Interaction of SR-4233 with Hyperthermia and Radiation in the FSAIIC Murine Fibrosarcoma Tumor System in Vitro and in Vivo

Terence S. Herman, Beverly A. Teicher, and C. Norman Coleman

Dana-Farber Cancer Institute [T. S. H., B. A. T.] and Joint Center for Radiation Therapy [T. S. H., B. A. T., C. N. C.], Boston, Massachusetts 02115

ABSTRACT

The effects of SR-4233 (3-amino-1,2,4-benzotriazine-1,4-dioxide), a hypoxic cell cytotoxic agent, were assayed against the FSAIIC murine fibrosarcoma in vitro and in vivo alone and in conjunction with hyperthermia and radiation. In vitro, a concentration of 500 μM of SR-4233 upon exposure of the cells for 1 h decreased the survival of hypoxic cells by about 1 log more than euoxic cells at 37°C and pH 7.40. At the same concentration at pH 6.45, this difference in cytotoxicity increased to about 3 logs. In conjunction with 42 or 43°C hyperthermia at pH 7.40, the killing of both euoxic and hypoxic cells was markedly increased (hypoxic > oxic), and the effect of hyperthermia on SR-4233 cytotoxicity was further increased at pH 6.45. SR-4233 proved to be an effective radiosensitizer of hypoxic cells in vitro, producing an enhancement ratio of 2.6 ± 0.2 at pH 7.40 and 2.7 ± 0.2 at pH 6.45. In vivo, however, SR-4233 (50 mg/kg) used with single dose radiation (10, 20, or 30 Gy) did not alter the slope of the radiation dose-dependent tumor growth delay curve but did produce a significant additive increase in tumor growth delay. Local hyperthermia (43°C, 30 min) plus SR-4233 (30 mg/kg) produced a tumor growth delay of 9.1 ± 2.2 days, whereas SR-4233 alone caused a tumor growth delay of only 1.7 ± 0.9 days and the hyperthermia, only 1.4 ± 0.7 days. The tumor growth delay increased to 28.2 ± 4.4 days with the addition of daily radiation (3 Gy for 5 days) to SR-4233 and hyperthermia given on treatment day 1 only. Hoechst 33342 dye-selected tumor subpopulation analysis at 24 h following treatment demonstrated that SR-4233 (30 mg/kg) was more toxic to dim (presumably hypoxic) cells by about 1.8-fold. The addition of hyperthermia to treatment with SR-4233 increased the killing of dim cells by about 5-fold but of bright cells by only 2-fold. Trimodality treatment with SR-4233, hyperthermia, and radiation increased the killing of bright cells by about 6.5-fold and of dim cells by about 16.5-fold as compared with radiation alone. These results indicate that SR-4233 might be used quite effectively with radiation and/or hyperthermia to treat tumors with significant hypoxic subpopulations.

INTRODUCTION

By radiobiological methods and by direct PO2 measurements, hypoxia is present in most animal solid tumor models. Gatembuy et al. (1) have measured the oxygen distribution in human squamous cell carcinoma metastases and correlated the oxygen tension in these masses with response to radiation therapy. Twelve of 31 tumors in this study had greater than 26% of their volume containing a PO2 less than 8 mm Hg. Vaupel et al. (2) observed in early human breast cancer xenografts growing s.c. in nude rats that, as tumor size increased, the O2 consumption rate rapidly decreased, reflecting progressively deficient blood supply.

Hypoxia has been shown to decrease the cytotoxic effects of several anticancer drugs (3) and radiation therapy (4). Use of a selective hypoxic cell cytotoxic agent, therefore, has good rationale both because many anticancer treatments are less effective in hypoxic environments and because most normal tissues are well oxygenated, and, thus, should be less affected than tumor cells by such drugs. SR-4233, as well as several other 1,2,4-benzotriazine-1,4-dioxides, have been shown to be potent hypoxic cell-selective cytotoxic agents (5-10).

