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Effects of Misonidazole on Tumor Cell Radiation Sensitivity and Potentially Lethal Damage Repair *In Vivo* and *In Vitro**

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Abstract—*The effects of misonidazole on the radiation response of a murine squamous cell carcinoma* in vivo *and on the radiation response of plateau phase cultures* in vitro *have been studied. From the analysis of the single cell survival curves for the* in vivo *tumors it has been concluded that misonidazole-induced effects are maximal during the first hour after injection, that a dose of* 0.05 mg/g *is marginally effective in reducing the survival following X-ray, but that a dose of* 0.5 mg/g *sensitizes both oxygenated* (1.4-fold) *and hypoxic* (1.7- to 2.0-fold) *tumor cells. In addition, it has been shown* in vivo *that a dose of* 0.5 mg/g *can inhibit the repair of potentially lethal damage.* In vitro *studies on plateau phase cultures of CHO cells have confirmed these* in vivo *observations*, i.e., 0.05 mg/ml *of misonidazole sensitized plateau phase cultures in air by a factor of* 1.2, *and inhibited the repair of potentially lethal damage. These data would suggest that misonidazole is not simply cytotoxic for, or a radiosensitizer of, hypoxic cells.*

INTRODUCTION

It is generally believed that the hypoxic cells within both animal and human tumors contribute to the relative radiation resistance of these tumors to radiation therapy [1], i.e., hypoxic cells can be up to three times more resistant than their well-oxygenated counterparts. Accordingly, methods which would overcome the contributions of hypoxia to radiation resistance should improve the effectiveness of radiotherapy. One of the more promising approaches to this problem involves pre-irradiation administration of drugs, such as the 2-nitroimidazole, misonidazole, which mimics the effects of oxygen on the sensitivity of hypoxic cells but can diffuse further from the capillary and reach the hypoxic cells because it is not consumed by the cells as rapidly as is oxygen [2].

In the course of investigating the effects of this drug in experimental radiotherapy *in vivo*, we observed that misonidazole preferentially sensitized hypoxic, as opposed to oxygenated, tumor cells, and that potentially lethal damage repair by tumors was inhibited in mice which received misonidazole prior to irradiation. Using plateau phase cultures of another tumor, this observation on misonidazole inhibition of potentially lethal damage repair has been confirmed. The results of these experiments are reported below.

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MATERIALS AND METHODS

In vivo *tumor system*

Equal numbers of male and female mice of the WHT/Ht strain were used both to carry the tumor and to assay for tumor cell survival. The tumor is a keratinizing squamous cell carcinoma which arose spontaneously within this strain, and has since been carried by subcutaneous passage. Further details regarding this tumor's characteristics have been published elsewhere [3, 4].

Ten to twelve days after the subcutaneous injection of $3–4 \times 10^4$ viable tumor cells, the tumors averaged 1 g in size and were treated as described below. Following treatment two tumors from a single mouse were pooled and

prepared into single cell suspensions as described by Hewitt [5]. The suspensions used in the present studies contained no detectable clumps, and satisfied the criteria for morphological viability [4].

In most studies, the tumors were harvested immediately after treatment (see also below), prepared into single cell suspensions, appropriately diluted, and then graded cell numbers (normally four to five) were transplanted into four axillary transplant sites on each of four mice. From the percentage of successful transplants as a function of cell dose, the number of cells required for 50% takes ($TD50$) was calculated, as were the 95% confidence limits [6]. For control tumors the $TD50$ was found to be 8.1 cells, and, therefore the surviving fraction for a given treatment equals 8.1 divided by the $TD50$ observed for cells harvested from the treated tumor.

In vivo *drug treatment and X-ray exposure*

Misonidazole or 1-(2-nitro-1-imidazolyl)-3-methoxy-2-propanol was kindly provided by Nippon-Roche Ltd. The drug was dissolved in saline at a concentration of 2 mg/ml and injected i.p. up to 6 hr before X-ray exposure. The two doses studied were 0.05 and 0.5 mg per g of body weight.

