Eur J Cancer Clin Oncol, Vol. 19, No. 4, pp. 527–532, 1983.
Printed in Great Britain.

0277-5379/83/040527-06$03.00/0

Effect of Misonidazole on the Radiosensitivity and Repair of Potentially Lethal Damage of L5178Y Ascites Tumor Cells*

KEISUKE NAKAGAWA,† HIROSHI TSUNEMOTO‡ and IKUO WATANABE||¶

†*Nihon University School of Dentistry at Matsudo, Matsudo, Chiba 273, Japan,* ‡*Division of Clinical Research and* ||*Division of Physiology and Pathology, National Institute of Radiological Sciences, Anagawa, Chiba 260, Japan*

Abstract—*The radiosensitizing effect of low concentrations of misonidazole was investigated by using L5178Y cells growing as an ascites tumor in DBA-2 mice. The cells were irradiated* in vivo *with graded doses of X-rays in the presence or absence of* 0.1–0.5 mg/g *body weight of misonidazole. Then cell survival was assayed* in vitro *by plating cells in soft agar medium. By analyzing the X-ray survival curves with or without misonidazole, the dose-modifying effects were determined. The results indicated that the slope and shoulder of the survival curves were greatly modified by the treatment with misonidazole. The dose-modifying factor in terms of the* D_0 *ratio between the drug-treated and untreated control cells was increased as the drug concentration was increased. Further, it was revealed that the isoeffect dose ratios, estimated by the linear quadratic equation of Chadwick and Leenhouts, were higher at a low-radiation dose range. This is due to the suppression of the shoulder region of survival curves for the drug-treated cells. The inhibition of the X-ray-induced repair of potentially lethal damage was apparent with* 0.1 mg/g *body weight of misonidazole. The inhibition became more effective as the drug concentration increased.*

INTRODUCTION

THE EXISTENCE of radioresistant hypoxic tumor cells is one of the major limiting factors in conventional radiotherapy for the local control of tumors. The accumulating experimental results have shown that misonidazole has an advantage in reducing the limiting factors by sensitizing hypoxic cells in tumor [1–4]. The X-ray-induced ionization in the target molecules and the chain of reactions of the irradiated molecules with those of electron-affinic sensitizers is assumed to be one of the main reactions for the phenomenon of hypoxic cell sensitization. However, in spite of the extensive works on the action of misonidazole, the effect of low concentrations of the drug on sensitization as well as on cellular repair of tumor cells from X-ray-induced potentially lethal damage (PLD) has not yet been clarified. Clinically, lower drug concentrations are desirable for patients in order to find relief from neurotoxicity.

In the present study we have confirmed that relatively low concentrations of misonidazole are still effective for sensitizing hypoxic L5178Y tumor cells. The experimental results indicated that drug concentrations as low as 0.1 mg/g body weight are effective not only for sensitizing hypoxic cells *in vivo* but also for inhibition of PLD repair.

MATERIALS AND METHODS

Animals and tumor

The experimental animals used were 8- to 10-week-old DBA-2 mice. The L5178Y ascites tumor cells, a subline of the tissue-cultured L5178Y cells, were grown in the peritoneal cavity of DBA-2 mice. The inoculum size was 1×10^7 cells in 0.1 ml. About 10 days after transplantation, cells

Accepted 29 September 1982.
*This work was supported in part by the Grant-in-Aid from the Ministry of Education, Science and Culture and the Ro-Research Society.
¶To whom requests for reprints should be addressed.

were fully grown in the peritoneum and reached a plateau phase.

Assay method

Small cell samples were drawn from the experimental animals, diluted with Fischer's medium to a desired concentration and inoculated into a soft agar medium consisting of Fischer's medium supplemented with 15% horse serum and 0.125% agar. The cultures were then incubated for 2 weeks in a humidified 5% CO_2 gas atmosphere. The surviving fraction of cells was determined by counting the number of surviving cell colonies relative to that of the untreated controls.

Drug

Misonidazole, kindly provided by Nippon Roche Ltd, was dissolved in physiological saline at a concentration of 25 mg/ml immediately before use. An appropriate volume of misonidazole solution was directly injected in the peritoneal cavity of each tumor-bearing mouse.

