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ORIGINAL ARTICLE

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# Modification of the antitumor activity of chemotherapeutic drugs by the hypoxic cytotoxic agent tirapazamine

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**Abstract** *Purpose*: Preclinical studies were performed to examine the interaction of the hypoxic cell toxin tirapazamine (TPZ), a benzotriazine di-*N*-oxide, with several chemotherapeutic agents, including carboplatin, cyclophosphamide, doxorubicin, etoposide, 5-fluorouracil (5-FU), taxol, and navelbine. *Methods*: The modification by TPZ of the antitumor drug activity and the effect of schedule were determined with an in vivo/in vitro clonogenic assay using well-established RIF-1 murine tumors transplanted into C3H mice. *Results*: Additive, or greater than additive, tumor cell killing was observed when TPZ was combined with carboplatin, cyclophosphamide, doxorubicin, etoposide, 5-FU and taxol. With the exception of 5-FU there were only small, or no, enhancements of the systemic toxicities of the drugs by TPZ. The greatest enhancement of antitumor activity was with carboplatin, with the maximum effectiveness when TPZ was given 2–3 h before the carboplatin. The activity of cyclophosphamide, doxorubicin, etoposide and taxol were most enhanced when TPZ was given 24 h before the drug. Additional investigations with three-drug combination treatments using cisplatin and TPZ with either etoposide or navelbine indicated a substantial therapeutic gain from the addition of TPZ. *Conclusions*: The data for each of the drugs tested in combination with TPZ, with the exception of 5-FU, indicate that potential clinical benefit may be obtained from therapies combining TPZ with conventional chemotherapy.

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## Introduction

The bioreductive drug tirapazamine (3-amino-1,2,4-benzotriazine 1,4-dioxide; SR 4233, TPZ) was originally developed for use in combination with irradiation of solid tumors because of its selective toxicity towards hypoxic cells [2]. Hypoxic cells are present in both rodent [14] and human tumors [19] and are widely believed to be responsible for treatment failure in radiotherapy [6, 11, 15]. We have combined TPZ with fractionated irradiation of murine tumors and have shown a substantial selective enhancement of the radiation effect on the tumors [3–5]. These results formed the basis for the current phase II studies of this drug with radiotherapy. However, the potential for TPZ in the clinic has been considerably expanded by our recent findings that this drug can significantly enhance the cytotoxicity of cisplatin in a schedule-dependent manner [7, 8]. Phase II and III clinical trials with TPZ combined with cisplatin are underway.

This report describes our preclinical investigations of the interaction of TPZ with other chemotherapeutic drugs. These include carboplatin, cyclophosphamide, doxorubicin, etoposide, 5-fluorouracil, taxol, and navelbine. There were varying degrees of potentiation and schedule dependence when TPZ was combined with these drugs, although, with the exception of carboplatin, none of these responses was found to be as large as that produced by TPZ and cisplatin [7]. However, since cisplatin treatment in the clinic is often combined with some of these drugs, such potentiations may be of importance. In this regard, we also investigated the effects of TPZ and cisplatin in three-drug combinations with either etoposide or navelbine. TPZ was found to

produce a major increase in the tumor cell kill by both combinations (cisplatin with etoposide and cisplatin with navelbine) and, in each case, the interactions appeared to be schedule-dependent.

## Materials and methods

### Animals and tumors

C3H/Km mice were housed under germ-free conditions. All studies were conducted according to the guidelines and directives set forth by the Stanford University Administrative Panel on Laboratory Animal Care. The RIF-1 fibrosarcoma was maintained according to a previously established protocol [18]. In brief, tumor cell monolayers, growing in Waymouth's medium supplemented with 15% fetal calf serum, were harvested with 0.05% trypsin. An inoculum from this suspension, consisting of $2 \times 10^5$ cells per 0.05 ml medium, was injected intradermally into the back of each mouse at a site approximately 2 cm from the base of the tail. Studies were initiated 2 weeks later when the mean tumor volume was approximately 150 $mm^3$.

