Cancer Chemother Pharmacol (2004) 53: 43–50
DOI 10.1007/s00280-003-0715-8

ORIGINAL ARTICLE

Erling T. Donnelly · Marianne Kelley · Sara Rockwell

Effects of RSR13 and oxygen on the cytotoxicity of cisplatin and carboplatin to EMT6 mouse mammary tumor cells in vitro and in vivo

Received: 24 April 2003 / Accepted: 27 August 2003 / Published online: 22 October 2003

Abstract *Purpose*: RSR13, 2-[4-[2-[(3,5-dimethylphenyl) amino]-2-oxoethyl]phenoxy]-2-methylpropanoic acid monosodium salt, allosterically modifies hemoglobin to increase tumor pO_2, increases the effect of radiation in animal tumor models, and is in phase III clinical trials as an adjuvant to radiotherapy. Cisplatin and carboplatin, two commonly used anticancer drugs have been used in combination with radiotherapy. Some studies have suggested that the cytotoxic effects of these drugs are altered in hypoxia. This study tested whether RSR13 plus oxygen breathing increased the cytotoxicity of cisplatin and carboplatin in vivo. *Methods*: Solid EMT6 tumors in BALB/c Rw mice were treated with cisplatin (5–30 μg/g) or carboplatin (5–200 μg/g) along with 150 μg/g RSR13 in combination with oxygen breathing. Tumor cell survival was assayed using clonogenic assays. The effects of pre- and posttreatments with RSR13 and oxygen breathing on the cytotoxicity of cisplatin or carboplatin were also examined. To assess whether RSR13 had direct effects on the cytotoxicity of the drugs, exponentially growing monolayers of EMT6 mouse mammary carcinoma cells were treated with graded concentrations of cisplatin or carboplatin for 2 h along with simultaneous (2 h) RSR13 treatments or with prolonged (22 h) pre- or posttreatment incubations with 100 μ*M* RSR13. *Results*: Single or multiple treatments with 150 μg/g RSR13 plus oxygen breathing had no effect on the viability of cells in EMT6 tumors in mice. After treatment with cisplatin or carboplatin, the tumor cell survival tended to be lower in oxygen-breathing mice especially at higher doses of cisplatin. Treatment with RSR13 plus oxygen breathing beginning 15 min before administration of the alkylating agents did not alter the cytotoxicity of cisplatin or carboplatin from that seen with oxygen breathing alone. Pretreatment with RSR13 plus oxygen at 22 and 14 h prior to administration of either cisplatin or carboplatin did not alter the effect of either alkylating agent. Treatment with RSR13 plus oxygen breathing beginning 15 min before administration of the alkylating agents and lasting for 2 or 5 h did not alter the cytotoxicity of either drug from that seen with oxygen breathing alone. The cytotoxicity of cisplatin was not altered by treatment with oxygen alone or with RSR13 plus oxygen for 5 h after cisplatin injection. For carboplatin, treatment with oxygen alone and with RSR13 plus oxygen for 5 h after injection increased to similar extents the response of the tumor cells compared to that seen with assays at 2 h. Neither short simultaneous treatments, prolonged pretreatment incubations, nor prolonged posttreatment incubations with RSR13 altered the survival of EMT6 cells in cultures treated with cisplatin or carboplatin. *Conclusions*: These findings indicate that RSR13 in combination with oxygen breathing does not alter the cytotoxicity of cisplatin or carboplatin when used simultaneously, as a pretreatment or as a posttreatment in vitro or in vivo. Our in vivo findings indicate trends that support previous findings that cisplatin is more cytotoxic to well-oxygenated tumor cells than to hypoxic tumor cells, and that this effect can be improved by improving tumor oxygenation, but the differences seen in our studies did not achieve statistical significance.

Keywords RSR13 · Cisplatin · Carboplatin · Oxygen delivery

E. T. Donnelly · M. Kelley · S. Rockwell (✉)
Departments of Therapeutic Radiology and Pharmacology,
Yale University School of Medicine,
New Haven, CT 06520-8040 USA
E-mail: sara.rockwell@yale.edu
Tel.: +1-203-7852963
Fax: +1-203-7857482

Introduction

RSR13, 2-[4-[2-[(3,5-dimethylphenyl)amino]-2-oxoethyl]phenoxy]-2-methylpropanoic acid monosodium salt, is a synthetic allosteric modifier of hemoglobin-oxygen binding [12]. RSR13 reduces the affinity of hemoglobin for oxygen by stabilizing the deoxyhemoglobin tetramer, thereby increasing oxygen delivery to hypoxic tissues [1, 12, 18, 24].

RSR13 has been shown to increase tumor and non-tumor tissue oxygenation in various animal models [2, 3, 13, 15], and to increase whole blood p50 in humans [23]. Previous studies in our laboratory and at other institutions have shown that RSR13 improves the response of solid tumors in experimental rats and mice to ionizing radiation [7, 15]. We have shown that 300 μg/g RSR13, combined with oxygen breathing, increases the cytotoxicity of radiation to EMT6 tumor cells, while oxygen breathing alone has no significant effect on the response of these tumors to radiation [15]. The nature of the change in the radiation dose-response curve is consistent with that expected from a decrease in the number of severely hypoxic cells in the tumor. In these same studies, RSR13 did not alter the survival curves for cells in tumors that were made fully hypoxic just before irradiation by asphyxiating the mice with nitrogen. This suggests that RSR13 has no direct effects on the tumor cells that alter their response to irradiation. The radiation dose-response curves from these studies were analyzed using rigorous mathematical techniques described previously to calculate the radiobiological hypoxic fractions of the tumors. Treatment with RSR13 plus oxygen reduced the hypoxic fraction to 9% from the value of 24% found for air-breathing mice in the same experiments. The hypoxic fraction for mice breathing oxygen but not treated with RSR13 was 24%, a value indistinguishable from that for air-breathing mice. This finding is in agreement with our findings from other studies showing only insignificant changes in the radiobiological fractions of EMT6 tumors in mice breathing oxygen, carbogen, or hyperbaric oxygen [11, 16, 17]. These findings support the hypothesis that RSR13 plus oxygen breathing improves the radiation response of tumors by improving tumor oxygenation, and that the results are significantly better than those produced by oxygen breathing alone [15].