Irradiation

The mice were irradiated without anesthesia, and X-irradiation was performed under the conditions of 200 kVp, 20 mA, target-tumor distance 63 cm, filtration with 0.5 mm Cu and 0.5 mm Al, half-value layer 1.2 mm Cu and 0.589 Gy/min dose rate. The interval between drug administration and X-irradiation was 30 min unless specified.

RESULTS

Examination of hypoxic condition

Cell survival was first examined under conditions of full growth of the tumor. Mice subjected to cervical dislocation were incubated at 37°C for various time intervals in order to establish the oxygen effect of the peritoneal environment for cell survival. The cessation of respiration after cervical dislocation renders the cells hypoxic, due to the interruption to the oxygen supply, and this may increase cell survival if a significant amount of oxygen existed at the time of cervical dislocation. The mice so treated were irradiated at 15 min intervals with 5.7 Gy of X-rays. Immediately after irradiation, ascites tumor cells were removed from the mice and cell survival was tested. Figure 1 shows the change in the fraction of surviving tumor cells as a function of incubation time. There was no significant difference in cell survival up to 60 min after cervical dislocation. The results suggest that the tumor cells are already in a hypoxic condition at the time of full growth in the peritoneum.

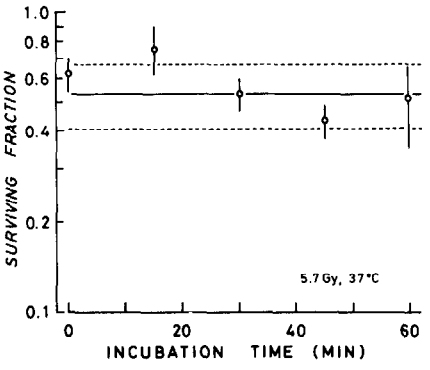

Fig. 1. Surviving fractions of L5178Y ascites tumor cells. The tumor-bearing mice were killed at time 0, incubated at 37°C for various time intervals and irradiated with 5.7 Gy of X-rays. The cells were harvested immediately after irradiation and plated in soft agar medium. Each point and bar represents mean and standard deviation. Horizontal solid and dotted lines indicate mean and standard deviation for pooled data throughout the 60-min period respectively.

Table 1. Plating efficiency of cells treated for 75 min with various concentrations of misonidazole at 37°C

Concentration of drug (mg/g body weight)	Plating efficiency (%)	Relative to control
Control	16.6 ± 10.1	1.00
0.1	15.5 ± 6.0	0.93
0.2	17.1 ± 8.8	1.03
0.5	17.1 ± 9.8	1.03

Cytotoxicity of misonidazole

In order to investigate the cytotoxicity of misonidazole itself, tumor cells fully grown in the peritoneal cavity were treated for 75 min with various concentrations of misonidazole. Then plating efficiency for each sample was tested in soft agar medium, as shown in Table 1.

The results indicate no significant cytotoxic effect of the drug until 75 min after administration, even at the highest dose used.

Cell killing in the presence of misonidazole

Three concentrations of misonidazole, 0.1, 0.2 and 0.5 mg/g body weight, were administered to the experimental mice intra-peritoneally 30 min before X-irradiation. After irradiation the tumor cells were harvested and inoculated into soft agar medium for the assay of survival. Survival was established at several different levels of X-ray doses in order to establish a dose-response relationship. The results are presented in Fig. 2.

Table 2 shows the parameters characteristic of each survival curve. These were obtained by fitting the survival data to multi-target [5] and linear quadratic [6] models. The survival curves for the misonidazole treated cells were character-

Table 2. Cell survival parameters and their confidence limits at 95%

Concentration of drug (mg/g body weight)	Multi-target model*		Linear quadratic model*	
	D_q (Gy)	D_0 (Gy)	α ($\times 10^3$)	β ($\times 10^6$)
0	2.46 ± 0.46	4.41 ± 0.42	0.44 ± 0.20	1.09 ± 0.28
0.1	1.62 ± 0.16	3.55 ± 0.11	1.35 ± 0.06	0.63 ± 0.40
0.2	0.49 ± 0.36	3.17 ± 0.30	2.63 ± 0.57	0.12 ± 1.01
0.5	-0.02 ± 0.10	2.38 ± 0.09	3.78 ± 0.33	0.23 ± 7.75

*See references [5] and [6].