In order to define further the capabilities of SR-4233, we have examined the effect of tumor microenvironmental conditions, including oxygenation level and pH, on the cytotoxicity of this drug alone and with hyperthermia or radiation in vitro and correlated these results with the toxicity of SR-4233 toward Hoechst 33342-defined tumor subpopulations in vivo. In addition, the tumor growth delay produced by SR-4233 with hyperthermia, radiation, and radiation plus hyperthermia was assessed.

MATERIALS AND METHODS

Drugs. SR-4233 was obtained as a gift from the Developmental Therapeutics Program of the National Cancer Institute (Bethesda, MD).

Cell Line. FSAIIC murine fibrosarcoma cells grow as monolayers in o-minimal essential medium (GIBCO, Grand Island, NY), supplemented with 10% fetal bovine serum (Sigma Chemical Co., St. Louis, MO). For experiments, FSAIIC cells were grown in plastic culture flasks and used when in exponential growth phase (11).

Production of Hypoxia. To produce hypoxia, the plastic flasks, containing exponentially growing monolayers in complete medium, were fitted with sterile rubber septa and exposed to a continuously flowing 95% N2/5% CO2 humidified atmosphere for 4 h at 37°C as previously reported (3). Parallel flasks were maintained in 95% air/5% CO2. At the end of 4 h, the drug or vehicle was added to the flasks by injection through the rubber septum without disturbing the hypoxia. Hypoxia was verified using a platinum electrode (Diamond General Corp., Ann Arbor, MI) at various points in the experimental procedure. The PO2 was found to be 2.2 ± 1.1 mm Hg across these measurements.

pH Alterations. The pH of the medium was adjusted using a 95% sodium bicarbonate/5% CO2 buffer system (11). For altered pH experiments, the original bicarbonate-buffered medium (pH 7.40) was replaced with media without NaHCO3. The deletion of bicarbonate resulted in media with a pH of 6.45. Flasks were then purged with either 95% air/5% CO2 for 4 h for normally oxygenated conditions or with 95% N2/5% CO2 for 4 h for hypoxic experiments as stated above (11). Representative flasks were monitored throughout the experimental procedures, and the pH of the media did not vary by more than 0.05 pH unit.

Heat Treatment. Exponentially growing cells were exposed to a temperature of 37, 42, or 43°C for 1 h in a Plexiglas water tank with a continuous in-flow and out-flow system controlled by a water temperature controller (Braun Thermomix 1460; Braun Instruments) (12). Cells underwent heating in sealed plastic flasks (Falcon Plastics) containing 5 ml of complete medium. Water temperature could be maintained at ±0.10°C (SD).

Radiosensitization Experiments. Exponentially growing cells were exposed to 500 μM concentrations of the SR-4233 in 25-cm2 flasks for a total of 1 h. Non-drug-treated controls were handled identically. Addition of the drug solution did not significantly alter the pH of the culture. Normally oxygenated or hypoxic conditions were maintained throughout the radiation treatment. Irradiation was carried out using a 125I radiosensitizing unit (Gammanect 40; Atomic Energy of Canada, Ltd., Oakville, ON). The drug was added to the cells immediately prior to irradiation. The radiosensitization effect was assessed by tumor growth delay and/or by Hoechst 33342 dye-selected tumor subpopulation analysis at 24 h following treatment.
SR-4233 IN COMBINED MODALITY REGIMENS

Pinawa, Manitoba, Canada) at a dose rate of approximately 1.05 Gy/min at 25°C. X-ray doses of 2.5, 5, 10, 15, or 20 Gy were used.

The enhancement ratio was calculated as the slope of the radiation survival curve on the exponential portion of the curve with the sensitizer present divided by the slope of the survival curve without sensitizer present with correction for drug cytotoxicity.

Cell Viability Determination. Cell viability was measured by the ability of single cells to form colonies in vitro, as described previously (3). Each data point per experiment represents the result of three different dilutions of cells plated in duplicate, and each experiment was repeated three to five times.

Tumors. The FSAI fibrosarcoma (13) adapted for growth in culture (FSAIIC) (14) was carried in C3H/FeJ male mice (Jackson Laboratories, Bar Harbor, ME). For the experiments, 2 × 10⁶ tumor cells prepared from a brei of several stock tumors were implanted i.m. into the legs of C3H/FeJ male mice 8–10 weeks of age.

