[CANCER RESEARCH 51, 3639-3642, July 15, 1991]

# Improvement in Therapeutic Ratio of Radiotherapy for a Murine Sarcoma by Indomethacin Plus Misonidazole[1]

Luka Milas,[2] Hisao Ito,[3] Toshitake Nakayama, and Nancy Hunter

*Department of Experimental Radiotherapy, The University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030*

## ABSTRACT

**In this study we asked whether the improvement in the therapeutic ratio of radiotherapy by indomethacin (INDO), which potentiates tumor radioresponse through stimulation of the immune system, could be further improved by combining it with the hypoxic cell radiosensitizer misonidazole (MISO). Mice bearing the syngeneic sarcoma fibrosarcoma (8 mm) in the leg were treated with single graded doses of γ-rays to the tumor or with irradiation combined with INDO, MISO, or both drugs. Local tumor control was the end point of tumor radioresponse. In addition, the effect of these drugs on radiation-caused hair loss and leg contractures was assessed. INDO increased tumor radioresponse by a factor of 1.31, but it did not affect either hair loss or leg contractures. MISO increased tumor radioresponse by a factor of 1.86, hair loss by a factor of 1.69, and leg contractures by a factor of 1.54, thus providing only a small therapeutic gain. The combined INDO plus MISO treatment increased tumor radioresponse by a factor of 2.72, which was more than the additive effect of the individual drugs. On the other hand, the combined treatment caused no additional hair loss compared to that caused by MISO only. Overall, our results show that INDO plus MISO treatment increased tumor radioresponse more than INDO or MISO alone and provided a significant therapeutic gain. Furthermore, they illustrate that combinations of two radiopotentiating agents with different mechanisms of action may improve the radiotherapeutic effect.**

## INTRODUCTION

In a series of recent publications, we reported that INDO,[4] an inhibitor of prostaglandin synthesis, augmented the radioresponse of PG-producing murine tumors (1-3). The effect was manifested in the prolongation of tumor growth delay, $TCD_{50}$ reduction, and delay of appearance of postirradiation recurrences. Under similar experimental conditions, INDO either did not influence the radioresponse of a number of normal tissues (2, 4) or protected some of them such as the hematopoietic tissue (5) and the lung (6). These observations show that INDO could improve the therapeutic ratio of radiotherapy.

Based on reports that PGs act as radioprotectors when given before radiation (7, 8), we initially hypothesized (2) that INDO augments tumor radioresponse by lowering the level of PGs in tumors. The hypothesis was not supported by our subsequent study showing that INDO augmented tumor radioresponse when given after irradiation was completed, which indicated that in order to potentiate tumor radioresponse the hypothetical radioprotective PGs need not be removed or reduced at the time of radiation exposure (3). However, the radiopotentiating effect of INDO was abolished or greatly reduced in mice the general immunocompetence of which was suppressed by whole-body irradiation or in nude mice that lack T-lymphocytes (3), implying that the observed radiopotentiation was mainly if not entirely mediated through the antitumor immune mechanisms augmented or elicited by INDO. In contrast, the antitumor activity of INDO alone, manifested by a slowing in tumor growth only, was not dependent on the immunocompetence of the tumor host. Instead, the effect was associated with the reduction in tumor vasculature as demonstrated by an intracutaneous assay for quantifying neoangiogenesis at the site of tumor cell inoculation (3). Hypothetically, suppressed angiogenesis would reduce tumor blood supply and consequently could lead to an increase in hypoxic cell content in tumors.

These observations suggest that tumor radioresponse could be increased even more if INDO is combined with an agent that augments tumor radioresponse through a mechanism different from that of INDO. Here, we tested whether this could be achieved by combining INDO with MISO, a prototype of hypoxic cell radiosensitizers that significantly increases tumor response to ionizing radiation (9, 10). Since INDO inhibits tumor angiogenesis (3), one might expect that the tumor radioresponse enhancement by the combination of INDO and MISO would even exceed the additive effect of these two agents administered separately, because the radiosensitizing effect of MISO is expressed under hypoxic conditions (10). Moreover, this combination would provide a higher therapeutic ratio than the individual treatments because INDO does not sensitize normal tissues to radiation (2, 4) but rather protects some of them (5, 6), whereas MISO can radiosensitize normal tissues (11).