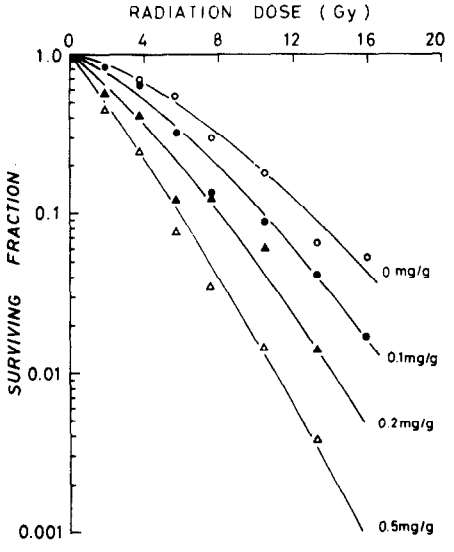

Fig. 2. Survival curves of hypoxic L5178Y ascites tumor cells irradiated with X-rays in the absence or presence of different concentrations of misonidazole. each point represents a mean value from 16 or more samples. Deviation bars were omitted for clarity. The solid lines were drawn based on the survival parameters presented in Table 1.

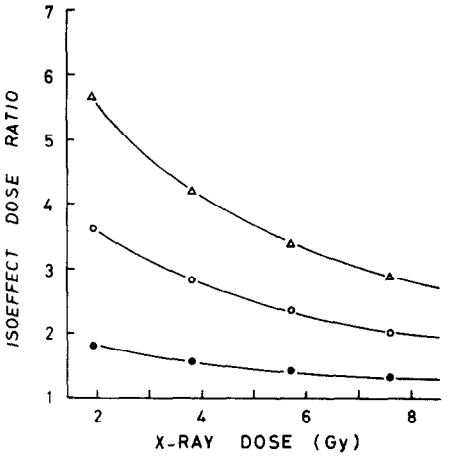

Fig. 3. Isoeffect dose ratios for each drug concentration against X-ray doses. The curves represent drug concentration at 0.1 (●), 0.2 (○) and 0.5 (△) mg/g body weight respectively (see text for detailed explanation).

ized by a reduction of D_0 and D_q doses when compared to the drug-free controls. The dose-modifying factor (DMF), in terms of the D_0 ratio (D_0 without drug/D_0 with drug), was found to vary proportionally as the drug concentration increased; namely, DMF was 1.3, 1.5 and 1.8 with doses of 0.1, 0.2 and 0.5 mg/g body weight respectively. In order to see the modification of the shoulder region more precisely, isoeffect dose ratio was determined. Namely, the theoretical surviving fraction for untreated cells after a certain dose (control dose) was calculated by using a linear quadratic equation [6] and the parameters α and β in Table 2. Then the theoretical radiation dose required by misonidazole treated cells to yield the same surviving fraction (treated dose) was estimated. The isoeffect dose ratio was determined by dividing the control dose by the treated dose. The resulting ratios were plotted against the control doses, as shown in Fig. 3.

The results in Fig. 3 demonstrate that the isoeffect dose ratios varied with both radiation dose and concentration of drug administered. The increase of the ratio at a low-radiation dose range is due to the suppression of the shoulder region of survival curves for the drug-treated cells.

Inhibition of cellular repair from X-ray-induced potentially lethal damage

To examine the effects of the drug on repair from PLD, tumor-bearing mice were killed and irradiated with 5.7 Gy of X-rays 30 min after misonidazole administration. They were incubated at 37°C for 6 hr. The tumor cells were harvested at 2-hr intervals and plated in soft agar medium for survival assay. Figure 4 shows the change in cell survival during 6 hr of post-irradiation incubation time. The survival for the cells treated with X-irradiation alone was increased as the interval between irradiation and the time of plating cells was prolonged. The result for the drug-free controls indicated that PLD repair took place, increasing cell survival maximally at 6 hr by a factor of 1.9. The cells which were treated with 0.1 mg/g body weight of the drug and 5.7 Gy of X-rays showed a slight increase in survival after 2 hr but plateaued until 6 hr. The maximal increase obtained was a factor of 1.4. On the other hand, cells treated with 0.5 mg/g body weight of the drug and 5.7 Gy of X-