Cisplatin is one of the most widely used drugs for the treatment of solid malignancies. The literature contains conflicting evidence regarding the effect of hypoxia on the cytotoxicity of cisplatin. Several studies have indicated that hypoxia enhances the cytotoxicity of cisplatin [4, 19, 20], while others have found that cisplatin is more cytotoxic under aerobic conditions [6, 10]. It has also been reported that the cytotoxicity of cisplatin is not influenced by the cellular oxygenation state [9, 22]. There have also been conflicting reports as to whether there is a differential radiosensitization of hypoxic and aerobic cells by cisplatin [4, 9, 20, 21]. The data in the literature suggest that the effects of oxygen on the cytotoxicity of cisplatin are complex and that they may involve not only the effects of the oxygenation at the time of treatment, but also the effects of posttreatment oxygen levels (perhaps through effects on repair of drug damage). We therefore included in our experiments groups exposed continuously to RSR13 plus oxygen for 5 h after treatment with the alkylating agents, to examine directly the effects of improved posttreatment tumor oxygenation.

There is also some evidence that the response of cells to platinum compounds may also be influenced by pretreatment variations in oxygenation. These most likely occur indirectly, and probably reflect changes in the physiology and proliferative patterns of the cells, the induction of stress responses, or changes in the antioxidant defenses, gene expression patterns or enzyme activities produced by the stress of transient cyclic exposures to hypoxia and oxygenation. We therefore included groups in our studies to assess whether multiple pretreatments with RSR13 and oxygen breathing, producing cyclic variations in tumor oxygenation, could alter the response of cells to cisplatin or carboplatin. This would model the situation that might occur if multiple treatments with RSR13 plus oxygen were used during a course of fractionated cancer therapy.

RSR13 is being tested in clinical trials as an adjuvant to radiotherapy and may be used in combination with chemotherapy and combined modality therapy. We therefore extended our previous studies examining the effect of RSR13 plus oxygen breathing on the response of tumors to radiation to examine the effects on the response of the tumors to cisplatin and carboplatin. These studies examined the effects of acute single treatments with RSR13 plus oxygen shortly before administration of the cytotoxic drug, the effects of multiple treatments with RSR13 plus oxygen given during the day before the cytotoxic drug, and a regimen of RSR13 plus oxygen that held the oxygenation continuously at an increased level for 5 h after drug treatment. Another set of studies used exponentially growing monolayers of EMT6 cells in cell culture to test for direct effects of 100 μ*M* RSR13 on the response of EMT6 cells to cisplatin or carboplatin, using both short simultaneous exposures and also prolonged pre- or posttreatment with RSR13, which might theoretically alter the cell physiology or the repair of drug damage, and thereby modify drug sensitivity.

Materials and methods

Drugs

RSR13, 2-[4-[2-[(3,5-dimethylphenyl)amino]-2-oxoethyl]phenoxy]-2-methylpropanoic acid monosodium salt, was provided by Allos Therapeutics (Westminster, Colo.). For cell culture experiments, RSR13 was dissolved in sterile, pyrogen-free, physiological saline. Carboplatin (Sigma-Aldrich, St. Louis, Mo.) was dissolved in Waymouth's medium while cisplatin (Sigma-Aldrich) was dissolved in DMSO. For in vivo use, RSR13, cisplatin and carboplatin were dissolved in sterile, pyrogen-free, physiological saline and injected intraperitoneally (i.p.).

Animals

All experiments were performed using male and female BALB/c Rw mice at 2.5–3.5 months of age. All protocols used with experimental animals were reviewed and approved in advance by the Yale Animal Care and Use Committee. All experiments were performed in full compliance with governmental, AAALAC, and institutional regulations and with the principles outlined in the USPHS Guide.

Tumor cells and tumors

The EMT6 mouse mammary tumor cell line (subline Rw) was used for both the in vitro and in vivo experiments. The origins and characteristics of the cell line and the techniques used to propagate and assay the cells and tumors are described in detail elsewhere [11, 14, 16, 17].

Tumors in mice

Tumors were produced by inoculating 2×10^5 cells, harvested from exponentially growing cell cultures, intradermally into the flank, and were used for experiments approximately 2 weeks later at volumes of about 100 mm^3 [14, 15]. Mice receiving oxygen were placed in gassing boxes which were then flushed with 100% oxygen. In the first set of in vivo experiments, mice were held under 100% oxygen from the time of drug administration until cell survival was assayed. In the experiments examining the effect of prolonged treatments with RSR13 and oxygen, the mice were held under 100% oxygen for 90 min following each of the first two RSR13 injections and from the time of the third RSR13 injection until cell survival was assayed. The air-breathing mice were kept in their cages.

Tumor cell survival was assayed using a colony formation assay. Tumors were explanted sterilely, and a single cell suspension was created by mincing and trypsinization [14, 15]. Cells were counted under a phase contrast microscope using trypan blue to determine the number of cells damaged during the suspension process, and were plated for colony formation as described below. Surviving fractions were calculated using the plating efficiencies of cells from untreated control tumors plated on the same day.

