ABSTRACT

Tirapazamine (SR 4233), a benzotriazine di-N-oxide, a potent and selective killer of hypoxic cells, is currently in Phase I clinical trials with the expectation that it will be combined with radiation therapy. However, because of the likelihood that hypoxic tumor cells may also be resistant to some commonly used chemotherapeutic agents, we have tested tirapazamine in combination with cisplatin (c-DDP) in the mouse RIF-1 tumor. A large, schedule-dependent enhancement of tumor cell killing was observed both in vivo and in vitro, with a maximal response observed when the SR 4233 was given 2–3 h before c-DDP. Assay of serum blood urea nitrogen levels following treatment with these two drugs indicates that SR 4233 does not enhance the kidney damage which can result from high doses of c-DDP. Leukopenia induced by the two drugs in combination was equal to that predicted from an additive effect of the responses to the individual drugs. Also, there was no change in the systemic toxicity of c-DDP (as judged by 50% lethal dose) when SR 4233 was combined with c-DDP at a dose and timing that produced the maximum tumor interaction. These observations point to a promising new combination therapy with considerable therapeutic advantage.

INTRODUCTION

Tirapazamine (3-amino-1,2,4-benzotriazine 1,4-dioxide; SR 4233; WIN 59075) is the lead compound in a new class of bioreductive anticancer drugs, benzotriazine di-N-oxides, which have a large preferential cytotoxicity to hypoxic cells. It is currently undergoing Phase I clinical testing. The drug is specifically toxic to hypoxic cells in tumors by a mechanism which involves one-electron reduction of the drug to a radical which kills the hypoxic cells by hydrogen abstraction from DNA, thereby producing double-strand breaks and chromosome aberrations (1). Since there is compelling evidence that viable hypoxic cells exist not only in transplanted rodent tumors (2) but also in human solid tumors (3–5) and that these hypoxic cells appear to adversely affect cure rates in radiotherapy (6–8), the primary intention has been to use tirapazamine in combination with fractionated radiotherapy. There are ample preclinical data which show that combining this drug with radiation schedules similar to those used clinically produces a large enhancement of radiation killing in murine transplanted tumors without any enhancement of the radiation response of surrounding normal tissues (9–11).

In addition to data showing that hypoxia affects the response of cells to ionizing radiation, there are also data which demonstrate that hypoxia can affect the response of cells to a variety of chemotherapeutic drugs because of the hypoxia per se, or because hypoxic cells tend to be furthest from viable blood vessels, or because hypoxic/nutrient-deprived cells tend to have slower rates of proliferation (12–15). If it is assumed that hypoxia can affect the response to chemotherapy, then we would expect an increase in the tumor cell kill by any hypoxia-limited chemotherapeutic agent if the agent were combined with a drug selectively toxic for hypoxic cells. This was the initial rationale for the combination of the nitroimidazole hypoxic cytotoxic agents (metronidazole and misonidazole) with various anticancer drugs; initial results with one drug (doxorubicin) demonstrated that this theory appears to hold (16). Success has encouraged several investigators to study the interaction of nitroimidazole hypoxic cytoxins with a variety of anticancer drugs, and it was soon found that a therapeutic gain could be achieved when these agents were combined with various anticancer drugs, particularly the alkylating agents cyclophosphamide and melphalan and the nitrosoureas 1,3-bis(2-chloroethyl)-1-nitrosourea and 1-(2-chloroethyl)-1-cyclohexyl-1-nitrosourea (17). However, it also became clear that the therapeutic gain produced was not the consequence of selective killing of hypoxic cells by the nitroimidazoles but appeared to be by a mechanism involving the potentiation of alkylating agent-induced DNA cross-links by metabolites of the nitroimidazoles (18, 19).