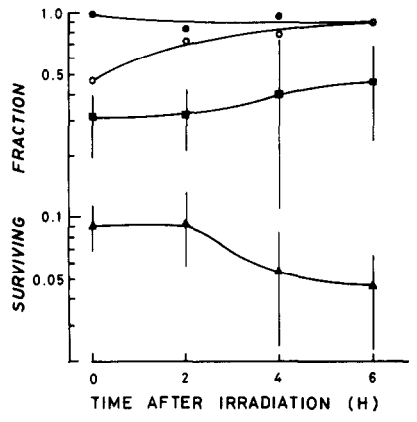

Fig. 4. Change in fraction of surviving cells by repair from potentially lethal damage. The top curve (●) represents the cells treated with misonidazole (0.5 mg/g) alone. The second curve (○) indicates the time-dependent increase of the surviving fraction after 5.7 Gy of X-rays. The third (■) and fourth (▲) curves represent the time-dependent change in the surviving fraction after 5.7 Gy X-rays in the presence of 0.1 and 0.5 mg/g body weight misonidazole respectively. Standard deviation bars for X-ray or misonidazole administration alone were omitted for clarity.

rays showed a decrease in survival, down to 50% of the initial level after 2 hr. Although the experiments in Fig. 4 were repeated 4 times, there was much fluctuation in survival values. The results in Fig. 4 indicate that PLD repair in X-irradiated cells was partially suppressed by the presence of misonidazole 0.1 mg/g, but completely by 0.5 mg/g.

DISCUSSION

Radiosensitizing effects of misonidazole on hypoxic mammalian cells have been examined by the use of various lines of tissue-cultured and transplantable tumor cells [7–23]. By using L5178Y ascites tumor cells, we took technical advantage of the following factors: cells were easily made hypoxic by cervical dislocation; the drug was administered *in vivo* and irradiation was *in vivo*; and quantitative assay of cell survival was possible by plating cells in soft agar medium. It was found that the hypoxic condition was automatically achieved by using cells at full density of growth. As shown in Fig. 1, the results indicated that there was no significant difference in surviving fractions between 0 and 60 min of incubation at 37°C in dead mice.

The drug doses we used in the present study were found to bring about no significant cytotoxic effect as measured by cell survival at 75 min after drug administration. Although a slight reduction of cell survival was observed after a long incubation time (2–6 hr) with misonidazole 0.5 mg/g, it was not statistically significant. The dose of 0.1 mg/g body weight for mice is presumably equivalent to about 100 mg/kg for the human body. Dische *et al.* [24] have reported that the human body can tolerate 30–120 mg/kg/fraction for repeated treatment over 3- to 4-week periods. The dose we used in the present study (0.1 mg/g) would be an applicable one for human radiotherapy.

The survival curves obtained from misonidazole-treated cells were characterized not only by a reduction of the D_0 dose but also by a reduction of cell survival at the shoulder region. It has been shown by Wong *et al.* [25] and Whitmore *et al.* [26] that reduction of survival at the shoulder region may be due to cytotoxic effects of misonidazole metabolites. Thus it may be reasonable to assume that misonidazole metabolites reduced survival at the shoulder region and caused high isoeffect dose ratios at the low-radiation dose range.

