Eur J Cancer Clin Oncol, Vol. 25, No. 7, pp. 1099–1104, 1989.
Printed in Great Britain

0277–5379/89$3.00 + 0.00
Pergamon Press plc

Chemosensitization at Reduced Nitroimidazole Concentrations by Mixed-function Compounds Combining 2-Nitroimidazole and Chloroethylnitrosourea

R. TIMOTHY MULCAHY,* JERRY J. GIPP,* ALAIN CARMINATI,† JEAN-LOUIS BARASCUT† and JEAN-LOUIS IMBACH†

**Department of Human Oncology, University of Wisconsin, Madison, Wisconsin* 53792, *U.S.A., and †Laboratoire de Chimie Bio-Organique, U.A.* 488 *au CNRS, U.S.T.L., Place Bataillon,* 30460 *Montpellier Cedex, France*

Abstract—*A mixed-function compound (I-278) combining 2-nitroimidazole and chloroethylnitrosourea has been shown to be greater than 2-fold more toxic to hypoxic HeLa–MR cells than to cells similarly exposed under aerobic conditions, consistent with chemosensitization of nitrosourea toxicity by the 2-nitroimidazole Misonidazole (MISO). However, in the case of I-278, the enhancement resulted from micromolar concentrations of 2-nitroimidazole as opposed to the millimolar quantities required for a similar enhancement by MISO. These experiments provide evidence (1) that the enhanced hypoxic toxicity of I-278 is not attributable to additional, independent hypoxic cell killing by the nitroimidazole group and (2) that the interaction between the two functions under hypoxic conditions results in increased crosslink formation typical of chemosensitization. The data strongly suggest that the chemosensitizing efficiency of nitroimidazoles can be dramatically improved by covalent linkage to a chloroethylating species.*

INTRODUCTION

As DESCRIBED in a previous report [1], mixed-function compounds which combine a 2-nitroimidazole and a chloroethylating nitrosourea functionality (NI–CENU), are preferentially toxic towards hypoxic HeLa–MR cells (Fig. 1). A comparison of the doses of one such compound I-278 (structure shown in Fig. 2), required to reduce cell survival to 0.01 under aerobic or hypoxic conditions revealed the compound to be greater than 2-fold more toxic in the absence of oxygen. Interestingly, the magnitude of enhancement was very similar to that typically observed when the 2-nitroimidazole, Misonidazole (MISO; 1.0 mM) was used to sensitize HeLa–MR cells to another chloroethylating nitrosourea, 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU) [2], which generates the same alkylating intermediates as the NI–CENU. However, in the current experiments only micromolar quantities of 2-nitroimidazole were required to produce this effect when administered as part of the mixed-function compound. If, as previously suggested, the enhanced activity of I-278 in hypoxia represents an interaction btween the two functions as occurs in chemosensitization the results suggest a possible advantage for the development and use of mixed-function chemosensitizing agents. However, as the preferential hypoxic toxicity of I-278 could be attributable to effects other than an interaction between the nitroimidazole and chloroethylating moieties (chemosensitization), experiments were designed to clarify the nature of the effect. These experiments provide evidence (1) that the enhanced hypoxic toxicity of I-278 is not attributable to additional, independent hypoxic cell killing by the nitroimidazole group; (2) that the interaction between the two functions under hypoxic conditions results in increased crosslink formation typical of chemosensitization and (3) strongly suggest that the chemosensitizing efficiency of nitroimidazoles can be dramatically improved by covalent linkage to a chloroethylating species.

Accepted 24 March 1989.
Reprint requests to R. Timothy Mulcahy Ph.D. at the address above.

MATERIALS AND METHODS

Drugs

The structures of the NI–CENU compounds and the oxazolidinone derivative are shown in Fig. 2.