## MATERIALS AND METHODS

**Mice.** We used inbred male or female C3Hf/Kam mice, bred and maintained in our own specific-pathogen-free mouse colony. They were 3 to 4 months old at the beginning of the experiments and were housed 3 to 5/cage. Within each experiment, mice of the same sex were used.

**Indomethacin.** Mice were given INDO (Sigma Chemical Co., St. Louis, MO) or vehicle (0.5% ethanol and 5% phosphate-buffered saline) in the drinking water. INDO was dissolved in absolute ethanol and diluted in distilled water containing 5% phosphate-buffered saline to achieve a final INDO concentration of 35 μg/ml. Water bottles were changed every 3 days. Treatment of mice started when tumors were 6 mm in diameter and was continued for 10 days.

**Misonidazole.** The hypoxic cell radiosensitizer MISO (obtained from the Drug Synthesis and Chemistry Branch, National Cancer Institute, Bethesda, MD) was dissolved in Ringer's solution and injected i.p. at a dose of 1 mg/g body weight 30 min before irradiation.

**Tumor Response to Radiation.** Experiments were performed using the immunogenic methylcholanthrene-induced FSA, which is syngeneic to C3Hf/Kam mice. Single-cell suspensions were prepared by trypsin digestion of nonnecrotic tumor tissue (12). Viability of cells was more than 95% as assessed by phase-contrast microscopy and trypan blue exclusion. Tumors were generated by injecting $5 \times 10^5$ viable FSA cells

Received 1/7/91; accepted 5/7/91.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This investigation was supported by NIH Research Grant CA-06294. Animals used in this study were maintained in facilities approved by the American Association for Accreditation of Laboratory Animal Care and in accordance with current regulations and standards of the United States Department of Agriculture and Department of Health and Human Services.

[2] To whom requests for reprints should be addressed, at Department of Experimental Radiotherapy, The University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Blvd., Houston, TX 77030.

[3] Present address: Department of Radiology, Keio University, School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo 160, Japan.

[4] The abbreviations used are: INDO, indomethacin; MISO, misonidazole; FSA, fibrosarcoma; $TCD_{50}$, dose of radiation yielding local tumor control in 50% of animals; PG, prostaglandin.

into the right thighs of mice. When tumors grew to 6 mm in diameter, the mice were treated with INDO or vehicle daily for 10 consecutive days. Tumor growth was determined by measuring 3 mutually orthogonal diameters with a Vernier caliper. When tumors grew to 8 mm, they were exposed to single doses of γ-radiation delivered from a dual-source $^{137}Cs$ irradiator at a dose rate of 7.5 Gy/min. During irradiation, the unanesthetized mice were immobilized in a jig, and the tumor was centered in a circular radiation field 3 cm in diameter. Mice were checked for the presence of tumors at the irradiated site at 2- to 7-day intervals for up to 120 days after irradiation. The effect of radiation with or without INDO and MISO was expressed by the $TCD_{50}$. $TCD_{50}$ was computed by the logit methods of analysis (13).

**Response of Normal Tissues to Radiation.** Hair loss (epilation) was examined on irradiated legs of mice in the $TCD_{50}$ experiment 35 days after irradiation. Only mice having no recurrent tumors were used for the determination of radiation-induced hair loss. At each irradiation dose point, the number of mice having 100% epilation was scored. The dose of radiation yielding complete hair loss in 50% of animals was then determined by the logit method of analysis (13).

Radiation-induced leg contracture (reduction in the leg extension) was also determined on mice in the $TCD_{50}$ assay that had no recurrent tumors. Measurements were made using a ruled Lucite jig 120 days after irradiation (14), and the length of the irradiated leg was subtracted from that of the nonirradiated leg.