When the tumor cells are irradiated in an unfavorable condition for cell growth and plating of cells is delayed after X-irradiation, the cell survival usually increases as a function of post-irradiation incubation time [27]. This phenomenon is operationally defined as the cellular repair from PLD given by ionizing radiation. The maximal increase of cell survival was by a factor of 1.9 when the cells at full density of growth were irradiated with 5.7 Gy of X-ray alone. In the presence of 0.1 mg/g misonidazole, the repair factor expressed as the ratio of maximal survival increase was found to be reduced to 1.4. Further, in the presence of misonidazole 0.5 mg/g the survival was reduced to 50% of the initial level (0 hr) within 4–6 hr after irradiation. Although the reason why such an unexpected reduction of cell survival took place is not clear at present, it may be reasonable to assume that doses higher than 0.5 mg/g body weight of misonidazole or its metabolites strongly suppressed PLD repair, but also reduced survival of hypoxic L5178Y cells during a long incubation period after irradiation. Suppression of PLD repair has been reported by Guichard *et al.* [28] and Sakamoto and Aritake [29]. Their results indicate that PLD repair was inhibited by factors of 1.3 and 1.23 with doses of misonidazole of 1.0 mg/g and 0.1 mg/g body weight in the case of human melanoma cells in nude mice [28], and by a factor of about 3 with a dose of 0.05 mg/ml in the case of Chinese hamster CHO cells [29]. On the contrary, McNally and Sheldon [30] reported that PLD repair occurred in MT solid tumor cells whether or not the misonidazole was present and did not affect the DMF. To explain the discrepancy between our results and theirs, experimental procedure must be considered as an important factor. Namely, in McNally and Sheldon's procedure the mice were allowed to survive until cell survival was assayed.

Since the half-life of the drug injected into mice is about 2 hr [31], the effective dose might decrease during the 6-hr repair time in their procedure. In our procedure the mice were killed immediately prior to irradiation so that they might retain the drug at a high concentration throughout the 6-hr repair time. Taking into account the half-life of the drug in human blood plasma [26], our procedure might be better for delayed survival assay than by allowing the mice to survive.

In conclusion, low concentrations of misonidazole in the cellular environment are still effective in the inactivation of hypoxic cells by the combined action of radiosensitization and inhibition of PLD repair.

Acknowledgements—We thank Drs S. Shibata and K. Fukuhisa for their permission to use computer program (EDCAL2) and helpful assistance at the computation.

REFERENCES

1. ADAMS GE. Hypoxic cell sensitizers for radiotherapy. *Int J Radiat Oncol Biol Phys* 1978, **4**, 135–141.
2. FOSTER JL. Differential cytotoxic effects of metronidazole and other nitro-heterocyclic drugs against hypoxic tumor cells. *Int J Radiat Oncol Biol Phys* 1978, **4**, 153–156.
3. DENEKAMP J, FOWLER JF. Radiosensitization of solid tumours by nitroimidazoles. *Int J radiat Oncol Biol Phys* 1978, **4**, 143–151.
4. DISCHE S. Hypoxic cell sensitizers in radiotherapy. *Int J Radiat Oncol Biol Phys* 1978, **4**, 157–160.
5. OKADA S. Survival curves and target theory (definitions of D_{37}, D_0, D_q, and n). In: ALTMAN KI, GERBER GB, OKADA S, eds. *Radiation Biochemistry*. Academic Press, New York, 1970, Vol. 1, Appendix 2, 329–336.
6. CHADWICK KH, LEENHOUTS HP. A molecular theory of cell survival. *Phys Med Biol* 1973, **18**, 78–87.
7. SHELDON PW, FOSTER JL, FOWLER JF. Radiosensitization of C3H mouse mammary tumours by a 2-nitroimidazole drug. *Br J Cancer* 1974, **30**, 560–565.
8. BROWN JM. Selective radiosensitization of the hypoxic cells of mouse tumors with the nitroimidazoles metronidazole and Ro 7-0582. *Radiat Res* 1975, **64**, 633–647.
9. DENEKAMP J, HARRIS SR. The response of a transplantable tumor to fractionated irradiation 1. X rays and the hypoxic cell radiosensitizer Ro-07-0582. *Radiat Res* 1976, **66**, 66–75.
10. BROWN JM. Cytotoxic effects of the hypoxic cell radiosensitizer Ro 7-0582 to tumor cells *in vivo*. *Radiat Res* 1977, **72**, 469–486.
11. SHELDON PW, HILL SA. Hypoxic cell radiosensitizers and local control by X-ray of a transplanted tumour in mice. *Br J Cancer* 1977, **35**, 795–808.
12. SHELDON PW, HILL SA. Further investigations of the effects of the hypoxic-cell radiosensitizer, Ro-07-0582, on local control of a mouse tumour. *Br J Cancer* 1977, **36**, 198–205.
13. HILL RP, BUSH RS. The effect of misonidazole in combination with radiation dose fractionation. *Br J Cancer* 1978, **37** (Suppl. 3), 255–258.
14. DENEKAMP J, STEWART FA. Sensitization of mouse tumours using fractionated X-irradiation. *Br J Cancer* 1978, **37** (Suppl. 3), 259–263.
15. MCNALLY NJ, DENEKAMP J, SHELDON P, FLOCKHART IR, STEWART FA. Radiosensitization by misonidazole (Ro 07-0582). The importance of timing and tumor concentration of sensitizer. *Radiat Res* 1978, **73**, 568–580.
16. FLOCKHART IR, SHELDON PW, STRATFORD IJ, WATTS ME. A metabolite of the 2-nitroimidazole misonidazole with radiosensitizing properties. *Int J Radiat Biol* 1978, **34**, 91–94.
17. DENEKAMP J, HARRIS ST. Tests of two electron-affinic radiosensitizers *in vivo* using regrowth of an experimental carcinoma. *Radiat Res* 1975, **61**, 191–203.
18. YUHAS JM, YURCONIC M, KLIGERMAN MM, WEST G, PETERSON DF. Combined use of radioprotective and radiosensitizing drugs in experimental radiotherapy. *Radiat Res* 1977, **70**, 433–443.
19. HOFER KG, HOFER MG, IERACITANO J, MCLAUGHLIN WH. Radiosensitization of hypoxic tumor cells by simultaneous administration of hyperthermia and nitroimidazoles. *Radiat Res* 1977, **70**, 362–377.
20. STONE HB. Enhancement of local tumour control by misonidazole and hyperthermia. *Br J Cancer* 1978, **37** (Suppl. 3), 178–183.
21. RAUTH AM, CHIN J, MARCHOW L, PACIGA J. Testing of hypoxic cell radiosensitizers *in vivo*. *Br J Cancer* 1978, **37** (Suppl. 3), 202–205.