### Drugs

TPZ, provided by Sanofi-Winthrop, Malvern, Pa. was dissolved in normal saline at a concentration of 1 mg/ml. Carboplatin (Bristol Laboratories, Princeton, N.J.), cisplatin (Bristol Laboratories, Princeton, N.J.), cyclophosphamide (Mead Johnson, Evansville, I.N.), and 5-fluorouracil (SoloPak Laboratories, Elk Grove Village, Ill.) were dissolved in sterile water. Doxorubicin (Cetus, Emeryville, Ca.) was dissolved in sterile saline. Etoposide (Bristol Laboratories, Princeton, N.J.), taxol (Mead Johnson, Princeton, N.J.), and navelbine (Burroughs Wellcome, Research Triangle Park, N.C.) were either injected from stock or diluted with sterile saline, as necessary. All the drugs were injected intraperitoneally on the basis of animal body weight.

### Toxicity assays

Before studies with tumor-bearing animals were performed, pilot studies were conducted to determine drug tolerance levels. The toxicity of each drug in nontumor-bearing mice was assessed alone and in combination with simultaneous delivery of TPZ. For drug-alone toxicity determinations, the saline carrier in the absence of TPZ was also delivered to maintain equal hydration levels. For combination drug studies, the TPZ dose was kept constant, while the dose of the other drug was varied. All animals were observed at least twice daily, and any that were becoming moribund were killed. $LD_{50}$ values were calculated from computer logit determinations based on 30-day survival. Drug doses for tumor-bearing mice were then kept significantly below these values to avoid systemic toxicity.

### Tumor cell survival assay

Tumor cell survival was assessed using an in vivo/in vitro excision protocol. As described above, hydration levels were maintained with saline in mice not receiving TPZ. In brief, mice were killed 24 h after treatment with the drug being tested in combination with TPZ. Tumors, assayed individually, were immediately excised, minced, dissociated with an enzyme cocktail, and plated for clonogenic survival [18]. After 2 weeks incubation at 37 °C in a humidified atmosphere containing 5% $CO_2$, tumor cell colonies were stained with crystal violet and counted. The relative number clonogenic cells per 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 comparing the efficacy of combination drug treatments with those of individual drugs, additivity was defined as the product of the surviving fractions (relative clonogenic cells per tumor) for each of the individual drug treatments.

## Results

The systemic toxicity of the various drugs tested alone and in combination with TPZ is shown in Table 1. The toxicities of both carboplatin and cisplatin were unchanged by TPZ given 2.5 h before either drug, and the same was true for etoposide with simultaneous TPZ treatment. However, carboplatin toxicity was quite markedly reduced when given simultaneously with TPZ. The toxic effects of cyclophosphamide, navelbine, doxorubicin, taxol, and 5-fluorouracil, when given at the same time as TPZ, were increased by amounts varying from approximately 15 to 45%.

**Table 1** Toxicity of various chemotherapeutic drugs alone and in combination with TPZ in C3H/Km mice

| Drugs | $LD_{50}$ mg/kg (95% confidence limits) | | TPZ dose[a] (mmol/kg) | Timing of combination treatment |
|---|---|---|---|---|
| | Drug alone | TPZ + drug | | |
| Carboplatin | 117 (95–145) | 121 (115–127) | 0.27 | TPZ 2.5 h before |
| Carboplatin | 117 (93–145) | 256 (241–273) | 0.27 | Simultaneous |
| Cisplatin[b] | 17.8 (17.0–18.7) | 17.7 (16.8–18.7) | 0.35 | TPZ 2.5 h before |
| Cyclophosphamide | 372 (338–409) | 311 (280–345) | 0.27 | Simultaneous |
| Doxorubicin | 22.2 (18.7–26.3) | 17.8 (15.8–20.0) | 0.27 | Simultaneous |
| Etoposide | 58 (48–71) | 61 (47–79) | 0.13 | Simultaneous |
| Etoposide | 58 (48–71) | 58 (48–71) | 0.20 | Simultaneous |
| 5-Fluorouracil | 437 (418–458) | 285 (215–379) | 0.13 | Simultaneous |
| 5-Fluorouracil | 437 (418–458) | 233 (186–292) | 0.20 | Simultaneous |
| Taxol | 54 (46–62) | 37 (33–42) | 0.27 | Simultaneous |
| Navelbine | 21.2 (18.5–24.2) | 17.2 (15.9–18.5) | 0.27 | Simultaneous |

