ABSTRACT

The new bioreductive drug 3-amino-1,2,4-benzotriazine 1,4-dioxide (SR 4233) shows a high selective cytotoxicity for hypoxic cells, both in vitro and in tumors in vivo. In the present experiments, we have tested the hypothesis that this selective killing of hypoxic cells might be exploited by taking advantage of the fluctuating hypoxia in tumors by use of a multidose regimen of SR 4233 with multiple small doses of X-rays. We have tested four different transplantable mouse tumors using a standard fractionated protocol of 8 × 2.5 Gy in 4 days, using a well tolerated dose of SR 4233 given with each radiation dose. All of the tumors showed a substantial enhancement of cell killing by SR 4233 over that produced by radiation alone with dose-modifying factors of 1.5 to 3.0 for the different tumors. In all four tumors, the enhancement of cell killing was greater than that produced by a large dose of the hypoxic cell sensitizer SR 2508 given before each irradiation, thereby demonstrating the superiority of the approach of using a hypoxic cytotoxic agent rather than a radiosensitizer in fractionated radiation protocols. The data suggest that SR 4233 has considerable promise as an adjunct to standard radiotherapy.

INTRODUCTION

SR 4233 is currently undergoing preclinical investigation as a possible antitumor agent. This drug is both a potent and a selective killer of hypoxic cells in vitro (1–3). It also causes extensive tumor cell death in vivo when combined with radiation (4, 5) or with agents that induce tumor hypoxia (6, 7). Mechanistic studies have implicated the 1-electron reduction product of SR 4233, necessarily a free radical, as the likely toxic species (2, 8). Recent studies using electron spin resonance have unequivocally identified the formation of a free radical reduction product from SR 4233 under hypoxic conditions in vitro (4).

In addition to the hypoxic cytotoxicity of this drug, we have recently identified a second property of SR 4233: its ability to act as an aerobic radiosensitizer of cells exposed to the drug under hypoxic conditions either before or following the aerobic irradiation (9).

It is possible that both of these properties, hypoxic cytotoxicity and hypoxia-activated aerobic radiosensitization, might be exploited in a fractionated course of irradiation to a tumor. It was postulated (10) and more recently demonstrated (11) that hypoxia in rodent tumors can be a dynamic process, with cells undergoing periods of intermittent hypoxia and oxygenation. This fluctuating hypoxia would mean that tumor cells could be exposed to SR 4233 while under hypoxic conditions and either be killed or be sensitized to subsequent irradiation under well oxygenated conditions. The absolute requirement for hypoxia for both of these phenomena would prevent any cytotoxicity or radiation sensitization of the normally well-oxygenated normal tissues surrounding the tumor. We have recently reported that the addition of SR 4233 to radiation produces a large increase in cell killing of the SCCVII mouse carcinoma over that produced by radiation alone with no increased effect on early skin reactions or late leg contracture (5, 12, 13). However, in one of these studies (12), all of the increased cell killing over radiation alone could be accounted for by hypoxic cytotoxicity, whereas in the others in which smaller individual radiation and drug doses were given more frequently, the enhanced cell killing over radiation alone was attributed both to hypoxic cytotoxicity and to aerobic radiosensitization (5, 13).

We report here the results of further studies to analyze the interaction of SR 4233 with fractionated irradiation both in the SCCVII carcinoma and in three other mouse tumors of varying hypoxic fractions. We also report the effect of drug dose and timing interval between drug and radiation on the interaction between SR 4233 and irradiation and compare the radiation potentiation with that produced by the classic hypoxic cell radiosensitizer, SR 2508. In all four tumors, well tolerated doses of SR 4233 markedly potentiated the radiation-induced tumor cell killing and were more effective in enhancing radiation cell killing than high doses of SR 2508.

MATERIALS AND METHODS

Drugs. SR 4233 was synthesized by Dr. Michael Tracy of SRI International, Menlo Park, CA. SR 2508 was obtained from the Drug Synthesis and Chemistry Branch, National Cancer Institute, NIH, Bethesda, MD. Both were dissolved in physiological saline immediately prior to use with SR 4233 at a concentration of 0.71 mg/ml and SR 2508 at a concentration of 50 mg/ml. Both drugs and saline were injected i.p. in a volume of 0.02 ml/g in a fractionated protocol of 8 injections in 4 days (injections at 12-h intervals).

