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Combination of the Bioreductive Drug Tirapazamine with the Chemotherapeutic Prodrug Cyclophosphamide for P450/P450-Reductase-based Cancer Gene Therapy[1]

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ABSTRACT

Tirapazamine (TPZ) is a bioreductive drug that exhibits greatly enhanced cytotoxicity in hypoxic tumor cells, which are frequently radiation-resistant and chemoresistant. TPZ exhibits particularly good activity when combined with alkylating agents such as cyclophosphamide (CPA). The present study examines the potential of combining TPZ with CPA in a cytochrome P450-based prodrug activation gene therapy strategy. Recombinant retroviruses were used to transduce 9L gliosarcoma cells with the genes encoding P450 2B6 and NADPH-P450 reductase. Intratumoral coexpression of P450 2B6 with P450 reductase sensitized 9L tumor cells to CPA equally well under normoxic (19.6% O_2) and hypoxic (1% O_2) conditions. The P450 2B6/P450 reductase combination also sensitized 9L tumor cells to TPZ under both culture conditions. Interestingly, bystander cytotoxic effects were observed for both CPA and TPZ under hypoxia. Furthermore, TPZ exerted a striking growth-inhibitory effect on CPA-treated 9L/2B6/P450 reductase cells under both normoxia and hypoxia, which suggests the utility of this drug combination for P450-based gene therapy. To evaluate this possibility, 9L tumor cells were transduced in culture with P450 2B6 and P450 reductase and grown as solid tumors in severe combined immune deficient mice *in vivo*. Although these tumors showed little response to TPZ treatment alone, tumor growth was significantly delayed, by up to approximately four doubling times, when TPZ was combined with CPA. Some toxicity from the drug combination was apparent, however, as indicated by body weight profiles. These findings suggest the potential benefit of incorporating TPZ, and perhaps other bioreductive drugs, into a P450/P450 reductase-based gene therapy strategy for cancer treatment.

INTRODUCTION

Solid tumors are characterized by poor vascularization associated with regions of hypoxia and severe hypoxia (1). Oxygen is required for the cytotoxic effects of radiation and many cancer chemotherapeutic drugs, and, consequently, tumor hypoxia is linked to both radiation resistance and chemoresistance. Accordingly, hypoxic tumor cells are among the most difficult to treat using conventional cancer chemotherapeutics. TPZ[3] (also known as SR4233 and WIN50975; Ref. 2) is the lead compound of a novel series of bioreductive drugs that exhibit a high specificity for hypoxic tumor cells (3, 4). TPZ can be activated by various cellular reductases, including the flavoenzyme NADPH P450 reductase (5–7), by a one-electron reduction that yields the TPZ nitroxide radical (8). This radical causes DNA single- and double-strand breaks and has been implicated in the cytotoxicity of TPZ under hypoxic conditions (9, 10). Consequently, cellular levels of P450 reductase may be an important determinant of the sensitivity of hypoxic tumor cells to TPZ (6). TPZ radical can be further converted to the inactive two-electron reduction product SR4317 either by radical disproportionation or by a second one-electron reduction (11). Under aerobic conditions, TPZ radical is rapidly reoxidized concomitant with the conversion of molecular oxygen to superoxide radical and other reactive reduced oxygen species, which mediate the cytotoxic effects of TPZ under aerobic conditions (12). Mouse liver microsomal P450 enzymes can metabolize TPZ to its inactive, two-electron reduction product (13, 14), which suggests that P450 enzymes serve to inactivate TPZ (11). The possibility that P450 metabolism of TPZ may contribute to drug activation via one-electron reduction, a reaction catalyzed by P450 enzymes with certain xenochemical substrates under anaerobic or hypoxic conditions (15, 16), has not been examined.