Cell culture studies

Cell culture experiments were performed using exponentially growing monolayers of EMT6 cells in 60-mm polystyrene Petri dishes (Corning, Corning, N.Y.). The cells were grown in Waymouth's medium (GIBCO, Grand Island, N.Y.) supplemented with 15% serum [a 1:1 mixture of fetal bovine serum (Gemini Bio-products, Woodland, Calif.) and fetal clone (Hyclone, Logan, N.Y.)], penicillin/streptomycin (Gemini Bio-products), fungizone (GIBCO), and gentamicin (Gemini Bio-products). Dishes were plated with 1×10^5 cells and the cultures were incubated at 37°C in a humidified atmosphere of 95% air/5% CO_2 for 4 days before treatment. On the day of treatment, the medium was removed, 5 ml fresh medium was added, and the dishes were allowed to equilibrate for 1 h prior to administration of drug. At the end of the treatment, the medium was removed, the cells were washed twice with 0.05% trypsin, trypsinized, and suspended. The single-cell suspension was counted and diluted, and the cells were plated at low densities into 60-mm Petri dishes containing Waymouth's medium and incubated for 14 days. Colonies were fixed, stained, and counted as described previously [5]. Each experiment included untreated control cultures, as well as vehicle-treated control cultures that were subjected to all of the experimental manipulations. Surviving fractions were calculated relative to the untreated controls.

Results

Effect of RSR13 plus oxygen breathing on the response of tumors to cisplatin

The experiments shown in Fig. 1 examined the viability of cells from tumors treated with increasing doses of cisplatin (5–30 μg/g). Cell viability was assayed 2 h after injection of drug. Three experimental groups were compared at each dose of cisplatin: air-breathing mice, oxygen-breathing mice, and mice receiving 150 μg/g RSR13 plus oxygen. The mice in the cisplatin and RSR13 plus oxygen group were injected with RSR13 i.p. 15 min before the i.p. injection of cisplatin, and were held under 100% oxygen for the 2 h between injection of cisplatin and assay of tumor cell survival.

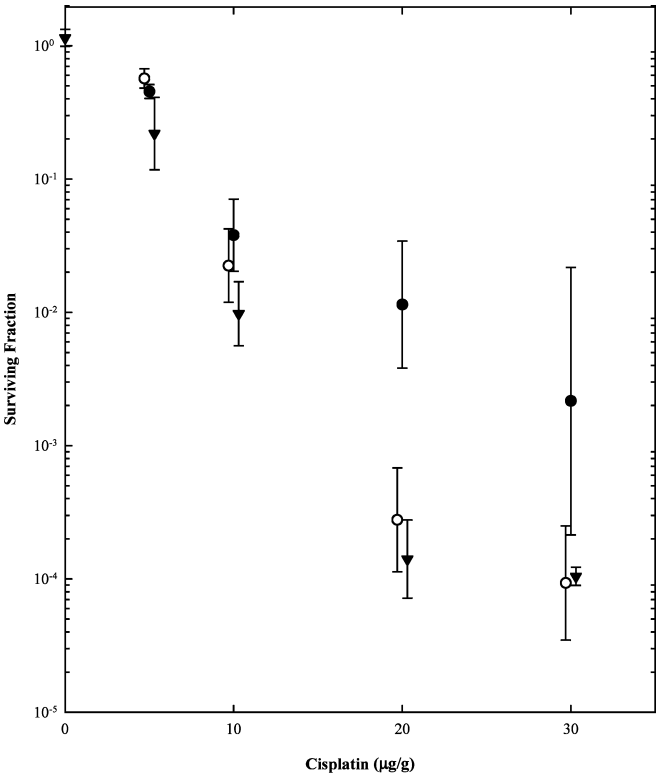

Fig. 1 Effect of treatment with cisplatin on the viability of cells in EMT6 tumors (● cisplatin only, air-breathing mice; ○ cisplatin only, oxygen-breathing mice; ▼ RSR13 plus cisplatin plus oxygen breathing). RSR13 at 150 μg/g was injected 15 min before injection of the cisplatin. Oxygen-breathing animals were held in 100% oxygen from the time of the first drug injection until the time of assay 2 h after injection of cisplatin. The data are presented as the means ± SEM of three to five independent determinations

Administration of 150 μg/g RSR13 plus oxygen breathing without cisplatin had no effect on the viability of the tumor cells (Fig. 1). There was a cisplatin dose-dependent decrease in the survival of cells in tumors. Tumor cell survival tended to be lower when the mice breathed oxygen, especially at high doses of cisplatin. However, the differences among the groups overall did not reach statistical significance ($P > 0.05$). Administration of RSR13 did not alter the effect over that obtained with oxygen alone.

A series of experiments were performed to assess whether multiple or longer treatments with RSR13 plus oxygen breathing altered the response of tumors to cisplatin (Fig. 2). Mice pretreated with RSR13 and oxygen were given 150 μg/g RSR13 22 h before cisplatin, then placed in 100% oxygen for 90 min. A second injection of 150 μg/g RSR13 was given 8 h later (14 h prior to the administration of cisplatin) and was followed by 90 min