Tumor Growth Delay Experiments. When the tumors were approximately 100 mm³ in volume, treatment was initiated. Animals received either a single dose of 50 mg/kg of SR-4233 or the same volume of vehicle (0.2–0.3 ml of phosphate-buffered normal saline) i.p. Radiation was delivered 1 h after drug or vehicle administration as single doses at 10, 20, or 30 Gy with a ¹³⁷Cs radiation unit GammaCel 40 at a dose rate of approximately 0.88 Gy/min to the tumor-bearing limb (the whole body received less than 2% of the total dose).

For hyperthermia ± radiation studies, in those groups receiving drug, SR-4233 (30 mg/kg) was injected as a single dose i.p. In those groups receiving hyperthermia, heat was delivered as a single dose locally to the tumor-bearing limb by immersion in a specially designed Plexiglas water bath at 44°C which allowed the centers of tumors to reach 43 ± 0.2°C in about 3 min as measured by a digital read-out thermistor (Sensortech, Inc., Clifton, NJ) placed into the center of the tumor in selected control animals as previously described (15). No anesthetic was used. Air from an electric fan was directed at the animals to prevent whole body heating. Hyperthermia was delivered immediately following i.p. injections. Radiation was delivered at 3 Gy immediately following the end of hyperthermia on day 1 and at 3 Gy for the next 4 days as described above. The progress of each tumor was measured three times weekly until it reached a volume of 500 mm³. Tumor growth delay was calculated as the days taken by each individual tumor to reach 500 mm³ compared to the untreated controls (16). Untreated FSAIIC tumors reached 500 mm³ in 13.4 ± 1.6 days. Each treatment group had seven animals, and the experiment was repeated three times. Days of tumor growth delay are the mean ± SEM for the treatment group compared to the control.

Data on the delay of tumor growth were analyzed with a BASIC program for the Apple IIe microcomputer. The program derives the best fit curve for each set of data and then calculates the median, mean, and standard error of the mean for individual tumor volumes and the day on which each tumor reached 500 mm³. The radiation dose-modifying factor was calculated as the slope of the radiation growth delay curve between the 10- and 30-Gy dose with SR-4233 present divided by the slope without use of the drug. Statistical comparisons were made with the Dunn multiple comparisons test (14).

FSAIIC Tumor Subpopulation Studies: Tumor Growth and Hoechst 33342 Labeling. When the FSAIIC tumor volumes were approximately 100 mm³ (about 1 week after tumor cell implantation), animals were treated with a single dose of SR-4233 (30 mg/kg), alone or with a single dose of hyperthermia (43°C, 30 min) and/or with radiation (10 Gy) delivered locally to the tumor-bearing limb as described above.

At 24 h posttreatment, the dye, Hoechst 33342 (2 mg/kg), was injected i.v. The mice were sacrificed 20 min after dye injection, tumors were excised, and single cell suspensions were prepared under sterile conditions for the colony-forming assay (17–20). To remove contaminating erythrocytes, 0.17 M NH₄Cl was added to the tumor cell suspension for 3 min at room temperature just after filtering through gauze. The cells were then washed once with a minimal essential medium supplemented with 10% fetal bovine serum, filtered through a syringe fitted with a 40-μm nylon mesh filter to remove cell clumps, counted, and centrifuged at 200 × g. Cells were then resuspended at a concentration of 2 × 10⁶ cells/ml in complete medium. No significant difference in the number of cells recovered in experimental versus untreated tumors was found. The number of cells per tumor (1.3–1.7 × 10⁶ cells) was independent of the interval between treatment and excision.