Mice and Tumors. Female C3H/Km and BALB/c mice, 3–4 months old, bred and held under defined flora conditions in the Stanford Radiation Biology Mouse Facility, were used in these experiments. The four transplantable tumors used in these studies were the SCCVII/St carcinoma, the RIF-1 sarcoma, and the EMT6/St and KHT/Ro tumors. The SCCVII carcinoma is a tissue culture-adapted cell line of a squamous cell carcinoma which arose spontaneously in the abdominal wall of a C3H mouse in the laboratory of Dr. H. D. Suit, Massachusetts General Hospital, Boston, MA. The derivation of the cell line and the details of its handling have been published (14). Details of the derivation and handling of the EMT6 and RIF-1 tumors have also been described previously (15, 16). The KHT/Ro tumor was obtained from Dr. Dietmar Siemann, University of Rochester. Details of the handling and characteristics of this tumor have been described elsewhere (17). All the tumors were transplanted in the lower back of their syngeneic hosts (BALB/c for the EMT6/St and C3H/Km for the other 3 tumors) by intradermal inoculation of 2 × 10^6 cells in 0.05 ml. Treatment of the tumors was started when they reached an average diameter of 7–9 mm.

Irradiation Conditions. For irradiation, the unanesthetized mice were placed in individual lead boxes with the tumors protruding through a cut out portion at the rear of each box. The irradiation was delivered using a 250-kVP X-ray machine at a dose rate of 1.69 Gy/min (added filtration of 0.35 mm copper, 15 mA, target to tumor distance of 31 cm and a half-value layer of 1.3 mm copper). Control groups (no irradiation) received 8 saline injections or 8 injections of SR 4233 only;
treated groups received 8 doses of 2.5 Gy with saline or of SR 4233 (0.08 mmol/kg) 30 min before or immediately after each irradiation dose. To evaluate the extent to which hypoxic cells governed the tumor response to this regimen, a group in which the hypoxic sensitizer SR 2508 (1000 mg/kg) was injected 45 min prior to each radiation dose was included in each experiment.

Evaluation of Tumor Response. Tumor response was evaluated either with the in vivo/in vitro excision assay or by regrowth delay. For the excision assay, mice were killed 12 h after the final 2.5-Gy irradiation, their tumors were excised and dissociated with an enzyme cocktail, and the cells plated for clonogenic survival in vitro. Specific details of the clonogenic assay have been reported elsewhere (14–17). Briefly, however, for the SCCVII, EMT6, and RIF-1 tumors, cell survival was assayed by plating known dilutions of tumor cells into Petri dishes containing Waymouth's medium plus 15% fetal bovine serum to assay for colony formation. The dishes were incubated at 37°C for 12–14 days and then stained, and the colonies were counted. For the KHT/Ro tumor, known dilutions of cells obtained from the disaggregated tumors were mixed with lethally irradiated cells in 0.2% agar containing a minimal essential medium plus 10% fetal bovine serum. Plates were stained 17 days later by adding 0.05% p-iodonitrotetrazolium violet in saline and stained overnight. For all tumors, the relative clonogenic cells per tumor were calculated as the product of plating efficiency and the cell yield from the tumor compared to that of saline-injected, but otherwise untreated, controls run in parallel. The data were obtained from 1–3 identical experiments each with two mice/treatment group.

For the regrowth delay assay of the SCCVII, RIF-1, and KHT/Ro tumors, groups of mice were treated as described above, except that the mice were not sacrificed until their tumors reached at least 4 times their volume at treatment. The geometric mean values (and SE limits) were calculated for each group from the pooled results of two experiments for each tumor with five mice/treatment group. In the case of SR 4233 with radiation with the SCCVII tumor, 2 of the 10 mice were apparently cured of their tumors (i.e., the tumors had not reappeared by 150 days after irradiation). To calculate the median growth delay for this group, growth delays equal to the longest in the experiment were assigned to these two tumors and the median value regarded as a minimum.