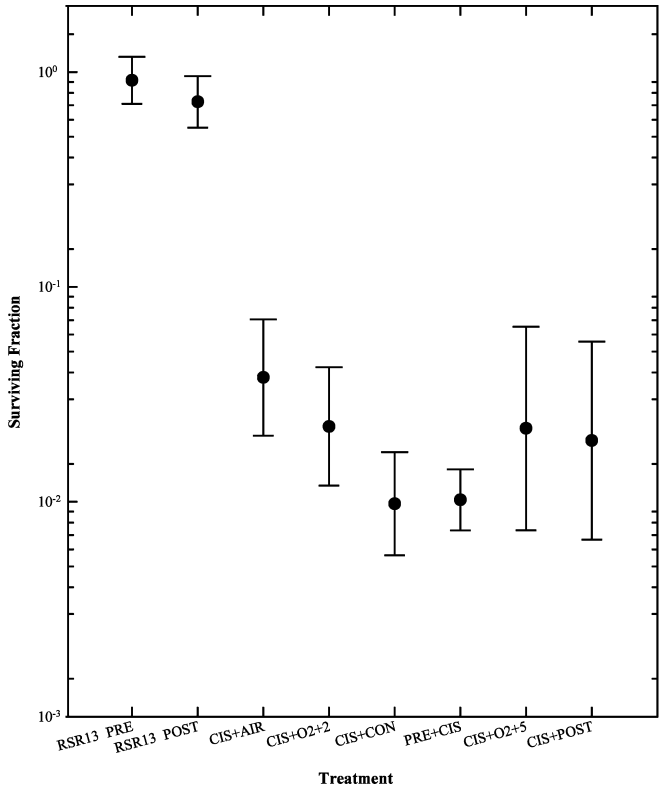

Fig. 2 Effect of multiple treatments with RSR13 plus oxygen before or after 10 μg/g cisplatin on the survival of cells in EMT6 tumors. Mice on the pretreatment regimen (*PRE*) received RSR13 plus oxygen 22 and 14 h before cisplatin, plus a third treatment 15 min before cisplatin treatment. Mice receiving the posttreatment regimen (*POST*) received RSR13 15 min before cisplatin and at 1.5 and 3.5 h after injection of cisplatin, and were held in 100% oxygen from the time of the first RSR13 injection until the time of assay 5 h after injection of cisplatin. Animals receiving RSR13 and oxygen, without cisplatin, on these same schedules were also examined (*RSR13 PRE*, *RSR13 POST*), as were animals treated with cisplatin plus 5 h of posttreatment oxygen breathing (*CIS + O2 + 5*). Data for mice treated with 10 μg/g cisplatin and breathing air (*CIS + AIR*) or oxygen (*CIS + O2 + 2*) and for mice treated with a single dose of RSR13 plus oxygen (*CIS + CON*) and assayed 2 h after injection of cisplatin are replotted from Fig. 1 for comparison. The data are presented as the means ± SEM of three to five independent determinations

of oxygen breathing. The mice were injected 15 min prior to cisplatin administration with a third dose of 150 μg/g RSR13 and the mice were then held in oxygen until cell survival was assayed 2 h after injection of 10 μg cisplatin. Pretreatment with RSR13 plus oxygen in conjunction with a 10 μg/g cisplatin treatment, as described above, did not alter the survival of the tumor cells from that seen with concurrent RSR13/oxygen/cisplatin treatments or with cisplatin alone in air-breathing or oxygen-breathing mice. The viability of EMT6 tumor cells was not altered by treatment with RSR13 and oxygen without cisplatin on this protracted multiple-treatment regimen.

The effect of posttreatment administration of RSR13 plus oxygen was examined using a regimen in which mice were treated i.p. with 150 μg/g RSR13 15 min before injection of cisplatin, and again injected with 150 μg/g RSR13 1.5 and 3.5 h after cisplatin administration. Mice were held in 100% oxygen from the first RSR13 treatment until assay of tumor cell survival 5 h after injection of 10 μg/g cisplatin. A control group of mice were injected three times with 150 μg/g RSR13 at the times specified above and were exposed to 100% oxygen for 5.25 h without cisplatin. As a control for the change in the time between injection of cisplatin and assay, a group of mice were injected with 10 μg/g cisplatin then held in 100% oxygen for 5 h before assay. The viability of the tumor cells from mice in the three groups receiving these posttreatments were similar to those of tumor cells from mice in the groups treated with cisplatin on the other regimens examined.

Effect of RSR13 and oxygen breathing on the response of tumors to carboplatin

As with cisplatin, the response of tumors to treatment with carboplatin was examined using three groups: air-breathing mice, oxygen-breathing mice, and mice receiving 150 μg/g RSR13 plus oxygen breathing beginning 15 min before carboplatin treatment. Preliminary studies used 5–30 μg/g of carboplatin but these doses were relatively ineffective in killing the EMT6 tumor cells as shown in Fig. 3. Subsequent experiments used higher doses of carboplatin (100–200 μg/g). Doses above 200 μg/g could not be given due to the solubility of the drug. Carboplatin alone produced a dose-dependent decrease in the viability of the tumor cells. Neither oxygen alone nor RSR13 plus oxygen altered the carboplatin dose-response curve.

Figure 4 shows the results of experiments assessing whether longer and multiple treatment regimens with 150 μg/g RSR13 plus oxygen breathing altered the response of tumor cells to carboplatin. The protracted treatment regimens used here were identical to those used in the cisplatin experiments described above. As seen in Fig. 4, these protracted treatments with RSR13 plus oxygen but without carboplatin did not produce a significant alteration in the viability of the EMT6 tumor cells. Pretreatment with RSR13 plus oxygen in conjunction with a 150 μg/g carboplatin treatment did not alter the survival of the tumor cells from that seen with concurrent RSR13/oxygen/carboplatin treatments or with carboplatin alone in air-breathing or oxygen-breathing mice.

The effect of posttreatment administration of RSR13 plus oxygen was examined using the regimen described above, in which mice were treated with 150 μg/g RSR13 15 min before injection of carboplatin, and again 1.5 and 3.5 h after carboplatin. Mice were held in 100% oxygen from the first RSR13 treatment until assay 5 h after injection of carboplatin. As a control for the change in the time between injection of carboplatin and assay, mice were injected with 150 μg/g carboplatin, then held in 100% oxygen for 5 h before assaying. There