## RESULTS

$TCD_{50}$ assays were performed with 8-mm FSA in normal mice or mice treated with INDO, MISO, or both. Table 1 shows $TCD_{50}$ values, and Fig. 1 shows tumor control curves at 120 days after tumor irradiation. INDO reduced the control $TCD_{50}$ value by a factor of 1.31, whereas MISO reduced it even more, by a factor of 1.86. The slopes of the radiation dose-response curves in control and MISO-treated animals were similar and steep. In contrast, the INDO curve had a shallow slope, indicating a significant heterogeneity in the tumor radioresponse of this agent. The combined treatment with INDO and MISO reduced the $TCD_{50}$ value by a factor of 2.72, which is more than the additive effect of INDO and MISO. The slope of the radiation response curve in this group was similar to that in the MISO group only.

The effect of the above treatments on radiation-caused hair loss is shown in Table 1. While the dose of radiation yielding complete hair loss in 50% of animals was not affected by INDO, it was significantly reduced by MISO (by a factor of 1.69). The combination of MISO and INDO was not more deleterious than MISO alone. Similarly, while INDO did not influence the severity of radiation-induced leg contractures, MISO significantly enhanced it (Fig. 2). At the contracture level of 4 mm, the enhancement ratio was 1.54. Also, MISO plus INDO was not more effective in this respect than MISO only. MISO not only increased the effect of radiation but also made the slope of the leg contracture response curve more steep.

## DISCUSSION

The observations described here showed that two agents, INDO and MISO, having different mechanisms for augmenting tumor radioresponse can be successfully combined to improve the efficacy of local tumor irradiation more than would be achieved by one agent. The therapeutic ratio was even more improved because INDO and MISO also differed in their action on radioresponse of normal tissues, in that INDO was noneffective while MISO increased the response, although to a lesser degree than that of tumors. We recently discovered that INDO potentiates tumor radioresponse through the immune system (3), whereas MISO is a highly investigated prototype of hypoxic cell radiosensitizing agents (9, 10).

When given as the only agent, INDO potentiated tumor radioresponse by a factor of 1.31, a degree of potentiation for the FSA tumor similar to that reported in our earlier publications using the $TCD_{50}$ assay (2, 3). However, as already reported (2, 3), the extent of this potentiation can vary from a factor of 1.26 to more than 2, depending on tumor type, choice of fractionation, timing of INDO administration in relation to tumor irradiation, the end point of tumor response, etc. It should also be noted that INDO can augment the radioresponse of PG-producing tumors only (1, 2). A significant feature of the INDO effect on tumor radioresponse is that it flattens the slope of the tumor cure response curve compared to that for tumors exposed to radiation only (Fig. 1). A similar effect was reported by us (9) and others (15) for *Corynebacterium parvum*, a potent

Table 1 *Effect of indomethacin and misonidazole on radiocurability of FSA tumors and on radiation-induced hair loss in the leg of C3Hf/Kam mice*

| | Radiocurability | | Hair loss | |
|---|---|---|---|---|
| Treatment[a] | $TCD_{50}$ (Gy) | EF[b] | $ED_{50}$ (Gy)[b] | EF |
| Control | 37.5 (36.3–38.8) | | 36.1 (32.6–38.0) | |
| INDO[c] | 28.7 (26.3–31.5) | 1.31 | 37.7 (35.8–39.2) | |
| MISO[d] | 20.2 (19.2–21.3) | 1.86 | 21.3 (19.2–22.6) | 1.69 |
| INDO + MISO | 13.8 (11.6–15.7) | 2.72[e] | 22.4 (20.8–24.1) | 1.61 |

[a] Mice with 8-mm FSA in the right hind thighs were exposed to single doses of radiation over that thigh. The data presented here are from 2–6 separate experiments.
[b] EF, enhancement factor; $ED_{50}$, dose of radiation yielding complete hair loss in 50% of animals.
[c] Treatment with INDO, 35 μg/ml in drinking water, was started when FSA tumors were 6 mm in diameter and was continued for 10 days.
[d] MISO (1 mg/g) was given i.p. 30 min before local tumor radiation.
[e] The enhancement factor for the combination of MISO plus INDO compared to INDO alone is 2.1.