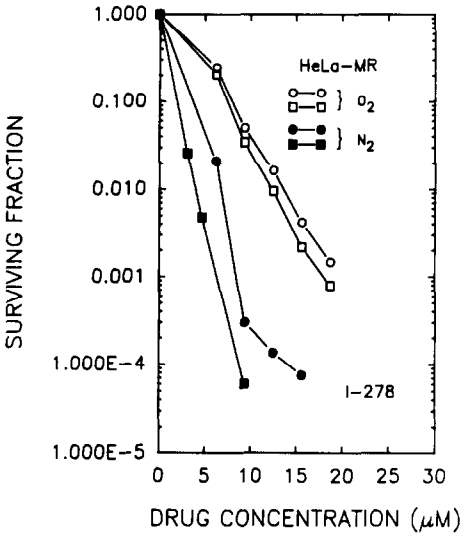

Fig. 1. Dose-response curves for HeLa-MR cells exposed to I-278 for 4 h under aerobic (open symbols) or hypoxic (closed symbols) conditions. The dose enhancement factors under hypoxic conditions are between 2.2 and 2.4.

The synthesis of these compounds has been described previously [3]. The compounds were initially dissolved in 100% dimethyl sulfoxide and diluted in culture medium at the initiation of cell treatment. Misonidazole was graciously provided by Dr. V. Narayanan of the Developmental Therapeutics program, DCT, NCI. MISO was dissolved directly in culture medium at the desired concentrations.

Cell line and drug treatments

HeLa–MR cells were used for these studies. Cells were maintained in exponential growth in BME medium supplemented with 10% fetal bovine serum and gentamycin (50 μg/ml). Cultures were incubated at 37°C 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 [4]. For aerobic exposures exponentially growing HeLa–MR cells were suspended in 10 ml of BME at a concentration of 1–2 × 10^5 cells per ml and transferred to glass treatment vials. For hypoxic exposures the cells were injected into 10 ml of medium in treatment vials only after the medium in the vials had been gassed for 3 h with a 97% N_2/3% CO_2 humidified gas mixture. Prior to being injected into the treatment vials at the conclusion of the 3 h 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°C to deplete oxygen by consumption.

To initiate drug exposure, various concentrations of the test compounds were diluted 100-fold by injection into appropriate treatment vials. Cells were incubated in the presence of drug(s) for 4 h at 37°C. 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 calculated as the ratio of plating efficiency of treated groups to that for controls.

Alkaline elution

Determination of DNA interstrand crosslinking was done by alkaline elution essentially as described by Kohn *et al.* in a review of the technique [5]. Cells

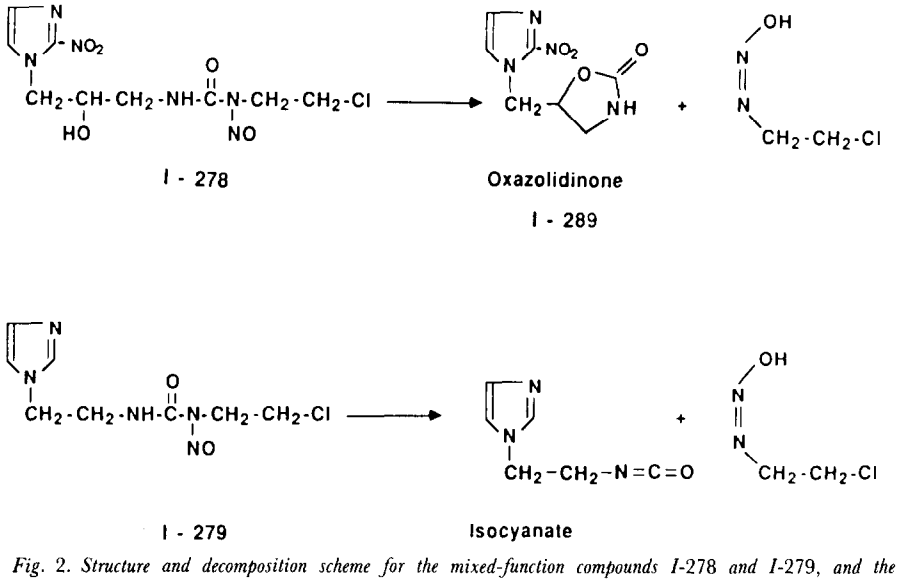

Fig. 2. Structure and decomposition scheme for the mixed-function compounds I-278 and I-279, and the oxazolidinone derivative I-289. Both mixed-function compounds generate the same alkylating species.