The ability of SR 4233 to selectively kill hypoxic cells in animal tumors now enables the question to be addressed of the potential of combining an agent specifically cytotoxic to hypoxic cells with commonly used anticancer drugs. For the initial studies, we chose c-DDP, both because of its wide application in cancer chemotherapy and because it has been shown to produce a large, selective killing of aerobic cells in a solid tumor model (15). This combination of tirapazamine and cisplatin has been tested previously by Holden et al. (20), who reported that the combination resulted in a slightly greater than additive cell killing at the highest dose of cisplatin used (20 mg/kg). They also showed no significant increase in cytotoxicity to bone marrow granulocyte-macrophage colony-forming units over that caused by cisplatin alone. These investigators, however, considered only one time interval between tirapazamine and cisplatin, that of the two drugs given simultaneously. Since we have observed, both for fractionated irradiation and large single doses, that tirapazamine given simultaneously with radiation produces the least interaction between the two modalities (9, 21) and because a time-dependent interaction has also been reported between cisplatin and radiation (22), we decided to investigate whether there was any schedule dependence of the interaction between tirapazamine and c-DDP. Our results show a marked schedule dependence for enhancement of the tumor response with no apparent increase in the systemic toxicity of cisplatin, as judged by the LD50 of cisplatin, by serum BUN levels, and by peripheral WBC counts.

MATERIALS AND METHODS

Animals and Tumors. The RIF-1 fibrosarcoma in C3H/Km mice (housed under defined flora conditions) was maintained alternately in vivo and in vitro, according to a previously established protocol (23). Tumor cell monolayers, growing in Waymouth's medium supplemented with 15% fetal bovine serum, were harvested with 0.05% trypsin. From this suspension, 2 × 105 cells in 0.05 ml medium were inoculated intradermally in the midline of the back of each mouse at a site approximately 2 cm from the base of the tail. Experiments were begun 2 weeks later when the mean tumor volume was approximately 200 mm3.

Drugs. SR 4233 was supplied by Sterling Drug, Inc. For animal studies, the drug was dissolved in normal saline at a concentration of 1 mg/ml and injected...
colonies were stained and counted after 2 weeks of incubation at 37°C in a 5% CO₂ humidified atmosphere. The number of relative clonogenic cells/tumor was calculated as the product of plating efficiency and tumor cell yield for treated tumors relative to that for control untreated tumors assayed in parallel.

For studies of cells in vitro, RIF-1 cells were seeded into 60-mm glass Petri dishes in Waymouth’s medium with 15% fetal bovine serum at a concentration of 2 × 10⁴ cells/dish. The experiments were performed 4–5 days later when there were approximately 10³ cells/dish at the time of treatment. The growth medium was then replaced with 2 ml of medium without serum, containing SR 4233 at a concentration of either 2 or 4 µg/ml. In each experiment, groups were included in which treatment with SR 4233 and c-DDP were performed both simultaneously and with an interval between the two treatments. In those groups in which there was an interval between treatments, the cells were rinsed twice immediately after the exposure to SR 4233 and then the medium was replaced with full growth medium until the time for the second treatment (with c-DDP), which was also performed in medium without serum. Both the exposure to SR 4233 and to c-DDP were for 1 h under hypoxic conditions. To achieve hypoxia, the dishes were loaded into specially fabricated, prewarmed aluminum gassing chambers which were placed on a shaking table and connected to a gassing manifold composed of a vacuum outlet line and in let lines for air or nitrogen (+ 5% CO₂). Hypoxia was achieved in the aluminum chambers through a series of 5 alternate evacuations in 2–3 min to 0.1 atmosphere followed by gassing with nitrogen (+ 5% CO₂). After gassing, the chambers were sealed and incubated for 1 h at 37°C. Measurement of the oxygen level in the medium using a Clarke electrode showed that hypoxia was achieved rapidly (in approximately 10 min with an average pO₂ level during the 1 h exposure of less than 200 ppm oxygen). Immediately after the treatment with c-DDP, the cells were trypsinized, counted, replated in plastic Petri dishes in Waymouth’s + 15% fetal bovine serum, and incubated for 14 days at 37°C in a 5% CO₂ humidified atmosphere; then the colonies were stained with crystal violet and those containing 50 or more cells were counted.