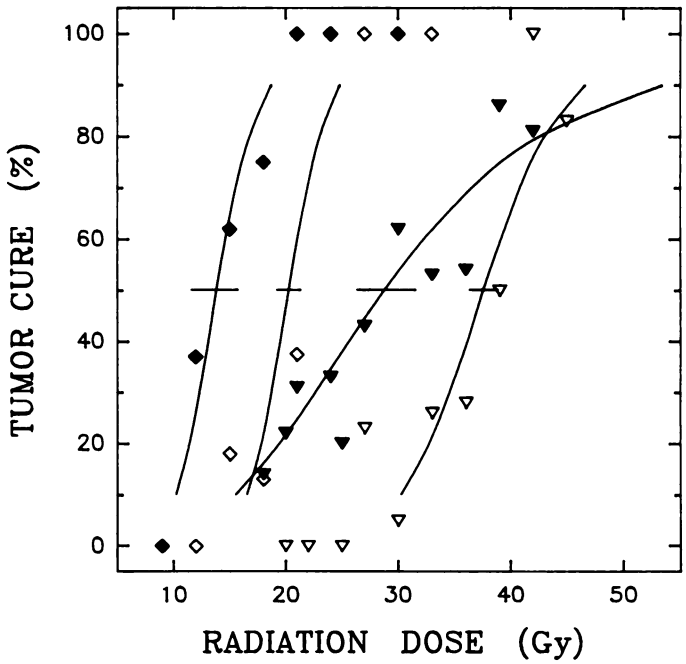


Fig. 1. Radiation dose-response curves for local tumor control of 8-mm FSA tumors growing in the legs of mice treated with radiation alone (▽) or with radiation plus INDO (▼), MISO (◇), or both INDO and MISO (◆). *Error bars*, $TCD_{50}$, 95% confidence limits. Treatment with INDO, 35 μg/ml in drinking water was started when FSA tumors were 6 mm and was continued for 10 days. MISO (1 mg/g) was given i.p. 30 min before local tumor irradiation. *Points*, data from 2–6 separate experiments.

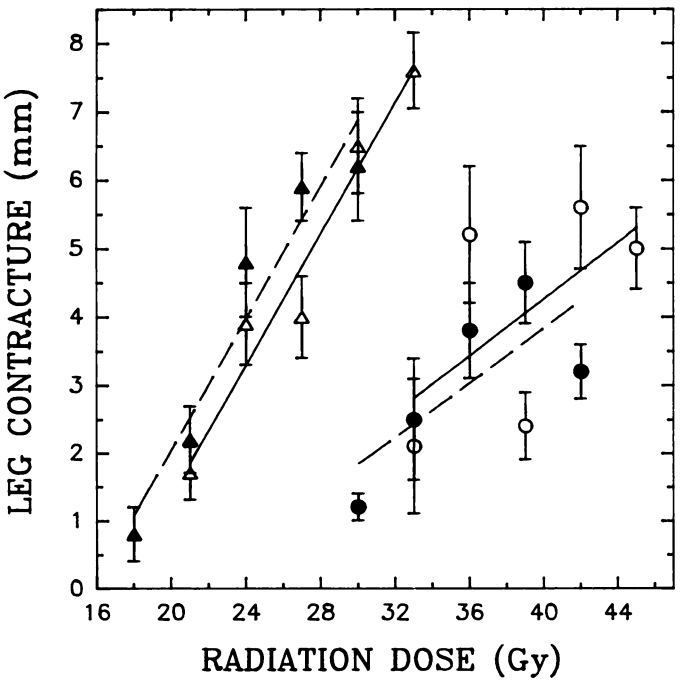


Fig. 2. Extent of leg contractures at 120 days after exposure of legs to single doses of radiation alone (○) or in combination with INDO (●), MISO (△), or both (▲). *Points*, mean leg contracture of 4 to 16 mice at a given radiation dose; *bars*, SE. *Straight lines* were obtained by linear regression analysis of the data. *Dashed lines*, INDO data; *solid lines*, data from mice not treated with INDO. The data presented here are from 3 separate experiments.

biological response modifier with strong antitumor activity. The observed shallow slope of tumor radioresponse caused by INDO implies greater heterogeneity in tumor radiocurability as compared to the control, and it probably reflects a wide variation in which antitumor immune mechanisms it elicits among tumor-bearing hosts. Such wide variation was demonstrated in mice exposed to *C. parvum* and local tumor irradiation (9).