were labelled with [^{14}C]thymidine at 0.02 μCi/ml for 48 h and for an additional 24 h in medium free of [^{14}C]thymidine to establish the label in high molecular weight DNA. Reference cells were similarly labelled with [^{3}H]thymidine. To allow adequate time for crosslink formation, the magnitude of crosslinking was determined 16 h after drug treatment. This interval was slightly longer than the time required for maximal crosslink formation for cells treated with chloroethylating nitrosoureas. For elution assay, drug-treated cells and reference cells to be used as internal standards were combined and irradiated at 0°C with 4.0 Gy 250 kVp X-rays at a dose rate of 1.78 Gy/min. The cells were then lyzed on 0.8 μm polycarbonate filters with 1% sodium dodecyl sulfate, 0.020 M EDTA (pH 10), treated for 1 h with proteinase K (0.5 mg/ml) and eluted from the filter with 2% tetrapropylammonium hydroxide (pH 12.1) containing 0.02 M EDTA and 0.1% sodium dodecyl sulfate. Fractions (6 ml) were collected at hourly intervals for 6 h. Crosslink factors (CLF) were calculated for each group using the equation

$$\mathrm{CLF} = \log (f_{\mathrm{irr}}/f_0)/\log (f_x/f_0)$$

where f_{irr}, f_0 and f_x are the fraction of DNA remaining on the filter for the irradiated alone group, the unirradiated control and the irradiated-drug treatment group, respectively, when 30% of the reference DNA was retained on the filter.

RESULTS

One possible explanation for the improved killing of hypoxic HeLa–MR cells by I-278 (Fig. 1) is that the nitroimidazole moiety itself is extremely toxic under hypoxic conditions, even at extremely low concentrations. If this were the case, the enhanced effectiveness could merely represent the cumulative toxicity of chlorethylation combined with hypoxic toxicity of the 2-nitroimidazole moiety, as opposed to a true chemosensitizing interaction. Further, if the 2-nitroimidazole associated with I-278 was significantly more toxic than MISO toward hypoxic cells this might account for the apparent difference in chemosensitizing potency of the two nitroimidazoles. To examine these possibilities the aerobic and hypoxic toxicity of I-289, the 2-nitroimidazole oxazolidinone product generated upon the decomposition of I-278 to active alkylating species (Fig. 2), was determined. Exposure of HeLa–MR cells to doses of I-289 up to 1.0 mM for 4 h in air did not result in any measurable cell kill (data not shown). As is typical of 2-nitroimidazoles, drug treatment in hypoxia did prove more effective, but survival after exposure to 0.1 mM was 0.93 ± 0.07. Since this dose is 4–5-fold higher than the nitroimidazole doses present in the I-278 experiments, it is highly unlikely that the enhanced effect of this agent under hypoxic conditions represents additive toxicity of its two functions.

The additional effectiveness of I-278 in hypoxic cells is therefore suggestive of an interaction between the two functional moieties consistent with chemosensitization. As chemosensitization has been associated with an increased frequency of interstrand crosslinks detectable in the DNA of treated cells [6–9], the magnitude of crosslinking in HeLa–MR cells exposed to I-278 under aerobic and hypoxic conditions was compared. Representative alkaline elution profiles are shown in Fig. 3. The formation of interstrand crosslinks was evident in the DNA of cells treated with I-278 under aerobic or hypoxic conditions, but additional crosslinks ($\mathrm{CLF_{N_2}/CLF_{O_2}}$ = 1.68 ± 0.10) were formed in HeLa–MR cells treated in the absence of oxygen. In a related experiment (data not shown) exposure of HeLa–MR cells to 50 μM I-278 under hypoxic conditions resulted in CLF equivalent to that induced by an aerobic exposure to 100 μM I-278. The increase in CLF associated with hypoxic exposure to I-278 is therefore similar in magnitude to the corresponding enhancement of cytotoxicity. In contrast, additional crosslinks were not observed ($\mathrm{CLF_{N_2}/CLF_{O_2}}$ = 0.81 ± 0.08) (Fig. 4) in hypoxic cells treated with a related imidazole-nitrosourea (no nitro-group) com-