X-ray exposures were given on a 250 kVp unit operated at 20 mA. The dose rate of the beam, after filtration through 0.5 mm Cu and 1.0 mm Al, averaged 59 rad/min at the 65 cm target sample distance. In most experiments, the tumors were harvested immediately after treatment; however, in certain studies, to allow for analysis of potentially lethal damage repair, the harvesting of the tumors was delayed for 7–8 hr after therapy.

In vitro *drug treatment and X-ray exposure*

The V79-1 subline of Chinese hamster cells was used throughout these experiments, and was grown in a modification of Eagle's medium [7] supplemented with 15% FCS (EM-15). Five to six days after plating, the cells, growing in 60 mm petri dishes, reached the plateau phase and were used for the experiments. Misonidazole was dissolved in EM-15 (0.05 mg/ml) and added to the cultures 30 min before exposure. Either immediately after exposure or at intervals of up to 8 hr later, the medium was removed, the cells were washed, trypsinized, counted and diluted, and then plated in order to measure the fraction of cells which could form colonies. The surviving fraction is equal to the surviving fraction in treated cultures divided by that observed in control cultures.

X-irradiations for these *in vitro* studies were similar to those described above, except that the machine was operated at 200 kVp and 23 mA, and the dose rate averaged 58 rad/min.

RESULTS

Figure 1 shows the surviving fraction of clonogenic tumor cells plotted as a function of the dose of X-rays administered to control animals. The curve relating surviving fraction

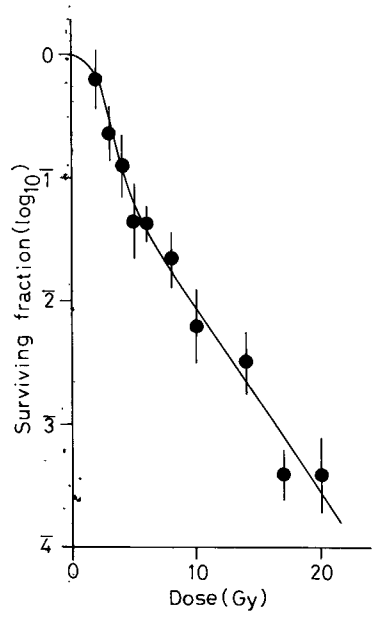

Fig. 1. *Surviving fraction of clonogenic squamous cell carcinoma cells assayed* in vitro *following exposure to X-rays* in vivo.

to dose consists of an initial steep ($D_0 = 1.20 \pm 0.17$ Gy) and a shallower ($D_0 = 3.13 \pm 0.31$ Gy) second component, with the transition point occurring at approximately 5 Gy.

To determine the optimal time for administration of misonidazole (0.5 mg/g of body weight) in order to obtain maximal radiosensitization, we administered this dose of misonidazole between 10 min (plotted as $t=0$) and 6 hr prior to a single dose of 10 Gy. As shown in Fig. 2, maximal reduction of the surviving fraction was obtained between 10 min and 1 hr after drug administration. By the sixth hour after injection the effects of misonidazole pre-treatment had all but disappeared (compare Figs. 1 and 2). We therefore selected 30 min between misonidazole injection and exposure for all further studies.

Figure 3 is a replot of the X-ray curve from Fig. 1 (solid line) compared to the single cell survival curves for tumors derived from mice which had received 0.05 mg/g or 0.5 mg/g of misonidazole 30 min before X-ray exposure.