RESULTS

Response as a Function of Number of Fractions. Fig. 1 shows the results of an experiment in which SCCVII tumors were irradiated twice daily with 2.5 Gy/fraction (12 h between treatments) with either physiological saline or SR 4233 (0.11 mmol/kg, 19.6 mg/kg) injected 30 min prior to each irradiation. Unirradiated mice were also given injections of either saline or SR 4233. The mice were sacrificed 12 h after either two, four, six, or eight of these radiation fractions. The dashed line marked “additive” in Fig. 1 is the product of the cell killing by radiation alone and by drug alone. This is the killing that would be expected if the irradiation and drug killings were acting independently on the same cell population in the tumor. It is clear that the actual cell killing caused by the addition of SR 4233 to the fractionated radiation course is considerably greater than that expected by additive cytotoxicity. Although this is consistent with radiosensitization, other interpretations are possible (see “Discussion”). The dose-modifying factor from the least squares regression lines comparing radiation plus drug with radiation alone is 2.97 ± 0.35 (SE).

Effect of Drug Timing. Fig. 2 shows the results of pooled experiments from fractionated courses of 8 × 2.5 Gy in 4 days (12 h between doses) in which a fixed dose of SR 4233 (0.08 mmol/kg) was given from 3 h before to immediately after irradiation. Each point shows a determination from an individual animal tumor. The “expected additive” of the radiation and drug treatment are shown and calculated as the product of drug only and radiation only survivals as discussed for Fig. 1. It is clear that, with the exception of drug given immediately prior to irradiation, the combination of drug plus radiation gives greater cell killing than that expected by additive cytotoxicity and that within the period tested there is not a large influence of timing on this effect.

Effect of Drug Dose. Fig. 3 shows the results of pooled experiments in which different doses of SR 4233 were given 30 min prior to each of 8 fractions of 2.5 Gy fractionated regimen. For survival to radiation (rad) alone; each point shows the data for a single tumor. Expected additive point, product of the mean killing by SR 4233 alone and that of radiation alone. The line has been fitted by eye. Pooled data from two experiments.

Comparison of Different Tumors. Four different transplanted mouse tumors were compared in a standard assay of fractionated radiation (8 × 2.5 Gy in 4 days) with SR 4233 (0.08 mmol/kg) given 30 min before each radiation dose. Inspection of Figs. 2 and 3 shows that this dose and timing produced a near
Although we did not perform dose-response curves as a function of delay studies. The results for the tumors are shown in Table 2.

For each of the four tumors is also shown in Fig. 4, as is the maximum enhancement of radiation cell killing in the SCCVII carcinoma.

Fig. 4 shows the data obtained for the tumors using excision assay as the end point for tumor cell killing. Also shown for each tumor is the effect of the hypoxic cell sensitizer SR 2508 given 45 min prior to each radiation dose at a concentration of 1000 mg/kg. Table 1 provides a summary of the data and a calculation of the dose-modifying factors for the addition of SR 2508 to radiation, assuming exponential cell killing as a function of a number of fractions in these studies, it is clear that for all the tumors a greater increase in growth delay was produced by the addition of SR 2433 than of SR 2508 to irradiation. Other experiments of growth delay as a function of radiation fraction number and size (not shown) demonstrate that these increases in growth delay are consistent with the dose modification factors obtained in the cell survival experiments.

**DISCUSSION**

In the present study, we have characterized the enhanced cell killing produced by the addition of SR 2433 to a fractionated irradiation protocol with doses per fraction similar to those used clinically. We have done this with four different mouse tumors differing considerably in their "radiobiological" hypoxic fractions. The EMT6 and the SCCVII tumors have 20–40% hypoxic cells (18), whereas the RIF-1 tumor has only approximately 1% hypoxic cells (19). The KHT/Ro tumor was chosen for these investigations because it has been reported that the hypoxic cells in this tumor are predominantly chronically rather than acutely hypoxic cells (17). We have also characterized for these four different tumors the extent of radiosensitization produced by a large dose of the electron-affinic hypoxic cell sensitizer SR 2508 given at the optimum time (45 min) prior to each radiation dose fraction. The tumor concentrations of SR 2508 produced by the dose used (1000 mg/kg) are considerably larger than that which could be achieved clinically and would be expected to produce a sensitizer enhancement ratio (SER) of the hypoxic cells of 2.0–2.2 (20). This SER would apply only to the hypoxic cells in the tumor and would be greatly diminished in a fractionated protocol with low radiation doses per fraction if extensive reoxygenation between fractions occurred (21).