Normal Tissues. Preliminary studies indicated that WBC counts reached a nadir on the third day after treatment with SR 4233 and c-DDP and then rose again to near control levels on day 5. We therefore performed a dose-response study on day 3 of c-DDP alone and SR 4233 + c-DDP, with the SR 4233 dose (0.27 mmol/kg) given 2.5 h before c-DDP. The results are shown in Fig. 4. They demonstrate that both agents produce a mild leukopenia and the combination produced an effect equal to that predicted from adding the responses to the individual drugs. It is of note that a decrease in leukopenia seen at the highest dose of cisplatin has also been reported and has been attributed to a possible hemocolection associated with nephrotoxicity (24).

Cell Survival. For animal studies, RIF-1 cell survival was evaluated according to an in vivo/in vitro excision assay. Mice were killed 24 h after c-DDP treatment; tumors were excised, minced, and dissociated with an enzyme cocktail (23); and cells were plated for clonogenic assay. Resultant tumor cell colonies were stained and counted after 2 weeks of incubation at 37°C in a 5% CO₂ humidified atmosphere. The number of relative clonogenic units was calculated as the product of plating efficiency and tumor cell yield for treated tumors relative to that for control untreated tumors assayed in parallel.

RESULTS

Tumors in Vivo. Fig. 1 shows the pooled results from two experiments in which 0.35 mmol/kg SR 4233 (63 mg/kg) was delivered to the tumor-bearing mice at various times over an interval from 3 h prior to 2 h after delivery of 8 mg/kg c-DDP; clonogenic survival was assessed 24 h later. Fig. 2 shows the results from another experiment with similarly treated mice in which the time interval between SR 4233 and c-DDP was extended to 24 h. Because of the large amount of cell killing observed at the nadir in Fig. 1 (which was on the border of the limits of the clonogenic assay), we reduced the dose of SR 4233 in the experiment shown in Fig. 2 from 0.35 to 0.27 mmol/kg (48.6 mg/kg). Despite this, the data from the three experiments show the same results: essentially additive toxicity when the drugs are given together; and a major cytotoxic interaction when the drugs are separated in time, with the maximal reduction in the number of clonogenic cells/tumor when SR 4233 treatment preceded c-DDP by approximately 2.5 h.

Fig. 3 describes the effect on relative clonogenic cells of treatment with various doses of SR 4233 given 2.5 h before either 4 or 8 mg/kg c-DDP. There was an approximately exponential reduction in tumor cell survival at both doses of c-DDP with increasing dose of SR 4233. The slopes of the two best fitting lines through the two sets of curves generated by these data were not statistically different.

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Assays of serum BUN were performed on day 6 after injection of SR 4233 and c-DDP, based on a preliminary investigation of the time...
for maximum increase in BUN following high doses of c-DDP. Fig. 5 shows the effect on serum BUN levels of c-DDP with and without pretreatment with SR 4233. These results show that SR 4233 in combination with c-DDP does not add to c-DDP kidney toxicity and may even protect at the highest dose tested.

As a further test of whether SR 4233 enhanced the systemic toxicity of c-DDP, we performed an LD_{50} experiment with c-DDP alone and with c-DDP given 2.5 h after injection of SR 4233. The LD_{50} for mice treated with 0.35 mmol/kg SR 4233 plus c-DDP was 17.7 mg/kg (95% confidence limit, 16.8–18.7 mg/kg), as contrasted with that for c-DDP alone which was 17.8 (17.0–18.7 mg/kg).

**In Vitro Experiments.** In an attempt to shed some light on the possible mechanism of the interaction of tirapazamine with cisplatin, we performed experiments with RIF-1 cells *in vitro*. In these studies, the cells were exposed to SR 4233 (2 or 4 μg/ml) for 1 h under hypoxic conditions and also exposed to cisplatin (2 μg/ml) for 1 h either simultaneously with the SR 4233 or up to 4 h later. The concentration of each agent was chosen to produce a similar level of cell killing of hypoxic cells as that of the RIF-1 tumors *in vivo*; for SR 4233, these surviving fractions obtained *in vitro* were 0.3 and 0.09 at 2 and 4 μg/ml, respectively, and for c-DDP, 3.5 × 10^{-3}. The results obtained for the drugs given simultaneously were not significantly different from the product of the survivals of the two agents given separately (i.e., compatible with additivity); whereas when the drugs were separated, there was more cell killing by a factor of up to 10^2.