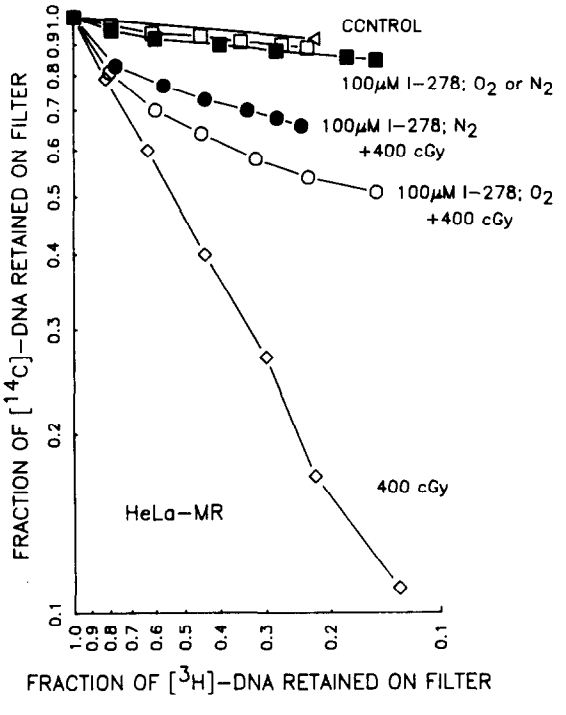

Fig. 3. Representative DNA alkaline elution profile for HeLa–MR cells exposed to I-278 under aerobic and hypoxic conditions, providing evidence of crosslink formation in cells treated aerobically (○) *and additional crosslinks in cells exposed in hypoxia* (●). *Cells were harvested for crosslink analysis* 16 *h after drug treatment.*

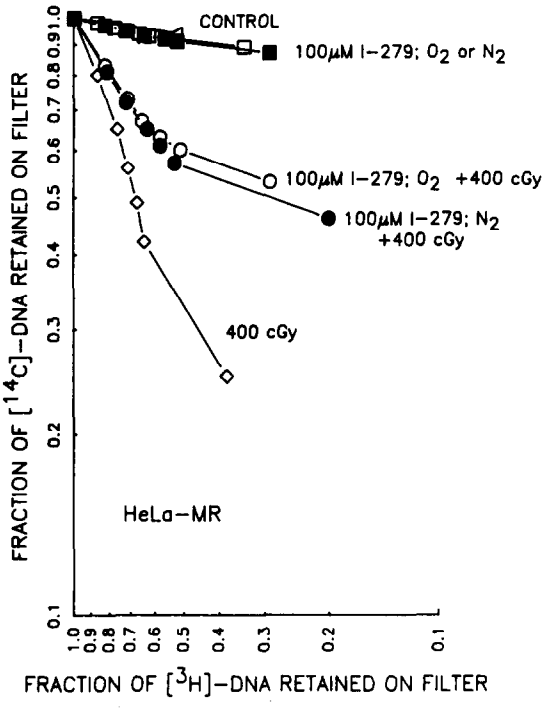

Fig. 4. Typical DNA alkaline elution profile for HeLa-MR cells exposed to I-279. Although DNA crosslinks were detected, similar levels were evident in the DNA of cells treated under either aerobic or hypoxic conditions. Alkaline elution was performed 20 h after the completion of exposure.

pound (I-279, Fig. 2), which did not exhibit any enhanced hypoxic toxicity (Fig. 5B). This compound generates the same chloroethylating species as I-278 (Fig. 2), has a similar half-life (Carminati *et al.*, unpublished) and is as toxic as I-278 in cells exposed in air (Fig. 5A).