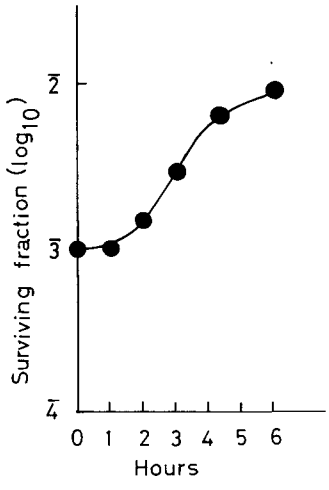

Fig. 2. Effect of increasing the time interval between misonidazole injection (0.5 mg/g) *and X-ray exposure* (10 Gy) *on the surviving fraction of clonogenic tumor cells.*

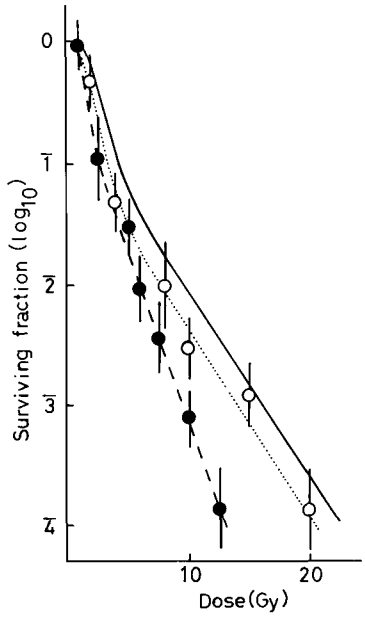

Fig. 3. Effects of misonidazole injection 30 min *before X-ray exposure on the single cell survival curve for clonogenic tumor cells.* (○: 0.05 mg/g, ●:0.5 mg/g)

Sensitization was barely detectable at the lower dose of misonidazole, 0.05 mg/g, but was readily apparent in both the low and high dose range following the administration of 0.5 mg/g. Table 1 provides the D_0 values for the initial and final components of these survival curves for drug-treated mice compared to the same for non-drug-treated controls. Also included in Table 1 are estimates of the degree of sensitization based on a comparison of the radiation doses required to produce surviving fractions of 0.1, 0.01 and 0.001. In summary, these data (Fig. 3 and Table 1) demonstrate that 0.5 mg/g of misonidazole can, when administered 30 min

Table 1. Sensitizer enhancement ratios (SER) for misonidazole determined from D_0 *ratios and from doses required to produce selected levels of survival*

Initial component		
D_0 (Gy)	1.06±0.18	0.83±0.19
SER	1.1	1.4
Final component		
D_0	2.94±0.39	1.56±0.25
SER	1.1	2.0
SER at $SF^a=0.1$	1.1	1.6
0.01	1.1	1.6
0.001	1.2	1.7

prior to X-ray exposure, reduce the surviving fraction of tumor cells in both the low (SER =1.4) and high (SER=1.7–2.0) radiation dose range, but that the lower dose, 0.05 mg/g, does not reduce the surviving fraction significantly.

Since these tumors were assayed immediately after exposure, it remained possible that at least part of the apparent sensitization resulted in fact from an interaction of the disaggregation procedure with the injury produced by misonidazole and/or X-rays. Of primary concern would be the possibility that residual misonidazole or an effect it induces might interfere with the repair of potentially lethal damage (PLD). To test for this we gave mice an injection of 0 or 0.5 mg/g of misonidazole, waited 30 min, irradiated them with a dose of 10 Gy, and, then, harvested the tumors up to 7 hr later for analysis of the surviving fraction of clonogenic tumor cells. The increase in the surviving fraction is associated with a delay between exposure and assay with the repair of PLD.

Figure 4 is a plot of the fraction of surviving clonogenic tumor cells as a function of the time which elapsed between exposure and assay. In mice receiving both 0 and 0.5 mg/g of misonidazole, PLD repair is apparent; the overall increase in the surviving fraction is approximately 16-fold in the absence of misonidazole pre-treatment, but only 6-fold in its presence (Fig. 4).