[a] TPZ $LD_{50}$ 0.44 mmol/kg
[b] Data from reference 7

TPZ enhanced carboplatin antitumor activity to the greatest degree when TPZ preceded carboplatin by 2–3 h (Fig. 1). This was in evidence for treatment with TPZ (0.27 mmol/kg) and carboplatin given at 60% of their individual $LD_{50}$ levels, but was more clearly shown when a much larger dose of carboplatin (150 mg/kg) was combined with the TPZ. Thus, the schedule dependence of the enhancement of tumor cell kill by TPZ was similar to that observed previously for cisplatin for this same tumor type [7].

Among the seven drugs tested with TPZ, the activities of cyclophosphamide, doxorubicin, etoposide, and taxol were most changed when TPZ was given 24 h before the other drug (Fig. 2). At all testing times, i.e. TPZ treatment given from 24 h before to 3 h after cyclophosphamide, TPZ increased cyclophosphamide tumor cell kill beyond additivity (Fig. 2A) This response was relatively constant over time, although pretreatment with TPZ 24 h before cyclophosphamide produced a slightly greater cell kill than treatment at other times. In contrast, treatment with TPZ showed significant enhancement beyond additivity only when given 24 h prior to the other drug for doxorubicin, etoposide and taxol.

TPZ enhancement of etoposide activity was only in evidence if TPZ was given the day before etoposide (Fig. 2C). However, as shown in Table 1, the absence of additional systemic toxicity following the combination of etoposide and TPZ indicates the potential for therapeutic enhancement with this drug combination.

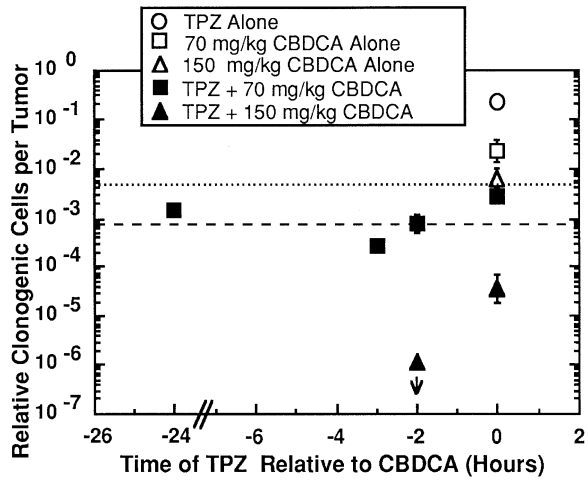


**Fig. 1** Number of clonogenic RIF-1 cells per tumor relative to control tumors. RIF-1 tumor-bearing mice were treated with TPZ (0.27 mmol/kg) alone, carboplatin (*CBDCA*) alone, or a combination of the two drugs according to the various schedules shown. The mice were sacrificed 24 h after CBDCA. Three independently assayed tumors per point, except for the point at − 2 h for TPZ + 150 mg/kg CBDCA, for which cell survival for two of three tumors was too low to be measured. *Bars* indicate standard errors. Simple additivity for TPZ with 70 mg/kg CBDCA is indicated by the *dotted line* and that for TPZ with 150 mg/kg CBDCA is the *dashed line*

Therefore, further tumor studies were conducted using multiple doses of TPZ and etoposide (Fig. 3A). Etoposide (27 or 35 mg/kg) was given on 3 consecutive days and two different dose schedules were examined with