The ability of the nitroimidazole moiety of I-278 to enhance the toxicity of the nitrosourea function at very low concentrations could result either from its direct linkage to the alkylating agent or its potency as a chemosensitizer. To evaluate this latter possibility, attempts were made to enhance the toxicity of I-279 with concentrations of I-289 comparable to those encountered in the I-278 experiments. As shown in Fig. 6b, the hypoxic toxicity of I-279 was not enhanced by combination with either 10 or 100 μM I-289 nor was the frequency of crosslinks increased (data not shown). Figure 6A also provides evidence that the toxicity of I-279 could indeed be enhanced by an effective chemosensitizing nitroimidazole, in this case MISO. Further evidence of the lack of sensitizing effectiveness of micromolar doses of I-289 was provided by experiments in which doses as high as 100 μM also failed to enhance CCNU toxicity (data not shown). These experiments suggest that the nitroimidazole group liberated by the decomposition of I-278 is not a highly effective sensitizing agent capable of inducing large DEFs at micromolar concentrations and, therefore, the large preferential hypoxic toxicity of I-278 is not due to the exquisite chemosensitizing potency of the nitroimidazole function *per se*.

DISCUSSION

Exposure of cells to mixed-function nitroimidazole–chloroethylnitrosourea (NI–CENU) compounds under hypoxic conditions results in enhanced cytotoxicity relative to comparable aerobic exposures. While similar in expression to chemosensitization of nitrosoureas by MISO, the enhancements observed with the NI–CENUs occur at much lower nitroimidazole concentrations. These experiments provide evidence that the large preferential hypoxic toxicity observed with the NI–CENU cannot be accounted for merely on the basis of the toxic

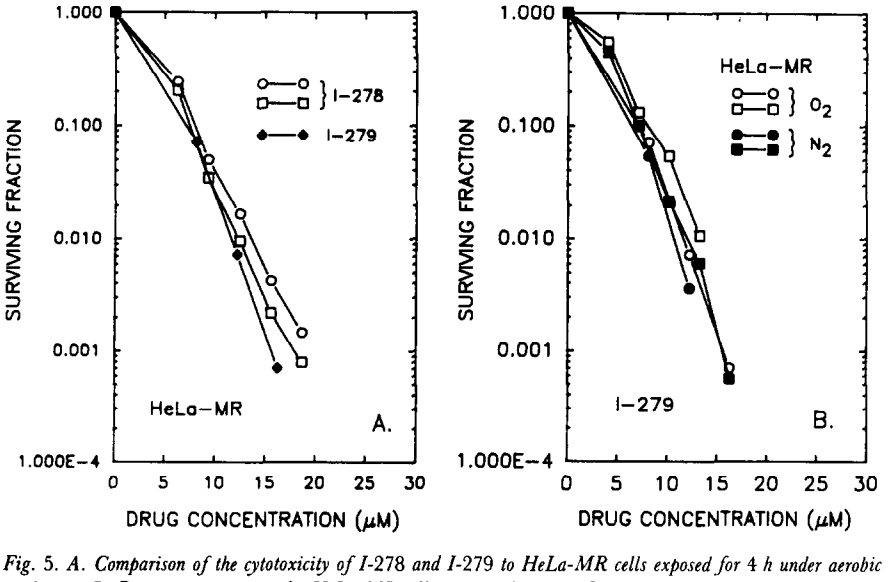

Fig. 5. A. Comparison of the cytotoxicity of I-278 and I-279 to HeLa-MR cells exposed for 4 h under aerobic conditions. B. Dose-response curves for HeLa-MR cells exposed for 4 h to I-279 under aerobic (open) or hypoxic (closed) conditions, demonstrating lack of preferential hypoxic toxicity.