MISO augmented tumor radioresponse more efficiently than INDO. The enhancement factor was 1.86, which is similar to that reported earlier by us for the same tumor (9). MISO is particularly effective as a specific sensitizer of hypoxic cells in the single radiation dose $TCD_{50}$ assay, because tumor cell survival in this assay reflects the survival of hypoxic cells. The improvement in tumor radiocurability by the combination of MISO with INDO exceeded the additive effects of individual treatments; the enhancement factor was 2.72 compared to the additive-action factor of 2.44 (obtained by multiplying the enhancement factor of 1.31 produced by INDO by that of 1.86 produced by MISO).

The reasons for the more than additive effect of the INDO plus MISO combination have not been investigated in the present study. Based on the currently available information on the activity of INDO and MISO a number of factors could account for this. Because INDO suppresses tumor vascularization (3) it is reasonable to assume that it could lead to an increase of hypoxia in tumors. This condition should result in greater radiosensitization by MISO. Another possibility is that due to its antiangiogenic effect, INDO could increase the volume of necrosis in the tumor and thus reduce the number of clonogenic cells, which would then require a lower dose of radiation to be inactivated. This possibility, however, appears unlikely as the necrotic fraction of 8-mm FSA, treated or untreated with INDO, is less than 2%.[5] On the other hand, the INDO-treated tumors do contain fewer cells in actively proliferating phases of cell cycle than the same-size untreated tumors (1), which would indirectly favor the possibility that INDO increases the hypoxic cell fraction. MISO was more effective in INDO-treated than in untreated mice; it decreased the $TCD_{50}$ by a factor of 2.1 in the former and by a factor of 1.86 in the latter.

The present study investigated the effect of the INDO and MISO combination on tumor response to large single doses of ionizing radiation. Whether the observed augmentation of tumor radioresponse will hold in settings in which tumors were exposed to fractionated irradiation with a range of doses used in the clinic is not known and warrants further investigation. However, based on earlier studies with both INDO and MISO, one could anticipate a significant improvement in tumor radiocurability. The MISO-induced tumor radiosensitization to fractionated radiation treatment is generally reduced (10). On the other hand, INDO was significantly more effective in increasing tumor radioresponse to fractionated as compared to single-dose irradiation (3).

The combination of INDO and MISO augmented normal tissue injury by ionizing radiation, notably hair follicle injury, which was responsible for radiation-induced hair loss, and the injury of tissues responsible for radiation-caused leg contractures. By itself, INDO did not alter the radioresponse of these tissues. Only MISO augmented it, by a factor of 1.69 for hair loss and 1.54 for leg contractures. These values remained unchanged when INDO and MISO were combined. The radiation dose-response curve for leg contractures was shallower for mice exposed to radiation only than that for MISO-treated mice. Since MISO augmented the radioresponse at higher doses of irradiation, hypoxic cells in the normal tissues of the leg most likely caused the slope of the leg contracture curve to be shallow.

These observations show that treatment with MISO alone would result in only a small therapeutic gain since, in spite of a remarkable increase in tumor radiocurability, normal tissue response was also augmented, although not as much as tumor response. On the other hand, although INDO enhanced tumor radiocurability less effectively than MISO, it did not influence the radioresponse of the two normal tissues. Thus, its therapeutic gain was slightly higher than that of MISO. However, a remarkable therapeutic benefit was achieved by combining the two agents. The combination increased tumor radiocurability more than the additive action of the individual agents, whereas normal tissue injury remained the same as that caused by MISO alone. The therapeutic gain could have been even greater if hematopoietic tissue and the lung were used for the assessment of normal tissue damage since these two tissues are protected by INDO against radiation injury (5, 6). We conclude, therefore, that agents differing in their mechanisms for augmenting tumor radioresponse can be successfully combined to improve the therapeutic gain of local tumor irradiation.