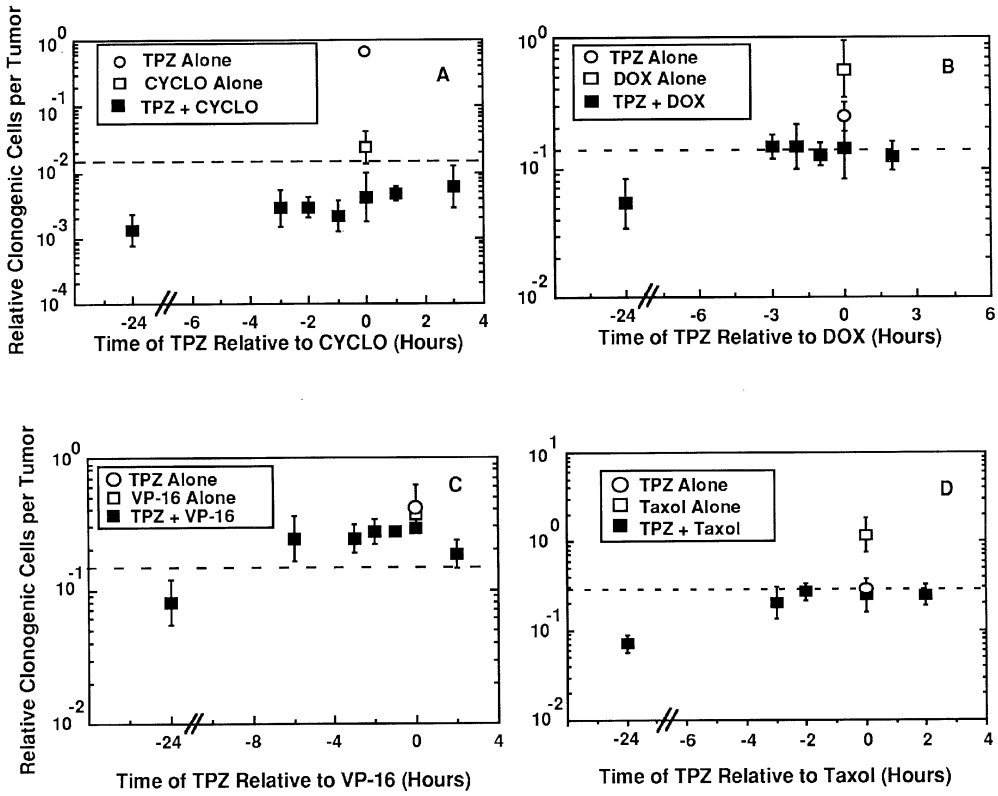


**Fig. 2A–D** Number of clonogenic RIF-1 cells per tumor relative to control tumors. RIF-1 tumor-bearing mice were treated with TPZ (0.27 mmol/kg) alone, and (**A**) cyclophosphamide (100 mg/kg, *CYCLO*) or (**B**) doxorubicin (12 mg/kg, *DOX*), (**C**) etoposide (35 mg/kg, *VP-16*), or (**D**) taxol (22 mg/kg) alone, or a combination of TPZ and one of these drugs according to the various schedules shown. The mice were sacrificed 24 h after the drug treatment. Each point is derived from the three independently assayed tumors; *bars* standard errors. Simple additivity for TPZ and each drug is indicated by the *dashed line*