The most important finding of this study is the confirmation and extension of the previously reported finding (5) that SR 2433, when added to a fractionated radiation protocol, produces extensive enhancement of the cell killing over that by radiation alone. Our data show that this effect is a robust phenomenon; it occurred in all of the different experiments with fractionated radiation with the SCCVII tumor, whether the variable was the number of dose fractions (Fig. 1), the interval between drug injection and irradiation (Fig. 2), or the drug dose (Fig. 3). It is also apparent from Fig. 4 that the effect of SR 2433 in enhancing radiation killing is not limited to the SCCVII tumor, but occurred with all four tumors.

Fig. 4 illustrates another important conclusion from these studies. In the experiments illustrated in Fig. 4, the effect of SR 2433 was compared with that of a very high (and clinically unusable) dose of SR 2508 (1000 mg/kg) given prior to each radiation fraction. For two of the tumors (SCCVII and RIF-1), there was little or no radiosensitization by SR 2508 in the fractionated protocol. For the other two, the addition of SR 2508 produced 1 log of additional cell killing over radiation alone (corresponding to a dose modification factor of approximately 1.4). However, for all the tumors, the enhanced cell killing produced by SR 2433 was greater than that produced by SR 2508. Interestingly, there appears to be an inverse correlation between the extent of radiosensitization by SR 2508 and the radiation potentiation by SR 2433: the tumors which showed little or no radiosensitization by SR 2508 (SCCVII and RIF-1) produced the largest enhancement of radiation damage by SR 2433, whereas the ones which showed radiosensitization by SR

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5 J. M. Brown, unpublished data on the hypoxic fraction of the SCCVII tumor.
2508 were the ones which showed the least potentiation by SR 4233.

Another conclusion which can be drawn from the present data is that SR 4233 is active whether given prior to or following each radiation dose. This can be seen in Fig. 2 for the SCCVII tumor and also in Fig. 4 for both the SCCVII and EMT6 tumors. In both instances, there appears to be a slightly greater effect when SR 4233 is given 30 min prior to irradiation compared to immediately afterwards, but this difference is only minor. This demonstrates that the action of SR 4233 in vivo is different from that of a traditional electron-affinic radiosensitizing agent such as SR 2508.6 The fact that the drug is not acting as a typical hypoxic cell sensitizer is also evident from the lack of effect of SR 2508 in the SCCVII and RIF-1 tumors. Since SR 2508 is not inherently inactive against these two tumors when the same drug dose is used with a single large dose of radiation (5, 22), it is likely that the lack of significant radiosensitization is attributable to extensive and rapid reoxygenation between each dose fraction. As we mentioned above, these two tumors are the ones which show the greatest potentiation of radiation damage by SR 4233. This suggests a possible mechanism of action of the drug as outlined below.

The rationale for these experiments was based on the fact that if tumor cells fluctuated in and out of hypoxia, then SR 4233 could enhance radiation killing both by killing the hypoxic cells and by radiosensitizing any of the hypoxic cells not killed by drug exposure. This effect would be at a maximum if most of the hypoxic cells in the tumor were of this intermittent or acute type, and, as has been already pointed out, the presence of such hypoxic cells could provide a simple explanation for reoxygenation (10). Thus, SR 4233 would appear to take advantage of tumor reoxygenation and indeed would give greater potentiation of radiation killing the more extensive the reoxygenation. This is precisely the opposite of the situation with a traditional hypoxic cell sensitizer, for which radiosensitization becomes less as the opportunity for reoxygenation increases (21).

In Figs. 1–4 we have indicated the expected cytotoxicity that would occur if radiation and SR 4233 were acting as independent cytotoxic agents on the same cell population. The expected additive, assuming exponential cell killing by both agents, is the product of the two surviving fractions. However, it is clear from all of the data that the actual killing produced by the combination of radiation and SR 4233 is considerably greater than that predicted by this method of adding the two cytotoxicities. This additional cytotoxicity could be interpreted as resulting from radiosensitization. However, this is not the only explanation. If it is assumed that the tumors consist of two separate populations of cells, one aerobic and one hypoxic, and that radiation and SR 4233 kill different fractions of these populations (as we know they do), then the expected additive should be calculated differently. Table 3 shows the difference resulting from adding the cytotoxicities for the data from Fig. 1. Additivity assuming a single tumor cell population is the mode used in all of the figures and is obtained by multiplying together the survival to each agent alone. However, it is assumed that killing by SR 4233 is entirely of the hypoxic population and the extent of cell killing by SR 4233 in each fraction defines the size of the hypoxic population, then the additive cytotoxicities can be calculated as shown in Table 3. This is actually a slight overestimate, since it assumes that radiation kills only aerobic cells. However, it is clear that the two methods of calculating additivity give different results and that the actual survival of combining the two agents is very nearly predicted by the method of adding cytotoxicities considering two separate populations.