Flow Cytometry and Sorting. The fluorescence of the cells from tumors was analyzed and sorted using the Coulter Epics V instrument (Hialeah, FL). Hoechst 33342 dye intensity was measured using an argon ion laser with excitation at 350–360 nm (40 mW power), and emission was monitored with a 457-nm long pass and a 530-nm short pass filter. The fluorescence distributions were divided into 10 fractions based on Hoechst 33342 intensity. Two sort fractions of cells were collected, one containing the brightest 10% of cells and the other containing the dimmest 20% of cells. No significant difference in the fluorescent patterns of the sorted cellular populations at 24 h after treatment was observed as compared with cells from untreated control tumors. The cells were washed once with complete medium. After 1 week, colonies were stained with crystal violet, and colonies of >50 cells were counted manually. The plating efficiency for the unsorted population was 15.5 ± 2.7%. For the 10% brightest cells, the plating efficiency was 9.5 ± 1.8%, and for the 20% dimmest cells, the plating efficiency was 6.4 ± 1.4%. The survival results are expressed as the surviving fraction ± SE of the treated bright and dim fractions compared to the bright and dim untreated controls, respectively.

**RESULTS**

At normal pH (pH 7.40) and 37°C, SR-4233 was much more cytotoxic toward hypoxic FSAIIC cells, such that exposure to a 500 μM concentration of SR-4233 for 1 h killed about 1 log of normally oxygenated cells and about 2 logs of hypoxic cells (Fig. 1). At elevated temperature, there was an increase in the cytotoxicity of SR-4233 to both normally oxygenated and hypoxic FSAIIC cells. At pH 7.4, approximately 3.5 logs of both normally oxygenated and hypoxic FSAIIC cells were killed upon exposure to 500 μM SR-4233 for 1 h at 42°C. Increasing the temperature during drug exposure to 43°C resulted in an additional marked sensitization of hypoxic cells to SR-4233 but almost no change in the killing of normally oxygenated cells compared to the killing seen at 42°C.

SR-4233 proved much more toxic to hypoxic FSAIIC cells at...
at normal pH (pH 7.40) or acidic pH (pH 6.45). No drug, oxygenated cells; ○, no drug, hypoxic cells; ●, drug, oxygenated cells; □, drug, hypoxic cells. Points, means of 3 independent determinations ± SE (bars). The curves were corrected for the cytotoxicity due to drug alone. Points on the y-axis, drug-alone killing.

**Fig. 2.** Radiation survival of FSaIC cells in the presence of 500 μM SR-4233 at normal pH (pH 7.40) or acidic pH (pH 6.45). ○, no drug, oxygenated cells; ●, no drug, hypoxic cells; ○, drug, oxygenated cells; □, drug, hypoxic cells. Points, means of 3 independent determinations ± SE (bars). The curves were corrected for the cytotoxicity due to drug alone. Points on the y-axis, drug-alone killing.

**Fig. 3.** Growth delay of the FSaIC fibrosarcoma produced by radiotherapy and SR-4233. SR-4233 was administered before irradiation. Treatment groups were: radiation with 10, 20, or 30 Gy/carbogen (●); SR-4233 (50 mg/kg) and the irradiation (■); irradiation and then SR-4233 (△). Points, means of 3 independent experiments; bars, SEM.

pH 6.45 and 37°C than at pH 7.40 and 37°C. In contrast, the cytotoxicity of SR-4233 in normally oxygenated cells was not affected by the acid extracellular pH. As the temperature was increased to 42°C and then to 43°C, the cytotoxicity of SR-4233 toward hypoxic cells further increased, whereas the cytotoxicity of SR-4233 toward normally oxygenated cells at pH 6.45 and elevated temperature increased to a lesser extent.

SR-4233 at a concentration of 500 μM was a highly effective radiosensitizer of hypoxic FSaIC cells at both normal pH (pH 7.40) and acidic pH (pH 6.45) (Fig. 2). SR-4233 did not alter the radiation response of normally oxygenated FSaIC cells at either pH. The enhancement ratios under hypoxic conditions were 2.55 ± 0.16 at pH 7.4 and 2.70 ± 0.18 at pH 6.45. Growth delay in FSaIC fibrosarcoma tumors produced by single dose radiation with or without SR-4233 (50 mg/kg) prior to or after radiation therapy is shown in Fig. 3. Administration of the SR-4233 alone resulted in about 2.7 days of TGD. Treatment with SR-4233 prior to radiation caused no significant alteration in the slope of the tumor growth curve. Not surprisingly, when SR-4233 was administered immediately after radiation, the slope of tumor growth curve was also unchanged. In vivo SR-4233 (50 mg/kg) displayed no discernable radiosensitizing properties but was additive with radiation in producing TGD whether administered prior to or after the radiation treatment.

