send reprint requests to: Dr. Michael E. McManus, Building 37, Room 3B27, National Cancer Institute, Bethesda, Md. 20205.