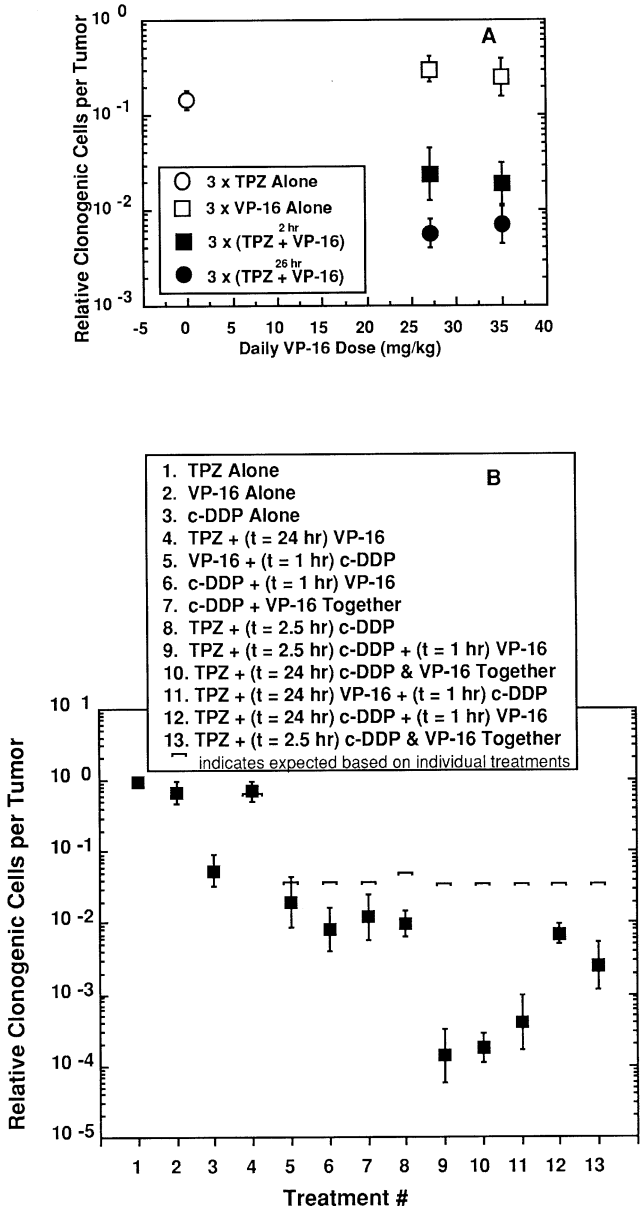


**Fig. 3 A** Number of clonogenic RIF-1 cells per tumor treated with three daily doses of 0.22 mmol/kg TPZ alone, 27 or 35 mg/kg etoposide (*VP-16*) alone, or a combination of the two drugs. TPZ was given either 26 or 2 h before each treatment with VP-16. Each point is derived from three independently assayed tumors; *bars* standard errors. **B** Number of clonogenic RIF-1 cells per tumor treated with 0.27 mmol/kg TPZ alone, 35 mg/kg etoposide (*VP-16*) alone, 11 mg/kg cisplatin (*c-DDP*) alone, or combinations of two or three of these drugs as shown. Each point is derived from the two to six independently assayed tumors; *bars* standard errors

either a 2-h or 26-h interval between TPZ and etoposide. While both schedules produced a greater tumor cell kill than that expected from the individual treatments, the 26-h schedule, in which the first treatment with TPZ was given the day before the first etoposide treatment, was the more effective. A possible reason for the fact that this daily course of TPZ and etoposide showed a more than additive cell kill, while the single treatments separated by less than 6 h showed a less than additive cell kill (Fig. 2C), is that with daily injections, separations between TPZ and etoposide include 24-h intervals between the two drugs, which do produce greater than additive cell kill in single treatments.

Because etoposide is frequently used in conjunction with cisplatin in the clinic, further studies were conducted with etoposide, TPZ, and cisplatin in three-drug combination schedules. Treatment doses were determined based on previous toxicity studies in which the three drugs, given together, were well tolerated and caused no deaths (data not shown). Figure 3B shows the combined results from two experiments. Although the TPZ activity with cisplatin alone in this study was somewhat less than that usually observed in our laboratory, the data clearly indicate that TPZ enhanced the tumor cell kill produced by cisplatin given with etoposide (groups 9, 10 and 11 compared with groups 5, 6 and 7).

Tumor cell kill induced by 5-fluorouracil was enhanced by TPZ at each of the time intervals tested (Fig. 4A). However, in view of the degree of toxicity of the combination of the two drugs (Table 1), a further study was conducted to compare antitumor activity at equitoxic doses. As is shown in Fig. 4B, a dose of 280 mg/kg 5-fluorouracil alone, which is 60% of the $LD_{50}$ value, produced the same amount of tumor cell kill as did TPZ with 150 mg/kg 5-fluorouracil (60% of the combination $LD_{50}$ value). Thus no therapeutic benefit was obtained from this drug combination.

An experiment was conducted to compare the effects of TPZ, navelbine, and cisplatin given alone or together in various combinations and schedules (Fig. 5). This study was conducted at well-tolerated dose levels determined from previous toxicity studies (data not shown). The effect of TPZ given simultaneously with navelbine was slightly greater than the predicted additive effect. In addition, when navelbine was given either with cisplatin or 4 h before cisplatin, the cell kill was also somewhat greater than that expected from additive toxicities. In contrast when TPZ was added to this combination using two different schedules, i.e. both navelbine and TPZ given 2.5 h before cisplatin (group 9), or navelbine given 1.5 h before TPZ which was followed 2.5 h later with cisplatin (group 10), the cell kill was more than 100 times greater than that predicted from the three individual treatments. As expected from the modest effect of TPZ on navelbine alone (group 4), most of the enhancement of the two-drug (cisplatin and navelbine) combination was the result of the enhancement by TPZ of the antitumor effect of cisplatin (compare group 8 with groups 9 and 10).