The results of these *in vitro* experiments are shown in Fig. 6. In order to compare one experiment to another, the data have been normalized to produce a value of 1 for the combined survival with the drugs given simultaneously. It can be seen that there are similar kinetics of enhancement of cell killing as observed in the *in vivo* results, although the absolute magnitude of the effect of splitting the two doses is less than that observed *in vivo*. To check that the interaction between the two agents depended on the presence of hypoxia, the experiments were repeated with 3 h between exposure of the cells to SR 4233 and cisplatin (2 μg/ml). Data show the ratio of the survival in the split dose group to that in the group in which the cells were exposed to the drugs simultaneously. Both exposures were under hypoxic conditions. Bars, SE.

**DISCUSSION**

In this investigation, we have demonstrated a large, schedule-dependent interaction between the hypoxic cytotoxic agent tirapazamine and the anticancer drug c-DDP. This interaction was no more than additive when the two drugs were given simultaneously, but when tirapazamine was given 2–3 h prior to the cisplatin injection, some 4–5 extra logs of cell kill were produced in addition to the 2 logs of cell kill produced by cisplatin alone. This represents an enormous
increase in tumor efficacy of cisplatin, and it is all the more remarkable that in the tests of the systemic toxicity of cisplatin (serum BUN and acute toxicity), the combination with the optimum separation for tumor efficacy (2.5 h) showed no enhancement compared to cisplatin alone. Thus, essentially all of the additional cell kill of the tumor cells translates into a therapeutic gain for this combination. It is also encouraging that recent studies with the SCCVII transplantable mouse carcinoma also show greater than additive cell kill for the combination of SR 4233 and cisplatin (data not shown).

One of our reasons for studying this combination of drugs was the finding reported by Grau and Overgaard (15) that despite killing a large proportion of the tumor cells in a solid tumor, cisplatin appeared to produce little or no killing of the hypoxic cells in the tumor. It is unlikely that this effect is due to an intrinsic resistance of hypoxic cells to cisplatin, since several authors have reported no differential between the sensitivity of hypoxic and aerobic cells in vitro (14, 25). Indeed, we also confirmed this result in our own experiments with RIF-1 tumors in vitro (data not shown). It would be possible, however, that the sparing of hypoxic cells in vivo could be the result of the lower cytotoxicity of slowly or nondividing cells to cisplatin compared to rapidly dividing cells (26, 27). If such a phenomenon occurs in vivo, then since tirapazamine has the property of selectively killing hypoxic and probably largely nondividing cells in vivo, one would expect a synergistic interaction of the two agents when used in vivo.

Although it is possible that part of the interaction in vivo could be the result of such a heterogeneous distribution of aerobic (rapidly proliferating) and hypoxic (slowly proliferating) cells in the tumors, it is unlikely that this distribution of cells could account for all of the effect, in view of our in vitro data. In these in vitro studies, the period for achieving hypoxia (5–10 min), combined with the total exposure under hypoxic conditions (1 h), is too short a time to expect any major changes in the proliferation rates of the cells. Thus, other explanations must be found to account for the interaction in vitro. One possibility would be depletions of intracellular glutathione, which plays a role in protecting against cisplatin toxicity (28, 29). However, at the doses of SR 4233 used, we have found little or no depletion of intracellular levels of glutathione; therefore, this explanation is unlikely. Further studies will be required to determine the mechanism of this interaction.

Whatever the mechanism is, however, it seems clear that hypoxia is necessary for the interaction. This was seen in the in vitro results and is consistent with the lack of potentiation of the systemic toxicity of c-DDP in the mouse. This requirement for hypoxia is a major advantage since it provides the basis for a tumor-specific interaction between the two drugs. In general, normal tissues are at an oxygen concentration above 10–15 mm Hg (3, 5). At these and higher oxygen partial pressures, the cytotoxicity produced by tirapazamine is very low (1). On the other hand, many tumors have a significant number of cells at oxygen concentrations below 10 mm Hg (3, 5) and at those partial pressures, the metabolism of SR 4233 to cytotoxic species is greatly increased.

REFERENCES


Tumor-specific, Schedule-dependent Interaction between Tirapazamine (SR 4233) and Cisplatin

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