P450 enzyme metabolism modulates the activity of several cancer chemotherapeutic agents, including the alkylating agent prodrugs CPA and ifosfamide, which are converted to therapeutically active DNA-alkylating metabolites after hydroxylation by hepatic P450 enzymes (17). A striking increase in the antitumor activity of CPA can be achieved using a prodrug activation-based gene therapy strategy designed to augment intratumoral expression of hepatic P450 enzymes belonging to the CYP2B and CYP2C subfamilies (18–21), which have a high capacity for CPA activation (22, 23) and are typically present at low levels in tumor tissue (24–26). The efficacy of this P450 gene therapy strategy can be further increased by coexpression of P450 reductase (27, 28), which is rate-limiting for many P450-dependent metabolic reactions. Because oxygen is a P450 cosubstrate and is required for all P450-catalyzed monooxygenase reactions, it is important to determine whether the low O_2 concentrations associated with tumor hypoxia compromise the efficacy of intratumoral P450/P450 reductase-catalyzed activation of prodrugs such as CPA.

In the present study, the impact of P450 and P450 reductase gene transfer on the chemosensitivity of tumor cells to CPA was evaluated under both hypoxic (1% O_2) and normoxic conditions (19.6% O_2). In addition, the impact of P450/P450 reductase gene transfer on the cytotoxicity of TPZ to tumor cells was evaluated both alone and when combined with the P450-activated prodrug CPA. As described below, P450/P450 reductase gene transfer sensitizes tumor cells to CPA both under normoxia and hypoxia. Moreover, the combination of the P450-activated prodrug CPA with the P450 reductase-activated bioreductive prodrug TPZ was shown to lead to a significant increase in tumor cell cytotoxicity *in vitro* and antitumor activity *in vivo* compared with that obtained using either drug alone. These findings demonstrate that the efficacy of cancer gene therapy using the P450/P450 reductase prodrug activation system can be substantially increased by combining a P450-activated prodrug with a P450 reductase-activated bioreductive drug.

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[3] The abbreviations used are: TPZ, tirapazamine (or SR4233), 3-amino-1,2,4-benzotriazine-1,4-di-*N*-oxide); SR4317, 3-amino-1,2,4-benzotriazine-1-oxide; CPA, cyclophosphamide; P450 (or CYP) cytochrome P450; hRED, human P450 reductase; rRED, rat P450 reductase; 9L/2B6/hRED, rat 9L gliosarcoma cells transduced with retrovirus encoding P450 2B6 and with retrovirus encoding hRED; 9L/2B6, 9L cells transduced with retrovirus encoding P450 2B6; 9L/pBabe, 9L cells transduced with the control retroviral vector pBabe-puro; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; SGD, specific growth delay (corresponds to the number of doubling times by which tumor growth is delayed by drug treatment); scid, severe combined immune deficient/deficiency.

MATERIALS AND METHODS

Chemicals. CPA, TPZ, hygromycin, and X-gal were obtained from Sigma Chemical (St. Louis, MO). Blasticidin *S*-hydrochloride was from ICN Biomedicals (Aurora, OH).

Construction of Recombinant Retroviruses. cDNA encoding hRED cloned into the *Eco*RI site of pUV1 (29) was obtained from Dr. F. Gonzalez (National Cancer Institute, Bethesda, MD). This cDNA was subcloned into the *Eco*RI site of pWZL-Blast, or pBabe Hygro (obtained from Millenium Pharmaceuticals, Cambridge, MA). These two retroviral vectors are based on the pBabe series (30) and encode either a blasticidin resistance or a hygromycin resistance gene transcribed from the viral 3′-long terminal repeat. The presence of the correct ATG initiation codon in the cloned P450 reductase cDNA was verified by DNA sequencing. CYP2B6 cloned into the retroviral vector pBabe-puro and the preparation of 9L/2B6 cells by retroviral transduction using this plasmid were described previously (28).