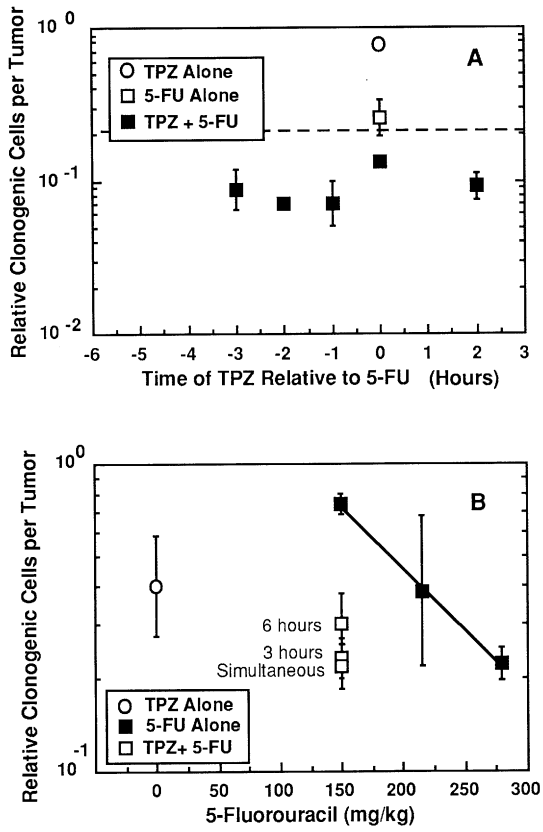


**Fig. 4** **A** Number of clonogenic RIF-1 cells per tumor relative to control tumors. RIF-1 tumor-bearing mice were treated with TPZ (0.20 mmol/kg) alone, 5-fluorouracil (150 mg/kg, *5-FU*) alone, or a combination of the two drugs according to the various schedules shown. The mice were sacrificed 24 h after 5-FU. Each point is derived from the three independently assayed tumors; *bars* standard errors. Simple additivity for TPZ and 5-FU is indicated by the *dashed line*. **B** Number of clonogenic RIF-1 cells per tumor relative to control tumors. RIF-1 tumor-bearing mice were treated with TPZ (0.20 mmol/kg) alone, 5-fluorouracil (*5-FU*) alone, or a combination of the two drugs with TPZ given either together with, or 6 or 3 h before, 5-FU. The mice were sacrificed 24 h after 5-FU. Each point is derived from the three independently assayed tumors; *bars* standard errors

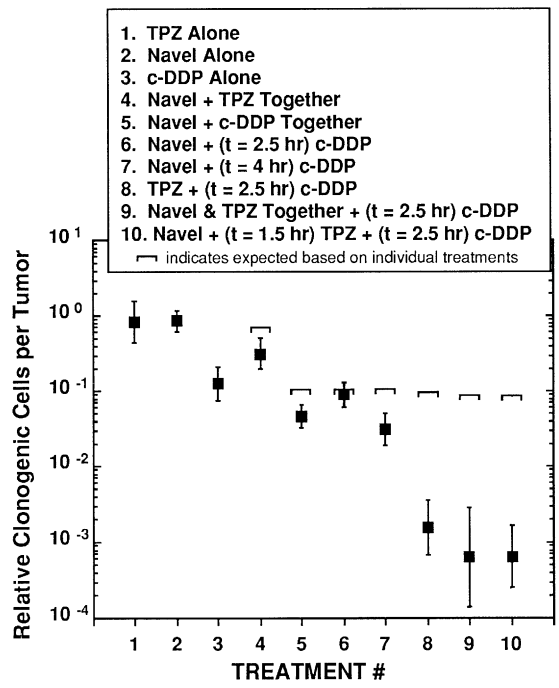


**Fig. 5** Number of clonogenic RIF-1 cells per tumor treated with 0.27 mmol/kg TPZ alone, 6 mg/kg navelbine (*Navel*) alone, 8 mg/kg cisplatin (*c-DDP*) alone, or combinations of two or three of these drugs as shown. Each point is derived from the three independently assayed tumors; *bars* standard errors