To verify that misonidazole does indeed interfere with PLD repair, without the complications of *in vivo* systems, we extended these studies to include plateau phase cultures of CHO cells. In Fig. 5(a) is plotted the single cell survival curve for CHO plateau phase cultures ($D_0=2.95\pm0.19$ Gy), as well as the surviving fraction as a function of the time between exposure and assay. Over the 8 hr interval tested, the surviving fraction increased by a factor of approximately three. Figure 5(b) is a similar plot for plateau phase

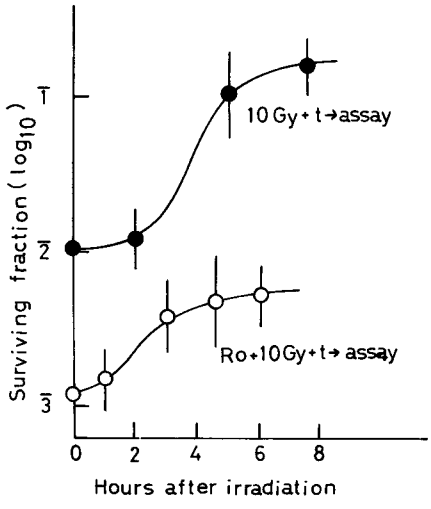

Fig. 4. Effects of increasing the interval between X-ray (10 Gy) and tumor harvest on the surviving fraction of clonogenic tumor cells in mice given no pre-treatment or 0.5 mg/g of misonidazole.

DISCUSSION

From the data presented above, it is readily apparent that misonidazole (0.5 mg/g) can reduce the fraction of clonogenic tumor cells which survive radiation exposure, and that this effect is observed in both the low and high X-ray dose range, although it is more pronounced in the latter. Further, although it could not be shown to be statistically significant, the data suggest that the lower dose of misonidazole, 0.05 mg/g, has similar although less pronounced effects.

The sensitizer enhancement ratios (SER) observed in the present experiments, 1.1–2.0 at a dose of 0.05 mg/g and 1.4–2.0 at a dose of 0.5 mg/g, are consistent with those which have been reported elsewhere in the literature. Peters [8], using a squamous cell carcinoma similar to that used in the present studies,

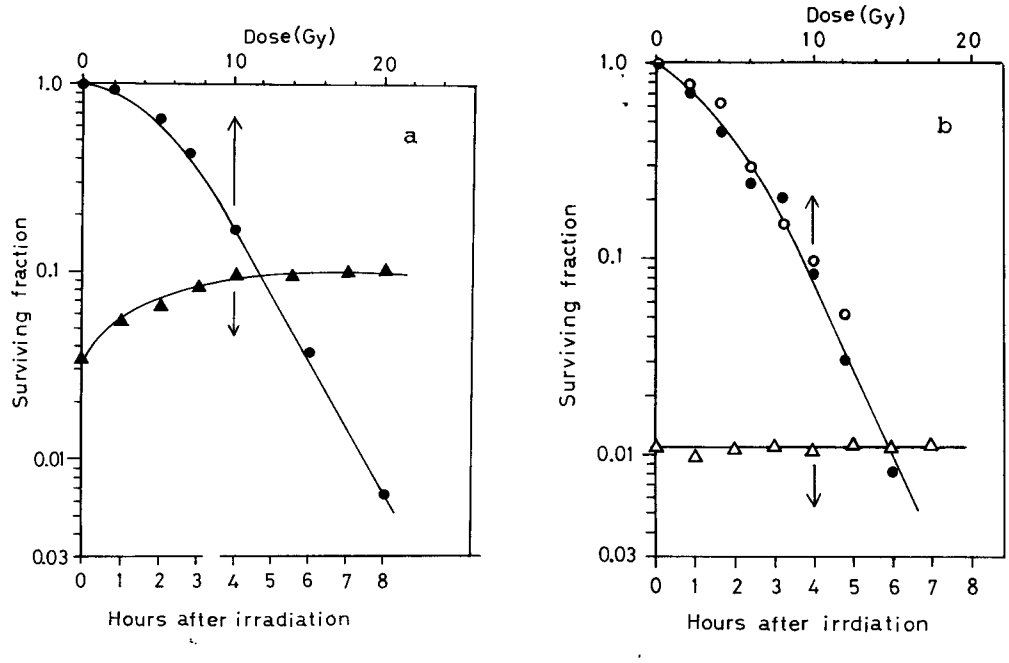

Fig. 5 (a). Survival of plateau phase cultures of CHO cells as a function of radiation dose (●) and the effects of varying the time between exposure and assay on the survival of cells exposed to 15 Gy (▲). (b). As in Fig. 5 (a), except that cultures were exposed to X-ray exposure. (Single cell survival = ●, ○; interval between exposure and assay = △.)