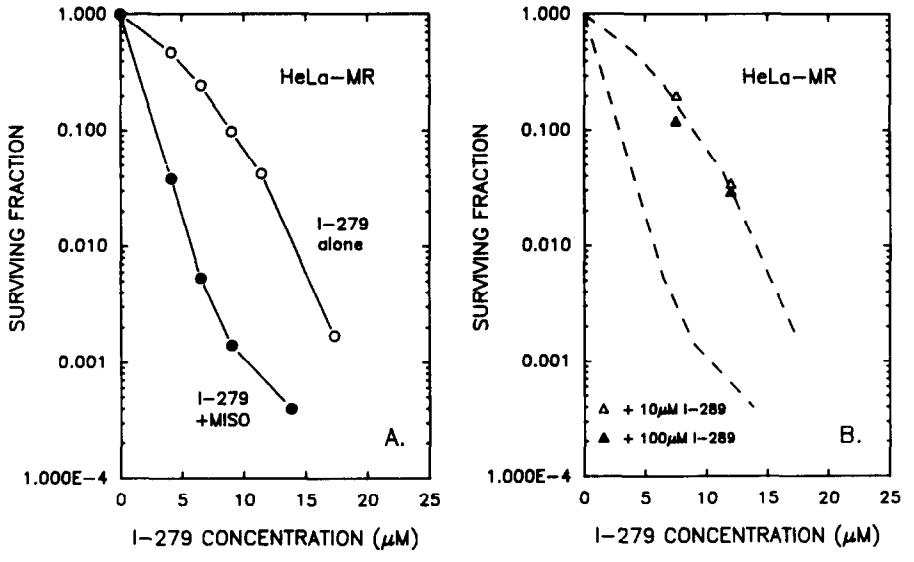

Fig. 6. A. Enhancement of the toxicity of I-279 by co-incubation with 1.0 mM MISO for 4 h under hypoxic conditions (i.e. chemosensitization). B. Lack of chemosensitization by 10 (Δ) or 100 (▲) μM doses of I-289. Dotted lines have been redrawn from Fig. 6A.

properties of the nitroimidazole. Rather, the data, particularly the crosslink data, suggest that the enhanced hypoxic cytotoxicity is a manifestation of chemosensitization. The results are therefore compatible with the hypothesis that the mixed-function combination provides an advantage favoring a chemosensitizing interaction at unusually low nitroimidazole concentrations.

The actual mechanism for this advantage is currently not clear. It could be argued that the enhanced hypoxic toxicity is the result of spatial cooperativity with the active alkylating moiety and chemosensitizing intermediate being liberated proximal to each other and an ultimate target, presumably DNA. This was indeed one of the initial rationales for the design of these agents. Alternatively, the advantage could be attributable to improved intracellular accumulation of the chemosensitizing intermediate resulting from its linkage with the more lipophilic nitrosourea. This later possibility certainly cannot be ruled out by the experiments described in this report. However, it seems unlikely that the enhanced hypoxic toxicity of I-278 could be entirely accounted for by improved nitroimidazole uptake. Based upon *in vitro* uptake data for MISO [10, 11], the peak intracellular concentration of nitroimidazole associated with a 1.0 mM exposure to MISO would be expected to be ~300 μM. Assuming that the intracellular NI–CENU concentrations rapidly approach extracellular levels as has been suggested for lipophilic CENUs [12], the highest intracellular nitroimidazole (I-289) concentration expected following exposure to I-278 at the concentrations used in the current experiments would be approx. 30 μM. Therefore, I-289 would have to be an order of magnitude more potent than MISO to produce equi-effective chemosensitization at these relative intracellular concentrations. This approximation is actually conservative. It assumes that I-278 behaves as other lipophilic nitrosoureas achieving a 1:1 ratio of intracellular/extracellular nitroimidazole concentration. For ratios less than 1.0, the difference in chemosensitizing potency between I-278 and MISO would be even more pronounced. Considering the data presented here and our previous experience with other 2-nitroimidazole chemosensitizing agents such a dramatic difference seems unlikely. Obviously this will have to be examined further with the availability of labelled compounds. However, even if the increased efficiency of the mixed-function NI–CENUs is due merely to enhanced intracellular delivery of the nitroimidazole function, it represents a potentially significant improvement in the design of drugs for use in a chemosensitizing strategy.

While the mechanism of action of the NI–CENU mixed-function compounds is not established, it does, however, differ significantly from that of the aziridine-nitroimidazole compound RSU-1069 and related analogs introduced by Adams *et al.* [13]. RSU-1069 is toxic to cells under aerobic and hypoxic conditions, but is significantly more toxic to cells in the absence of oxygen [13, 14]. It has been suggested that this differential toxicity is related to the monofunctional action of the aziridine group in the presence of oxygen and the drug's bifunctional nature upon reduction of the nitro-group in hypoxia [14]. Presumably an intact molecule is required for bifunctional activity. In contrast, in the case of the NI–CENUs, the alkylating moiety is itself bifunctional. Furthermore, the NI–CENU must dissociate

into two distinct molecules in order to generate the alkylating intermediates. Presumably the activity of these agents involves an interaction between the two compounds liberated upon decomposition of the parent compound.