In order to simulate more accurately the expected interaction between fractionated radiation and either SR 4233 or SR 2508, we have modeled the data in Table 3 using a computer program which assumes that the tumor has 33% hypoxic cells, all are killed by each fraction of SR 4233, and radiation killing follows a multitarget model for both aerobic and hypoxic cells with a slope difference ("oxygen enhancement ratio") between the
aerobic and hypoxic cells of 2.5. The parameter of the multitarget model was set so as to produce 38% cell killing of the tumor cells by a single dose of 2.5 Gy (i.e., as actually observed; Table 3). This computer simulation predicts an exponential survival curve for the radiation plus SR 4233 data reaching a surviving fraction of 9 × 10^{-5} after 8 fractions. This is to be compared to the "additive" line of Fig. 1 which predicts a surviving fraction of only 10^{-3} after 8 fractions. Thus, the actual data for SR 4233 plus fractionated radiation can be largely accounted for by complementary cytotoxicity of the two agents and makes it less likely that there is radiosensitization of aerobic cells as we have postulated in earlier publications (5, 13). However, we cannot rule out this latter possibility entirely, especially since a closer fit to the experimental data could be obtained if we assumed a larger proportion of hypoxic cells in the tumor with these "extra" hypoxic cells being radiosensitized. Nonetheless, even if (as we suspect) there is no aerobic radiosensitization, this does not mean that the complementary additivity of a hypoxic cell toxin, such as SR 4233, with radiation will not be extremely useful in clinical practice. In fact, it can be readily shown that far greater cell killing can be obtained from adding a hypoxic cytotoxin than from adding a radiation sensitizer to each radiation dose, even if the radiation sensitizer is so efficient as to produce full sensitization of the hypoxic cells (23). This means, in effect, that hypoxic cells in tumors are a therapeutic advantage when a hypoxic cytotoxin is combined with fractionated radiation: greater cell killing is produced in tumors if they have hypoxic cells (particularly, if these tumor cells reoxygenate after radiation) than if they are fully oxygenated.

Finally, it would be reasonable to speculate from the present data whether SR 4233, or an analog, would have potential as an adjunct with standard radiotherapy. We have previously shown that SR 4233 in the same fractionated regimen produces no enhanced radiation response of either an early skin reaction or late occurring contracture of the irradiated leg (5, 13). This is to be expected because the effects of SR 4233, whether they be hypoxic cytotoxicity or radiosensitization of aerobic cells, both depend on the presence of hypoxic cells. Thus, it would appear that the enhancement of radiation cell killing will be limited to tumors which have hypoxic cells and is therefore unlikely to occur in normal tissues. The other aspect of clinical relevance is whether the dose of SR 4233 needed to produce radiation enhancement can be given with each radiation dose in a multifraction regimen. We have performed experiments with daily doses of SR 4233 at dose levels from 0.04–0.08 mmol/kg/day. Mice can tolerate daily doses in this range (a range which produces the potentiation of the radiation effect) for up to 6 weeks with minimal ill effects.7 However, it is too early as yet to know whether effective doses will be tolerated in humans, although there is no reason at present to suppose that they will not be. It would appear, therefore, that SR 4233 is a drug of considerable promise for use with radiation therapy. In fact, because of the inherent advantage of killing rather than radiosensitizing hypoxic tumor cells, SR 4233 would appear to be superior to any classic hypoxic cell sensitizer, either in clinical use or in preclinical testing.

REFERENCES


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Potentiation by the Hypoxic Cytotoxin SR 4233 of Cell Killing Produced by Fractionated Irradiation of Mouse Tumors

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