cultures of CHO cells which had been exposed to 0.05 mg/ml of misonidazole for 30 min prior to X-ray. The D_0 for the single cell survival curve (2.51 ± 0.11 Gy) suggests a marginal (SER = 1.2) level of radiosensitization (Fig. 5), but the effects of misonidazole on PLD repair are readily apparent. The surviving fraction of tumor cells did not increase detectably when the assay was delayed as much as 7 hr after exposure (15 Gy).

reported an SER of 1.87 at a dose of 0.25 mg/g. Denekamp *et al.* [9] reported an SER for hypoxic epidermal cells in WHT/Ht mice of 1.12–2.05 over the dose range of 0.02–1.0 mg/g. Lastly, Fowler and Adams [10] reviewed the data of a number of investigators and found SER values ranging from 1.6 to 2.2 at a dose of 1 mg/g. In general, therefore, our results are consistent with past experience.

Where our data differ, however, is that we

have observed apparent sensitization in both the low dose, high survival and high dose, low survival ranges. The initial component of the survival curve for *in vivo* tumors is generally assumed to reflect the survival of well-oxygenated cells, while the final component is assumed to reflect the survival of hypoxic, and therefore radiation resistant, tumor cells [11–13]. The fact that we have observed apparent sensitization of the initial component of the cell survival curve would suggest either radiosensitization of well-oxygenated cells or a more complex interaction of misonidazole with irradiated cells than is normally assumed.

As a first step in investigating the mechanisms underlying these effects we have studied the effects of misonidazole on PLD repair, both *in vivo* (Fig. 4) and *in vitro* (Fig. 5b). In both systems, PLD repair inhibition was apparent, at least among the cells which survived 10 (*in vivo*) or 15 (*in vitro*) Gy. *In vivo* we can assume that these cells were hypoxic, but our plateau phase cultures were not hypoxic, yet PLD repair inhibition was apparent (Fig. 5b).

Published reports [14–17] on the effects of misonidazole on the radiosensitivity of hypoxic CHO cells have all included SER values (at comparable misonidazole concentrations) which are larger than those reported here, SER = 1.2, for our plateau phase CHO cells. Again, this would suggest that the effects we are observing are not a function of hypoxia, and that the 1.5-fold greater D_0 for plateau phase cells compared to exponentially growing ones (data not shown) is more likely to be a product of other than oxygenation status, which might also affect the sensitivity to misonidazole.

Although our studies on PLD repair *in vitro* have employed standard techniques [18–21] and obtained results similar to those of Little *et al.* [22] and Belli *et al.* [23], it is still not clear exactly how misonidazole has interfered with this process. Further study will be required to elucidate the nature of this mechanism.