Although the mechanism of action of the NI–CENU compounds is not well established, the experience with these compounds and that with the alkylating nitroimidazoles in the RSU-1069 series provides considerable impetus for the further development of mixed-function drugs for radiation and/or chemotherapy. With further refinements, these compounds may evolve to offer significant advantage over conventionally used agents and sensitization protocols.

Acknowledgements—The authors are grateful to Kevin Hickson for excellent technical assistance and to Peggy Ziebarth for assistance with the preparation of the manuscript. This work was supported by NIH Grant CA-42325.

REFERENCES

1. Mulcahy RT, Carminati A, Barascut J-L, Imbach J-L. Aerobic and hypoxic toxicity of a new class of mixed-function drugs associating nitroimidazoles and chloroethylnitrosourea in nitrosourea-sensitive (Mer^-) and -resistant (Mer^+) human tumor cells. *Cancer Res* 1988, **48**, 798–801.
2. Mulcahy RT. Misonidazole-induced chemopotentiation of 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea toxicity in O^6-methylguanine-DNA methyltransferase proficient (Mer^+) and deficient (Mer^-) cell lines. *Cancer Res* 1986, **46**, 2892–2897.
3. Carminati A, Barascut J-L, Chenut E, Bournut C, Mathé G, Imbach J-L. On a new class of mixed drugs associating nitroimidazoles and CENU: the NICE NU. *Anticancer Agents* 1988 **3**, 57–65.
4. Mulcahy RT. Effect of oxygen on misonidazole chemosensitization and cytotoxicity *in vitro*. *Cancer Res* 1984, **44**, 4409–4413.
5. Kohn KW, Ewig RAG, Erickson LC, Zwelling LA. Measurement of strand breaks and crosslinks by alkaline elution. In: Friedberg EC, Hanawalt PC, eds. *DNA Repair, A Laboratory Manual of Research Procedures*. New York, Marcel Dekker, Vol. I, 379–401.
6. Ramonas IM, Erickson LC, McManus ME. The effect of misonidazole on the cytotoxicity and DNA cross-linking activity of an activated sulfidocyclophosphamide in hypoxic mouse leukemia cells. *Mol Pharmacol* 1982, **22**, 175–181.
7. Taylor YC, Evans JW, Brown JM. Mechanism of sensitization of Chinese hamster ovary cells to melphalan by hypoxic treatment with misonidazole. *Cancer Res* 1983, **43**, 3175–3181.
8. Murray D, Meyn RE. Enhancement of DNA cross-linking activity of melphalan by misonidazole *in vivo*. *Br J Cancer* 1983, **47**, 195–203.
9. Mulcahy RT. Cross-link formation and chemopotentiation of EMT-6/Ro cells exposed to MISO after CCNU treatment *in vitro*. *Int J Radiat Oncol Biol Phys* 1986, **12**, 1389–1392.
10. Brown DM, Gonzalez-Mendez R, Brown JM. The kinetics of intracellular uptake of 2-nitroimidazoles: influence of partition coefficient, hypoxia and uncouplers of oxidative phosphorylation. *Radiat Res* 1981, **87**, 433–434.
11. Sutherland RM, Keng P, Controy PJ, McDermott D, Bareham BJ, Passalcqua W. *In vitro* hypoxic cytotoxicity of nitroimidazoles: uptake and cell cycle phase specificity. *Int J Radiat Oncol Biol Phys* 1982, **8**, 745–748.
12. Weinkam RJ, Deen DF. Quantitative dose response relations for the cytotoxic activity of chloroethylnitrosoureas in cell culture. *Cancer Res* 1982, **42**, 1008–1014.
13. Adams GE, Ahmed I, Sheldon PW, Stratford IJ. Radiation sensitization and chemopotentiation: RSU 1069, a compound more efficient than misonidazole *in vitro* and *in vivo*. *Br J Cancer* 1984, **49**, 571–577.
14. Stratford IJ, Walling JM, Silver ARJ. The differential cytotoxicity of RSU 1069: cell survival studies indicating interaction with DNA as a possible mode of action. *Br J Cancer* 1986, **53**, 339–344.