To examine the efficacy of SR-4233 in a combined modality regimen with hyperthermia and fractionated radiation, a dose of 30 mg/kg of SR-4233 was used (Table 1). The TGD produced by the hyperthermia treatment (43°C, 30 min) alone was about 1.4 days. SR-4233 (30 mg/kg) produced a TGD of about 1.7 days. When the administration of SR-4233 was followed by hyperthermia, the resulting TGD was strikingly increased to about 9.1 days, an increase of 5.4-fold over the drug alone. In these experiments, radiation was delivered daily in 3-Gy fractions for 5 days and produced a TGD of about 6.3 days. The combination of either hyperthermia or SR-4233 given with radiation on treatment day 1 only of the 5-day radiation protocol produced TGDs of about 8 days. The complete treatment regimen of SR-4233 followed by hyperthermia on day 1 and daily radiation, however, resulted in a TGD of about 28 days.

To determine the effectiveness of these various treatments on environmentally determined tumor subpopulations, FSaIC tumors were treated, and 24 h later the animals were given injections of a tracer amount of Hoechst 33342 (2 mg/kg) (Fig. 4). After 20 min, the animals were sacrificed, a single cell suspension of the tumors was prepared, and the cells were sorted on the basis of fluorescence intensity. The survival of the terminal populations was assessed by colony formation. The 10% brightest cells were felt to represent a population near to the tumor vasculature (enriched in oxygenated cells), and the 20% dimmest cells, a population distal from the tumor vasculature (enriched in hypoxic cells). As we have shown previously, hyperthermia (43°C, 30 min) was about 2.5-fold more cytotoxic toward the dim cell tumor subpopulation, and radiation (10 Gy) was about 2-fold more cytotoxic toward the bright cell tumor subpopulation (21, 22). SR-4233 (30 mg/kg) was about 1.8-fold more cytotoxic toward the dim cells than toward the bright cells. The addition of hyperthermia to treatment with SR-4233 increased the killing of the dim cells by about 5-fold and the killing of the bright cells by about 2-fold compared to SR-4233 treatment alone. The addition of SR-4233 to treatment with 10 Gy increased the killing of the bright tumor cell subpopulation by about 2.5-fold and the killing of the dim tumor cell subpopulation by about 5-fold compared to 10 Gy alone. The complete regimen of SR-4233 followed by hyperthermia and then 10 Gy was slightly more effective against the
dim tumor cell subpopulation than against the bright tumor cell subpopulation. This treatment combination was about 6.5- and 16.4-fold more effective in the bright and dim tumor subpopulations, respectively, than radiation alone.

**DISCUSSION**

There are few anticancer treatments which are more cytotoxic toward hypoxic cells. These include the quinone bioreductive alkylating agents mitomycin C and porfiromycin (3, 23, 24) and the 2-nitroimidazole radiosensitizing agents such as etanidazole and misonidazole (25–27). Although our data demonstrate that SR-4233 is not as potent a cytotoxic agent as is mitomycin C (3, 23), it is much more cytotoxic than etanidazole (28), and the cytotoxicity of SR-4233 toward hypoxic cells is markedly increased under acidic conditions, which are likely to coexist in hypoxic tumor subpopulations (25, 26).

It is probable that the hypoxic tumor subpopulation located distal from the tumor vasculature is an important therapeutic problem in the treatment of solid tumors (9, 22, 24–28). The 1,2,4-benzotriazine-1,4-dioxide, SR-4233, represents a promising new class of anticancer drugs which is capable of attacking hypoxic tumor subpopulations both through direct hypoxic cell-selective cytotoxicity and hypoxic cell radiosensitization (5–10). 1,2,4-Benzotriazines were synthesized previously by medicinal chemists in an effort to prepare antimalarial agents which would maintain the therapeutic activity of the quinoline ring system but would not be susceptible to hydroxylation (29, 30). Most of these compounds, including the 1-N-oxide, had little antimalarial activity (30).