Construction of 9L Gliosarcoma Cell Lines Expressing Human P450 Reductase cDNA by Retroviral Infection. Transfection of the ecotropic packaging cell line Bosc 23 (31) with human P450 reductase-encoding retroviral plasmid DNA, harvesting of the retroviral supernatant, and infection of 9L gliosarcoma cells (both 9L/pBabe control cells and 9L/2B6 cells) were carried out as described previously (28). Pools of blasticidin or hygromycin-resistant cells were selected using 3 μg/ml blasticidin *S* hydrochloride or 250 μg/ml hygromycin, respectively, for 2 and 3 days respectively. Drug-resistant pools of cells were propagated and then assayed for P450 reductase enzyme activity in isolated microsomes as described previously (28). A 4- to 5-fold increase in P450 reductase-catalyzed cytochrome C reduction ($\Delta A_{550\ nm}$ measured at 30°C) was obtained in both the 9L/hRED and the 9L/2B6/hRED pools of transfectants.

Cytotoxicity Assays. To evaluate the chemosensitivity of the P450- and P450/P450 reductase-expressing 9L tumor cells, cells were plated in triplicate at 4000 cells/well of a 48-well plate 18–24 h before drug treatment. Cells were then treated with drugs (0–1 mM CPA or 0–50 μM TPZ, as specified in each experiment) and incubated for 4 days unless otherwise indicated, in a tissue culture incubator maintained under hypoxic conditions (1% O_2, 5% CO_2, 94% N_2) or under normoxic conditions (19.6% O_2, 5% CO_2, 75.4% N_2). Cells remaining after this time were quantitated using a crystal violet/alcohol-extraction assay (28). Data are presented as cell number relative to drug-free controls, mean ± SD values for triplicate samples, unless indicated otherwise. Error bars not seen in the individual figures are too small to be visible.

Bystander Cytotoxicity Assay. 9L/lacZ cells (20) were plated in duplicate at 7×10^3 cells/well in a 12-well plate, and were mixed with an equal number of 9L/2B6/hRED cells or 9L/pBabe cells. Cells were treated with increasing doses of TPZ (0–2.5 μM) or CPA (0–1.5 mM) under hypoxia. 9L/lacZ cells were visualized by X-gal staining after 5 days of drug exposure, as follows. Cells were washed with PBS, then fixed for 5 min in 0.5 ml of PBS containing 2% formaldehyde and 0.05% glutaraldehyde. Cells were then washed in PBS and stained with 0.5 ml of staining solution containing 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM $MgCl_2$, and 1 mg/ml X-gal; dried; and then photographed. The β-galactosidase activity stain was then resuspended with 1 ml of DMSO and absorbance values were measured in a Lab Instruments SLT Spectra microtiter plate reader using a 650-nm filter.

Tumor Growth Delay Assay. 9L tumor cells to be used for tumor implantation (9L/pBabe or 9L/2B6/hRED) were grown in DMEM on 100-mm dishes until nearly confluent. The cells were trypsinized and resuspended in DMEM without fetal bovine serum to a concentration of 8×10^6 cells/ml and were kept on ice until injection. Four-week-old male ICR/Fox Chase/outbred immunodeficient scid mice (Ref. 32; Taconic Farms, Germantown, NY) weighing 23–26 g, were injected with 4×10^6 cells at each of two s.c. sites per animal. Cells were injected in a volume of 0.5 ml of serum-free DMEM using a 0.5-inch 29-gauge needle and a 1-ml insulin syringe. Tumor growth was monitored twice a week using Vernier calipers (Manostat Corp., Switzerland), and tumor surface areas were calculated. In one experiment, mice were given an initial i.p. injection of TPZ at 40 mg/kg of body weight 19 days after tumor implantation, at which time the tumor size was ~100 mm^2; a second injection of TPZ (40 mg/kg) was given about 4 weeks later (Experiment 1; Table 1, see below). In a separate experiment, CPA and TPZ were both administered 17 days after tumor implantation (tumor size ~100 mm^2): TPZ (40 mg/kg) was injected i.p. at $t = 0$ h, followed by CPA i.p. at $t = 2$ h and again at $t = 26$ h (150 mg CPA/kg body weight for each injection; Experiment 2; Table 1, see below). This schedule of CPA injection is the same one used previously with 9L/2B6 tumors (28). TPZ and CPA were solubilized in PBS at 2.4 mg/ml and 5 mg/ml, respectively. Both solutions were kept at 37°C until injection, to maintain drug solubility.