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REFERENCES

1. Kallman RF. The phenomenon of reoxygenation and its implication for fractionated radiotherapy. *Radiology* 1972; **105:** 135.
2. Adams GE. Prospects and problems with hypoxic radiation sensitizer. In: (Breccia A, Rimondi C, Adams GE eds.) *Radiosensitizers of Hypoxic Cells.* Amsterdam: Elsevier-North-Holland Biomedical Press, 1979: 13–29.
3. Hewitt HB, Chan DP, Blake ER. Survival of clonogenic cells of murine keratinizing squamous carcinoma irradiated *in vivo* or under hypoxic conditions. *Int J Radiat Biol* 1967; **12:** 535.
4. Hewitt HB, Sakamoto K. The comparative survival of clonogenic cells of a murine epithelioma after irradiation in mice breathing air, oxygen and carbon dioxide or hyperbaric oxygen. *Br J Radiol* 1971; **44:** 457.
5. Hewitt HB. The effect on cell survival of inhalation of oxygen under high pressure during irradiation *in vivo* of solid mouse sarcoma. *Br J Radiol* 1966; **39:** 19.
6. Litchfield JT, Wilcoxon F. A simplified method of evaluating dose–effect experiments. *J Pharmacol Exp Ther* 1949; **96:** 99.
7. Eagle H. Nutritional needs of mammalian cells in tissue culture. *Science* 1955; **122:** 501.
8. Peters LJ. Modification of the radiocurability of a syngeneic murine squamous carcinoma by its site of growth, by electron affinic drugs, and by ICRF 159. *Br J Radiol* 1976; **49:** 708.
9. Denekamp J, Michael BD, Harris SR. Hypoxic cell radiosensitizers: Comparative tests of some electron affinic compounds using epidermal cell survival *in vivo*. *Radiat Res* 1974; **60:** 119.
10. Fowler JF, Adams GE. Radiosensitization of hypoxic cells in solid tumor in mice. *Br J Radiol* 1975; **48:** 77.
11. Hewitt HB, Wilson CW. The effect of tissue oxygen tension on the radiosensitivity of leukemia cells irradiated *in situ* in the livers of leukemic mice. *Br J Cancer* 1959; **13:** 675.

12. Powers WE, Tolmach LJ. A multi-component X-ray survival curve for mouse lymphosarcoma cells irradiated *in vivo*. *Nature* (*Lond*) 1963; **197:** 710.
13. Sakamoto K, Lam GKY. Cell survivals of squamous carcinoma exposed to pions at various positions of depth dose curve and PLD repair of the tumors exposed at digital peak region to pions produced by the cyclotron at TRIUMF. *Radiat Res* (in press).
14. Ling CC, Michael HB, Epp ER, Peterson EC. Interaction of misonidazole and oxygen in the radiosensitization of mammalian cells. *Int J Radiat Oncol Biol Phys* 1980; **6:** 583.
15. Asquith JC, Watts ME, Patel K, Smithen CE, Adams GE. Electron affinic sensitization. V. Radiosensitization of hypoxic bacteria and mammalian cells *in vitro* by some nitroimidazole and nitropyrazoles. *Radiat Res* 1974; **60:** 108.
16. Millar BC, Fielden EM, Steele JJ. Effect of oxygen-radiosensitizer mixtures on the radiation response of Chinese hamster cells, line V. 79–753B, *in vitro*. *Radiat Res* 1980; **82:** 478.
17. Moore BA, Palcic B, Skarsgard LD. Radiosensitizing and toxic effects of the 2-nitroimidazole R0-07-0582 in hypoxic mammalian cells. *Radiat Res* 1976; **67:** 459.
18. Whitmore GF, Guyas S. Studies on recovery process in mouse L cells. *Natl Cancer Inst Monogr* 1967; **24:** 141.
19. Phillips RA, Tolmach LJ. Repair of potentially lethal damage in X-irradiated HeLa cells. *Radiat Res* 1966; **29:** 413.
20. Djordjevic B, Perez AG. Repair of damage in irradiated HeLa cells (abstract). *Radiat Res* 1970; **43:** 270.
21. Belli JA, Shelton M. Potentially lethal radiation damage: repair by mammalian cells in culture. *Science* 1969; **165:** 490.
22. Little JB, Hahn GM, Frindel E, Tubiana M. Repair of potentially lethal radiation damage *in vitro* and *in vivo*. *Radiology* 1973; **106:** 689.
23. Belli JA, Dicus GJ, Nagle W. Repair of radiation damage as a factor in preoperative radiation therapy. In: Vaeth JM, ed. *The Interrelationship of Surgery and Radiation Therapy in the Treatment of Cancer. Proceedings of the Fifth Annual San Francisco Cancer Symposium. Frontiers of Radiation Therapy and Oncology.* Basel: Karger, 1970: Vol. 5, pp. 40–57.