22. OVERGAAD J. Effect of misonidazole and hyperthermia on the radiosensitivity of a C3H mouse mammary carcinoma and its surrounding normal tissue. *Br J Cancer* 1980, **41**, 10–21.
23. MCNALLY NJ. The effect of an hypoxic cell sensitizer on tumour growth delay and cell survival. Implications for cell survival *in situ* and *in vitro*. *Br J Cancer* 1975, **32**, 610–618.
24. DISCHE S, SAUNDERS MI, LEE ME, ADAMS GE, FLOCKHART IR. Clinical testing of the radiosensitizer Ro 07-0582: experience with multiple doses. *Br J Cancer* 1977, **35**, 567–579.
25. WONG TW, WHITMORE GF, GULYAS S. Studies on the toxicity and radiosensitizing ability of misonidazole under conditions of prolonged incubation. *Radiat Res* 1978, **75**, 541–555.
26. WHITMORE GF, GULYAS S, VARGHESE AJ. Sensitizing and toxicity properties of misonidazole and its derivatives. *Br J Cancer* 1978, **37** (Suppl. 3), 115–119.
27. HALL EJ. *Radiobiology for the Radiologist*. Harper & Row Publisher Inc., New York, 1978, 131–134.
28. GUICHARD M, LANGEN-OMRI F, MALAISE EP. Influence of misonidazole on the radiosensitivity of a human melanoma in nude mice: time-dependent increase in surviving fraction. *Int J Radiat Oncol Biol Phys* 1979, **5**, 487–489.
29. SAKAMOTO K, ARITAKE S. Effects of misonidazole on tumor cell radiation sensitivity and potentially lethal damage repair *in vivo* and *in vitro*. *Eur J Cancer Clin Oncol* 1981, **17**, 825–830.
30. MCNALLY NJ, SHELDON PW. The effect of radiation on tumor growth delay, cell survival and cure of the animal using a single tumor system. *Br J Radiol* 1977, **50**, 321–328.
31. FLOCKHART IR, LARGE P, MALCOLM S, MARTEN TR, TROUP P. Pharmacokinetic and metabolic studies of the hypoxic cell sensitizer misonidazole. *Xenobiotica* 1978, **8**, 97–105.