## ACKNOWLEDGMENTS

We wish to thank Patricia Norfleet for her assistance in the preparation of the manuscript. We are grateful to Lane Watkins and his staff for the supply and care of the animals used in these studies.

[5] L. Milas, Y. Furuta, and N. Hunter, unpublished observations.

## REFERENCES


1. Furuta, Y., Hall, E. R., Sanduja, S., Barkley, T., Jr., and Milas, L. Prostaglandin production by murine tumors as a predictor for therapeutic response to indomethacin. Cancer Res., *48:* 3002–3007, 1988.
2. Furuta, Y., Hunter, N., Barkley, T., Jr., Hall, E., and Milas, L. Increase in radioresponse of murine tumors by treatment with indomethacin. Cancer Res., *48:* 3008–3013, 1988.
3. Milas, L., Furuta, Y., Hunter, N., Nishiguchi, I., and Runkel, S. Dependence of indomethacin-induced potentiation of murine tumor radioresponse on tumor host immunocompetence. Cancer Res., *50:* 4473–4477, 1990.
4. Milas, L., Nishiguchi, I., Hunter, N., Murray, D., Fleck, R., Ito, H., and Travis, E. Radiation protection against early and late effects of ionizing irradiation by the prostaglandin inhibitor indomethacin. *In:* Advances in Space Research, in press, 1991.
5. Nishiguchi, I., Furuta, Y., Hunter, N., Murray, D., and Milas, L. Radioprotection of hematopoietic tissues in mice by indomethacin. Radiat. Res., *122:* 188–192, 1990.
6. Fleck, R., and Travis, E. L. The effect of indomethacin on normal tissue radiation response (Abstract). *In:* 37th Annual Meeting of the Radiation Research Society, p. 78, 1989.
7. Hanson, W. R., and Ainsworth, E. J. 16,16-Dimethylprostaglandin $E_2$ induces radioprotection in murine intestinal and hematopoietic stem cells. Radiat. Res., *103:* 196–202, 1985.
8. Walden, T. L., Jr., Patchen, M., and Snyder, S. L. 16,16-Dimethylprostaglandin $E_2$ increases survival in mice following irradiation. Radiat. Res., *109:* 440–448, 1987.
9. Stone, H., and Milas, L. Modification of radiation response of murine tumors by misonidazole (R007-0582), host immune capability, and *Corynebacterium parvum.* J. Natl. Cancer Inst., *60:* 887–893, 1978.
10. Brown, J. M., Biaglow, J. E., and Hall, E. J. Sensitizers and protectors to radiation and chemotherapeutic drugs. Cancer Treat. Symp., *1:* 85–101, 1984.
11. Suit, H. D., Maimonis, P., Michaels, H. B., and Sedlacek, R. Comparison of hyperbaric oxygen and misonidazole in fractionated irradiation of murine tumors. Radiat. Res., *87:* 360–367, 1981.
12. Milas, L., Hunter, N., Mason, K., and Withers, H. R. Immunological resistance to pulmonary metastases in C3Hf/Bu mice bearing syngeneic fibrosarcoma of different sizes. Cancer Res., *34:* 61–71, 1974.
13. Finney, D. J. Quantal responses and the tolerance distribution. *In:* Statistical Methods in Biological Assay, pp. 454–456. London: Griffen, 1952.
14. Hunter, N., and Milas, L. Protection by *S*-2-(3-aminopropylamino)-ethylphosphorothioic acid against radiation-induced leg contractures in mice. Cancer Res., *43:* 1630–1632, 1983.
15. Suit, H. D., Sedlacek, R., Wagner, M., Orsi, L., Silobrcic, V. and Rothman, K. J. Effect of *Corynebacterium parvum* on the response to irradiation of a C3H fibrosarcoma. Cancer Res., *36:* 1305–1314, 1976.