Several triazines and hydrazines have antitumor activity. Dacarbazine, 5-methyl-(3,3-dimethyl-1-triazeno)imidazole-4-carboxamide, is a 1,2,3-triazine which undergoes metabolic activation by a microsomal, NADPH-dependent, mixed function oxidase, then a spontaneous tautomerization to eliminate a methylidiazonium ion (CH,N=N+), which can methylate nucleic acids (31–35). Procarbazine, N-(1-methylthethyl)-4-[2-methylhydrazino)methyl]benzamide, is a hydrazine which undergoes extensive metabolism through several pathways giving rise to chemically unstable species which also break down to a methylidiazonium ion or methyl radical and alkylate DNA (30–38).

SR-4233 is the oxidized form of the 1,2,4-benzotriazine, thereby bypassing metabolic steps which require oxygen, and, thus, can be metabolized by bioreductive mechanisms to both active and inactive species (39–41). Two reduction products, the 1-oxide and the 3-aminobenzotriazine, have been shown to be relatively nontoxic to both normally oxygenated and hypoxic cells (42). SR-4233 may also be metabolized through a ring opening mechanism leading to a chemical species, which, like dacarbazine and procarbazine, can break down further, with formation of a cytotoxic phenyldiazonium ion or an oxygen-inhibited phenyl radical.

Hyperthermia increased the cytotoxicity of SR-4233 to both well oxygenated and hypoxic cells in culture. The increased killing under hyperthermic conditions was more marked in the hypoxic cells. In vivo, the addition of hyperthermia (43°C, 30 min) to treatment with SR-4233 increased the TGD produced by SR-4233 by 5.4, which is a larger increase in TGD than seen with cis-diaminedichloroplatinum(II) (16), etanidazole (28), bleomycin (19), 1,3-bis(2-chloroethyl)-1-nitrosourea (23), or mitomycin C (23) in combination with this temperature in the same tumor system. In addition, from the Hoechst dye tumor subpopulation survival studies, it is clear that the greatest effect of the combination of SR-4233 plus hyperthermia is in the dim (hypoxic cell-enriched) tumor cell subpopulation as predicted by the in vitro data. It is notable that in our prior studies of the addition of etanidazole to cisplatin, hyperthermia, and radiation, improved killing of the dim tumor subpopulation also appeared to correlate with the production of an excellent TGD by the combination (28). The mechanism(s) underlying the positive cytotoxic interaction between hyperthermia and many anticancer drugs are largely unknown (43). It is quite conceivable, however, that chemical activation of SR-4233 is increased by the addition of thermal energy and/or that cellular repair systems are inhibited.

SR-4233 was also an effective radiosensitizer of hypoxic cells in vitro. A concentration of 500 μM SR-4233 produced dose modification of radiation cytotoxicity in hypoxic FSaIIC cells that was of the same magnitude as produced by 5 mM etanidazole. Unlike the 2-nitroimidazoles, etanidazole and misonidazole, and like cis-diaminedichloroplatinum(II) (44), the radiosensitizing potential of SR-4233 was not diminished under acidic pH conditions. In vivo, however, SR-4233, at a dose of 50 mg/kg, showed no radiosensitizing effect, as measured by slope change of the radiation dose-dependent TGD curves, but did add to the TGD produced by radiation. The cytotoxicity of the drug toward hypoxic cells probably accounted for most of the enhancement of the TGD produced with radiation.

Thus, SR-4233 is an effective new hypoxic cell cytotoxic agent in preclinical testing which, if toxicities prove tolerable, may increase the capabilities of radiation and/or hyperthermia to provide local control of unresectable malignancies.

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tion, SR-4233 or a related 1,2,4-benzotriazine-1,2-dioxide may be useful adjunctive treatments for use with chemotherapeutic agents against systemic solid tumors in humans.

REFERENCES


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