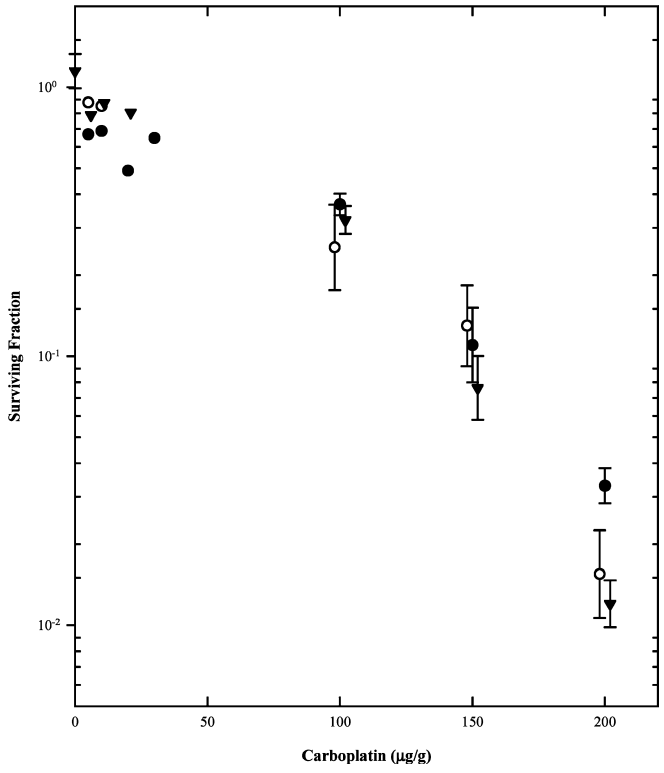

Fig. 3 Effect of treatment with carboplatin on the viability of cells in EMT6 tumors (● carboplatin only, air-breathing mice; ○ carboplatin only, oxygen-breathing mice; ▼ RSR13 plus carboplatin plus oxygen breathing). RSR13 at 150 μg/g was injected 15 min before carboplatin; oxygen-breathing animals were held in 100% oxygen from the time of the first drug injection until the time of assay 2 h after carboplatin. Points with error limits are means ± SEM of three or four independent determinations; points without error limits are single points or means of two determinations

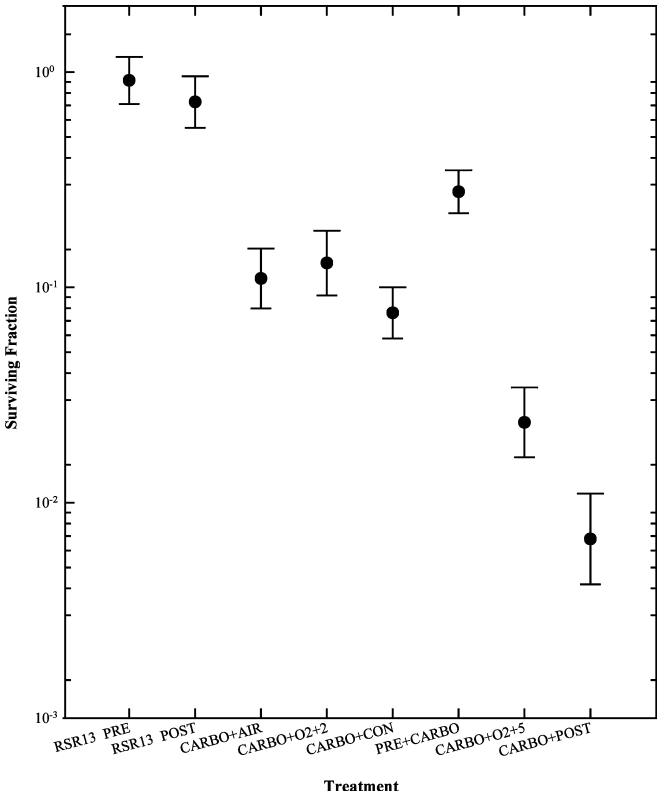

Fig. 4 Effect of multiple treatments with RSR13 plus oxygen before or after 150 μg/g carboplatin on the survival of cells in EMT6 tumors. Mice receiving the pretreatment regimen (*PRE*) received two treatments with RSR13 plus oxygen 22 and 14 h before carboplatin, plus a third treatment with RSR13 plus oxygen 15 min before carboplatin. Mice receiving the posttreatment regimen (*POST*) received RSR13 15 min before carboplatin, and at 1.5 and 3.5 h after injection of carboplatin and were held in 100% oxygen from the first RSR13 injection until the time of assay 5 h after injection of carboplatin. Animals receiving RSR13 and oxygen without carboplatin were also examined (*RSR13 PRE*, *RSR13 POST*), as were animals treated with carboplatin plus 5 h of oxygen breathing (*CARBO + O2 + 5*). Data for mice treated with 150 μg/g carboplatin and breathing air (*CARBO + AIR*) or oxygen (*CARBO + O2 + 2*), and for mice treated with a single dose of RSR13 plus oxygen (*CARBO + CON*) are replotted from Fig. 3 for comparison. The data are presented as means ± SEM of three to five independent determinations

was a marginally significant difference ($P = 0.05$) between tumor cell survival in the group treated with oxygen for 2 h after carboplatin injection and the group treated with oxygen for 5 h after carboplatin injection. The group treated with RSR13 plus oxygen and carboplatin on this posttreatment regimen was significantly different from each of the groups plated 2 h after carboplatin injection ($P < 0.05$), but was not significantly different from the group treated only with oxygen for 5 h after carboplatin injection. Analyses using the nonparametric Mann-Whitney *U*-test rather than Student's *t*-test gave similar results.

Effect of RSR13 on the toxicity of cisplatin in vitro

In the first series of cell culture experiments, shown in Fig. 5A, EMT6 cultures in exponential growth were treated for 2 h with increasing concentrations of cisplatin (5–30 μ*M*) with or without 100 μ*M* RSR13. This concentration of RSR13 was chosen based on data for the clinical pharmacokinetics of RSR13. Clinical doses of 100 mg/kg per day RSR13 achieve plasma concentrations of 449 ± 63 μg/ml (1.32 m*M*) [8]. As 95% of the drug is bound to serum proteins and 5% is present as free drug, the concentration of free drug in the blood is 66 μ*M*. The concentration of 100 μ*M* RSR13 used in these in vitro studies was thus chosen to approximate the maximal concentrations of free drug which could be achieved in patients using the current clinical regimens.