## Discussion

Presented here is a summary of a series of experiments designed to examine the interaction of the hypoxic cytotoxin TPZ with various chemotherapeutic drugs in clinical use against solid tumors. We performed the experiments because of the remarkable potentiation by TPZ of the antitumor efficacy of cisplatin observed in preclinical studies [7], the completion of several phase II, and initiation of phase III studies, of TPZ with cisplatin, and because cisplatin is often combined with these other anticancer agents. Not unexpectedly, the interaction of TPZ with the chemotherapeutic drugs studied showed a spectrum of responses from largely additive (doxorubicin, etoposide, and taxol) to more than additive (carboplatin, cyclophosphamide and 5-fluorouracil), and from little or no schedule dependency (cyclophosphamide and 5-fluorouracil) to a marked schedule dependency (carboplatin, doxorubicin, etoposide, and taxol). In the case of the latter three drugs (doxorubicin, etoposide, and taxol), we observed the maximum interaction when TPZ was given 24 h prior to the injection of the anticancer drug. The reason for such an unusual schedule dependency is not apparent, and further work is underway to investigate this. It is of note, however, that for many of the drugs, and particularly for cisplatin [7] and carboplatin, the least optimum schedule was simultaneous delivery with TPZ. This is reminiscent of previous studies [3, 20] in which it was found that simultaneous treatment with TPZ and irradiation are also least effective. This could be the result of blood flow changes caused by the bolus injection of TPZ, and such changes have in fact been reported for some tumors [9]. It is important to note, however, that when we simulated the 2-h delivery of TPZ that is used in the clinic, we obtained a similar enhancement of the cisplatin tumor cell kill, but the marked schedule dependency was not present [8]. This underlines the importance of determining the mechanism of action of the schedule dependency in order to predict whether it is likely to occur in the clinical situation.

The scheduling issue becomes much more difficult to analyze when more than two drugs are combined, as

illustrated in our experiments using TPZ and cisplatin with either etoposide or navelbine. Because of the complexity and number of possible schedules, it was necessary to limit the number of tested combinations. In addition, additivity expressed as a simple product of the survival for each individual drug may not adequately predict the expected interaction among the various drugs. However, several schedules for etoposide with cisplatin and TPZ appeared to be quite beneficial, although two of the five three-drug schedules were clearly less effective. Elsewhere [1] it has been shown that navelbine is most effective when given 4 h before cisplatin and our results agree with this, although when TPZ was added to the treatment, the effect was the same whether or not the navelbine was given with the TPZ 2.5 h before or 4 h before the cisplatin, with the TPZ given between the two chemotherapeutic agents.

Mechanism studies that might explain the enhanced tumor cell kill of chemotherapy by TPZ have not been completed. The initial rationale for this series of experiments, which began with our earlier studies using cisplatin and TPZ [7, 8], was the preferential cytotoxicity for hypoxic cells exhibited by TPZ, in conjunction with evidence that the activity of certain other drugs in solid tumors can be limited by reason of hypoxia [10, 12, 16, 17]. However, our in vitro cisplatin data [7] have shown that preferential killing of hypoxic tumor cells cannot account for all of the effect seen with TPZ and cisplatin. Other factors could clearly play a role. For example, TPZ may change the distribution of the accompanying drug or the efficiency with which the tumor cells repair DNA damage, as others have previously speculated [13]. In addition, TPZ may alter cell cycle distributions and thus change the sensitivity of the tumor to further drug treatment. This is consistent with our results with four of the drugs (cyclophosphamide, doxorubicin, etoposide and taxol), which showed maximum tumor cell kill when TPZ was given 24 h before each drug. However, until further studies are completed, the mechanism(s) by which TPZ interacts with chemotherapeutic drugs remains undefined. It is clear, however, that TPZ has considerable promise as an agent that can selectively enhance the antitumor effects of currently used anticancer drugs.

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