The survival of the cells treated with 100 μ*M* RSR13 alone was similar to the survival of cells from untreated control cultures. The survival curve for cells treated with increasing concentrations of cisplatin for 2 h showed a decrease in survival that was well approximated by an exponential survival curve, as shown in Fig. 5A. A concomitant 2-h treatment with 100 μ*M* RSR13 did not

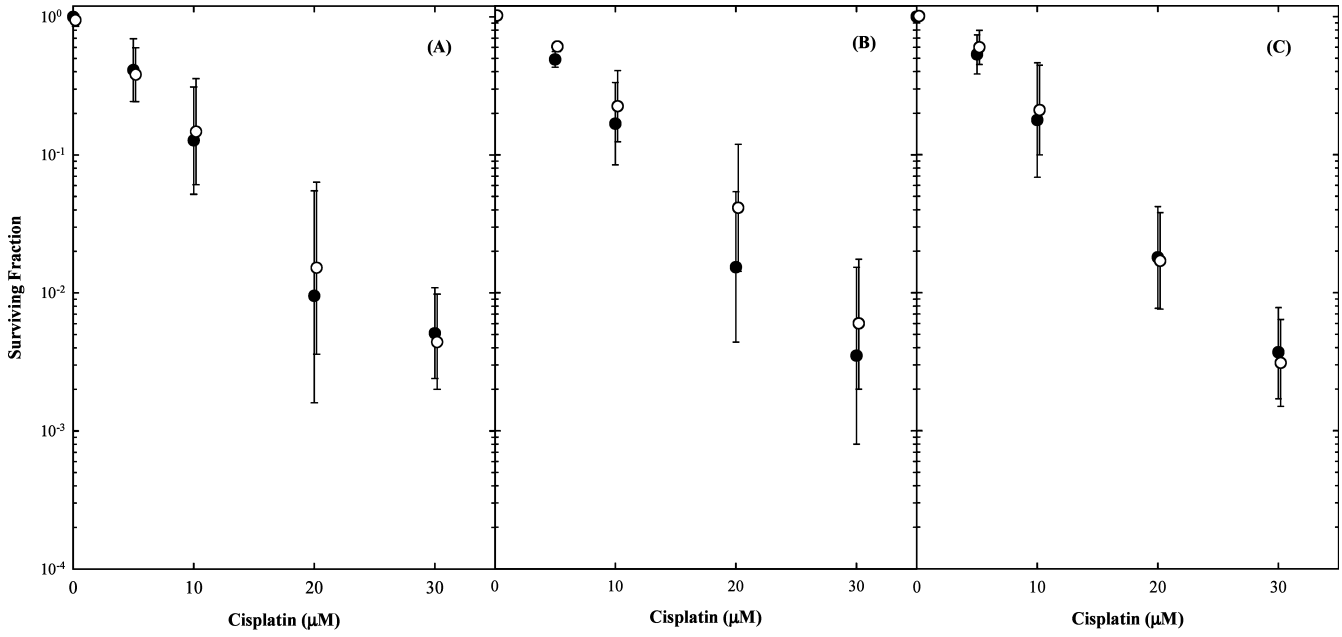

Fig. 5A–C Effect of cisplatin alone or with 100 μ*M* RSR13 on the survival of exponentially growing EMT6 cell cultures: **A** 2-h concurrent treatments; **B** treatment with cisplatin for 2 h alone or in combination with RSR13 for 22 h before cisplatin and also during the cisplatin treatment; **C** treatment with cisplatin alone for 2 h or in combination with RSR13 for 2 h during cisplatin and 22 h after cisplatin. Surviving fractions were calculated relative to untreated controls. Vehicle-treated controls subjected to all experimental manipulations were included with each experiment (● cisplatin only, ○ cisplatin plus 100 μ*M* RSR13). The data are presented as the means ± SEM of three of four independent determinations

alter the survival curve significantly. The survival curves for cisplatin alone and RSR13 plus cisplatin were indistinguishable within each of the individual experiments, as well as in the pooled data shown in Fig. 5A.

Figure 5B shows the results of experiments in which cultured cells were treated either with cisplatin alone for 2 h or with 100 μ*M* RSR13 for 22 h followed by a 2-h treatment with both 100 μ*M* RSR13 and cisplatin. Pretreatment with 100 μ*M* RSR13 for 22 h before cisplatin and during cisplatin exposure did not produce statistically significant changes in the survival curve in any of the individual experiments or in the pooled data.

The effect of treatment with cisplatin alone for 2 h and with cisplatin plus 100 μ*M* RSR13 for 2 h, followed by the removal of the cisplatin and incubation with 100 μ*M* RSR13 for an additional 22 h is shown in Fig. 5C. There was no discernible difference between the survival curves.

Effect of RSR13 on the toxicity of carboplatin in vitro

The experiments shown in Fig. 6 examined the effect of increasing concentrations of carboplatin (5–200 μ*M*) with or without 100 μ*M* RSR13 on EMT6 cell cultures. The three regimens examined tested the effects of a short simultaneous treatment with RSR13 (Fig. 6A), pretreatment plus simultaneous treatment with RSR13 (Fig. 6B) and simultaneous treatment plus posttreatment with RSR13 (Fig. 6C) using regimens identical to those described above. None of these treatments with RSR13 altered the response of the cells to carboplatin.

Discussion

In these studies, we used protocols which examined three ways in which RSR13 might affect the cytotoxicity of cisplatin or carboplatin in this mouse mammary carcinoma model. In our initial studies we sought to determine whether acute RSR13-mediated changes in tumor oxygenation modified the cytotoxicity of the platinum compounds. Secondly, we wanted to test whether administration of RSR13 plus oxygen breathing before treatment might induce alterations of gene expression, changes in cell proliferation, or other metabolic or physiological perturbations that may alter the response of the tumor cells to subsequent treatments with cisplatin or carboplatin. This would model the situation that might occur if multiple treatments with RSR13 plus oxygen were used during highly fractionated cancer therapy. Finally, we wanted to test whether prolonged treatment with RSR13 plus oxygen breathing after administration of the platinum compounds might alter the kinetics of the production, fixation, or repair of damage induced by the cytotoxic drug, and thereby alter the effect of the alkylating agents on the tumor cells.

Our cell culture studies shown in Figs 5 and 6 provided no evidence to suggest that 100 μ*M* RSR13 had any direct effect that altered the survival of EMT6 cells treated with cisplatin or carboplatin either for acute,

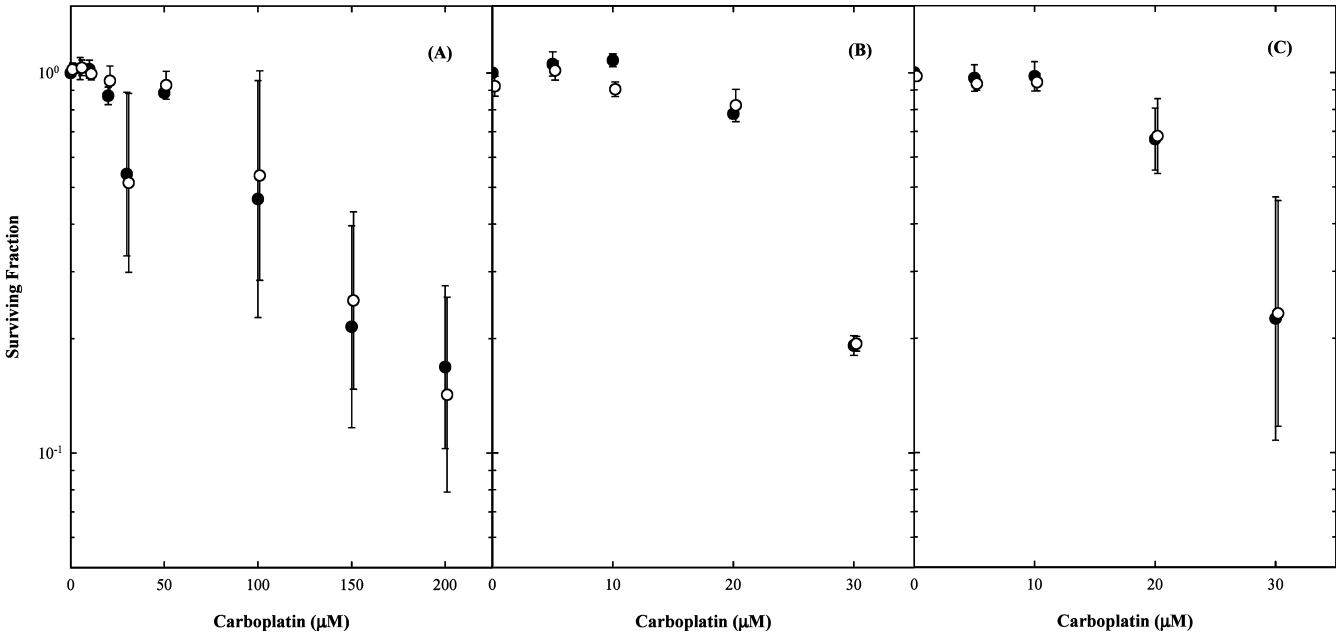

Fig. 6A–C Effect of carboplatin alone or with 100 μ*M* RSR13 on the survival of exponentially growing EMT6 cell cultures: **A** simultaneous RSR13 treatments; **B** pretreatment plus simultaneous RSR13 treatment; **C** simultaneous and posttreatment RSR13. Surviving fractions were calculated relative to untreated controls. Vehicle-treated controls subjected to all of the experimental manipulations were included with each experiment (● carboplatin only, ○ carboplatin plus 100 μ*M* RSR13). The data are presented as the means ± SEM of three independent determinations

simultaneous 2-h exposures, or for 24-h RSR13 exposures that provided lengthy pretreatment or posttreatment exposures to RSR13. Any effects of RSR13 see in vivo therefore must reflect changes in tumor oxygenation rather than direct effects of RSR13 on the activity of the alkylating agents or any direct interactions between the effects of RSR13 and those of cisplatin and carboplatin.

When mice were subjected to an oxygen-rich environment and the platinum drugs, the tumor cell survival tended to be lower; this was especially notable at higher doses of cisplatin. However, there was no evidence that treatment with RSR13 plus oxygen breathing beginning 15 min before administration of the alkylating agents modified the cytotoxicity of cisplatin or carboplatin from that seen with oxygen breathing alone. The effect of oxygen on cisplatin cytotoxicity was not consistent or statistically significant when analyzed over the entire length of the dose-response curve; however, the trend suggests an increase in the cytotoxicity of cisplatin in mice breathing oxygen. This agrees with the work of Grau and Overgaard who found that cisplatin is preferentially cytotoxic to well-oxygenated tumor cells in vivo [6].

There was no evidence that pretreatments with RSR13 plus oxygen at 22 and 14 h prior to administration of cisplatin or carboplatin altered the effect of either drug on the tumors.

The cytotoxicity of 10 μg/g cisplatin on tumor cells was not affected by treatment with either oxygen alone or RSR13 plus oxygen for 5 h after cisplatin injection. The effect of RSR13 plus oxygen treatments continuing after treatment with carboplatin is more difficult to interpret. The lower survivals seen in the groups treated with oxygen alone or with RSR13 plus oxygen groups and plated 5 h after injection of the 150 μg/g carboplatin could reflect either an effect of the changes in the posttreatment oxygenation or simply the effect of the delayed plating. Delayed plating might result in additional cell kill if carboplatin or active metabolites of carboplatin were still circulating in the mice at 2 h. In this case the difference between the findings at 2 and 5 h would be an artifact of the timing of the clonogenic assay, and would have little or no therapeutic significance. Alternatively, the effect could reflect a difference in the effectiveness of the drug due to the improvement in postinjection oxygenation. Further studies would be necessary to determine the mechanism underlying these posttreatment changes in measured cell survival and to assess whether they were of clinical significance.

In summary, these findings indicate that RSR13 in combination with oxygen breathing does not alter the cytotoxicity of cisplatin or carboplatin when used simultaneously, as a pretreatment or as a posttreatment either in vitro or in vivo. The findings from this series of experiments are consistent with those of previous studies showing that RSR13, with or without oxygen, does not produce direct cytotoxic effects [15]. The findings also provide some support to the previous work of Grau and Overgaard who showed that cisplatin is more cytotoxic to well-oxygenated tumor cells than to hypoxic tumor cells [6].

Acknowledgements This work was supported by a grant from Allos Therapeutics and Training Grant T32CA09085 from the NIH. We dedicate this paper to the memory of our co-author and collaborator, Marianne Nierenburg Kelley.

References

1. Abraham DJ, Wireko FC, Randad RS (1992) Allosteric modifiers of hemoglobin: 2-[4-[[(3,5-disubstituted anilino)carbonyl]methyl]phenoxy]-2-methylpropionic acid derivatives that lower the oxygen affinity of hemoglobin in red cell suspensions, in whole blood, and in vivo in rats. Biochemistry 31:9141
2. Amorino GP, Lee H, Holburn GE, Pashcal CB, Hercules SK, Shyr Y, Choy H (2001) Enhanced tumor oxygenation and radiation response by the allosteric effector of hemoglobin, RSR13. Radiat Res 156:294
3. Doppenberg EM, Rice MR, Alessandri B, Qian Y, Di X, Bullock R (1999) Reducing hemoglobin oxygen affinity does not increase hydroxyl radicals after acute subdural hematoma in the rat. J Neurotrauma 16:123
4. Douple EB, Richmond RC (1979) Radiosensitization of hypoxic tumor cells by cis- and trans-dichloroammineplatinum(II). Int J Radiat Oncol Biol Phys 5:1369
5. Evans D, Dice C (1993) Effects of cisplatin on the induction of apoptosis in proliferating hepatoma cells and nonproliferating immature thymocytes. Cancer Res 53:2133
6. Grau C, Overgaard J (1988) Effect of cancer chemotherapy on the hypoxic fraction of a solid tumor measured using a local tumor control assay. Radiother Oncol 13:301
7. Khandelwal SR, Kavanagh BD, Lin PS, Truong QT, Lu J, Abraham DJ, Schmidt-Ullrich RK (1998) RSR13, an allosteric effector of hemoglobin, and carbogen radiosensitize FSAII and SCCVII tumors in C3H mice. Br J Cancer 79:814
8. Kleinberg L, Grossman SA, Piantadosi S, Pearlman J, Engelhard H, Lesser G, Ruffer J, Gerber M (1999) Phase I trial to determine the safety, pharmacodynamics, and pharmacokinetics of RSR13, a novel radioenhancer, in newly diagnosed glioblastoma multiforme. J Clin Oncol 17:2593
9. Korbelik M, Skov KA (1989) Inactivation of hypoxic cells by cisplatin and radiation at clinically relevant doses. Radiat Res 119:145
10. Melvik JE, Pettersen EO (1988) Oxygen- and temperature-dependent cytotoxic and radiosensitizing effects of cis-dichlorodiammineplatinum(II) on human NHIK 3025 cells in vitro. Radiat Res 114:489
11. Moulder JE, Rockwell S (1984) Hypoxic fractions of solid tumors: experimental techniques, methods of analysis, and a survey of existing data. Int J Radiat Oncol Biol Phys 10:695
12. Randad RS, Mahran MA, Mehanna AS (1991) Allosteric modifiers of hemoglobin 1: design, synthesis, testing, and structure-allosteric activity relationship of novel hemoglobin affinity decreasing agents. J Med Chem 4:752
13. Richardson RS, Jordan MC, Haseler LJ, Tagore KS, Wagner PD (1998) Increased VO_{2max} with a right shifted HbO_2 dissociation curve at a constant O_2 delivery in dog muscle in situ. J Appl Phys 84:995
14. Rockwell S (1977) In vivo-in vitro tumor systems: new models for studying the response of tumors to therapy. Lab Anim Sci 27:831
15. Rockwell S, Kelley M (1998) RSR13, a synthetic allosteric modifier of hemoglobin, as an adjunct to radiotherapy: preliminary studies with EMT6 cells and tumors and normal tissues in mice. Radiat Oncol Investig 6:199
16. Rockwell S, Mate TP, Irvin CG, Nierenburg M (1986) Reactions of tumors and normal tissues in mice to irradiation in the presence and absence of a perfluorochemical emulsion. Int J Radiat Oncol Biol Phys 12:1315
17. Rockwell S, Kelly M, Irvin CG (1992) Effects of the perfluorochemical emulsion FMIQ on the radiation response of EMT6 tumors. Int J Radiat Biol 6:833
18. Safo MK, Moure CM, Burnett JC, Joshi GS, Abraham DJ (2001) High-resolution crystal structure of deoxy hemoglobin complexed with a potent allosteric effector. Protein Sci 10:951
19. Skov KA, Adomat H, Farrel NP, Matthews JB (1998) Assessment of toxicity of bis-platinum complexes in hypoxic and aerobic cells. Anticancer Drug Design 13:207
20. Stratford IJ, Williamson C, Adams GE (1980) Combination studies with misonidazole and a cis-platinum complex: cytotoxicity and radiosensitization in vitro. Br J Cancer 41:517
21. Sun JR, Brown JM (1993) Lack of differential radiosensitization of hypoxic cells in a mouse tumor at low radiation doses per fraction by cisplatin. Radiat Res 133:252
22. Teicher BA, Lazo JS, Sartorelli AC (1981) Classification of antineoplastic agents by their selective toxicities toward oxygenated and hypoxic tumor cells. Cancer Res 41:73
23. Wahr JA, Gerber MJ, Venitz J, Baliga N (2001) Allosteric modification of oxygen delivery by hemoglobin: effects of RSR13 on p50 in anesthetized patients. Anesth Analg 92:615
24. Wireko FC, Kellogg GE, Abraham DJ (1991) Allosteric modifiers of hemoglobin 2: crystallographically determined binding sites and hydrophobic binding/interaction analysis of novel hemoglobin oxygen effectors. J Med Chem 34:758