Tumor growth delay data were analyzed as described previously (33). Tumor doubling time was defined as the time in days required for the tumor to double in surface area. Tumor growth delay values were then calculated as the difference (in days) in tumor doubling time between the drug-treated and control groups. SGD values were calculated as $(T_2 - T_1) \div T_1$, where T_1 and T_2 are the times (in days) required for the control and the drug-treated tumors, respectively, to double in surface area. The SGD parameter provides an estimate of the number of doubling times by which tumor growth is delayed by drug treatment and facilitates comparisons of therapeutic responses between tumors that differ in their growth rates (33).

RESULTS

Transduction of Human P450 Reductase. 9L rat gliosarcoma cells transduced with the human P450 gene *CYP2B6* (9L/2B6 cells; Ref. 28) were infected with retrovirus particles engineered to express a full-length human P450 reductase cDNA. A pool of 9L cells transduced with human P450 reductase in the absence of P450 coexpression was obtained in a similar manner (9L/hRED cells). P450 reductase activity measured in isolated cell microsomes was increased ~4- to 5-fold by hRED transduction, to a level of ~100 nmol cytochrome C reduced/min/mg protein in both pools of transduced cells. Evaluation of the cytotoxicity of CPA toward 9L/2B6/hRED cells in comparison to 9L/2B6 cells under normoxic conditions (19.6% O_2) revealed a large increase in CPA cytotoxicity in response to retroviral transduction of human P450 reductase (Fig. 1*A*). No CPA cytotoxicity was observed in 9L/hRED cells, or in 9L/pBabe control cells (Fig. 1*A*). Thus, human P450 reductase gene transfer greatly increases the chemosensitivity of a tumor cell transduced with a cytochrome P450 gene, much in the same way as was shown previously for a rat P450 reductase gene (28). Rat and human P450 reductase exhibit 92% amino acid sequence identity (29). A similar degree of sequence conservation characterizes other mammalian P450 reductases, which are expected to behave similarly in this regard.

Impact of Hypoxia on P450/P450 Reductase-dependent CPA Cytotoxicity. To test whether the efficacy of P450/P450 reductase-based gene therapy is likely to be compromised by the hypoxic conditions found within solid tumors, the cytotoxicity of CPA toward 9L/2B6/hRED cells and 9L/2B6 cells was assayed under conditions of hypoxia (1% O_2). Fig. 1*B* shows that hypoxia does not significantly decrease the cytotoxic effect of CPA toward 9L/2B6/hRED cells or toward 9L/2B6 cells (compare Fig. 1*A*). Similar results were obtained in studies using 9L cells coexpressing rat P450 gene 2B1 and rRED (data not shown). Thus, P450-catalyzed prodrug activation is not impaired under hypoxic conditions.

Augmentation of Tumor Cell Cytotoxicity by Combination of a Bioreductive Drug with a P450-activated Prodrug. We next examined the cytotoxic effects of TPZ when treating tumor cells transduced with P450 in combination with P450 reductase. Fig. 2*A* shows that in cells cultured under normoxic conditions, transduction of P450 2B6 with P450 reductase substantially increased the cytotoxicity of TPZ toward 9L tumor cells. In cells grown in hypoxic conditions, in which TPZ is about 10-fold more active against 9L tumor cells, a significant chemosensitization was also obtained on transduction of P450/P450 reductase (Fig. 2*B*).

The P450-activated prodrug CPA and the bioreductive drug TPZ kill tumor cells by distinct mechanisms: DNA cross-linking, in the case of phosphoramide mustard derived from P450-activated CPA; and either DNA strand scission by TPZ radical (under hypoxic con-

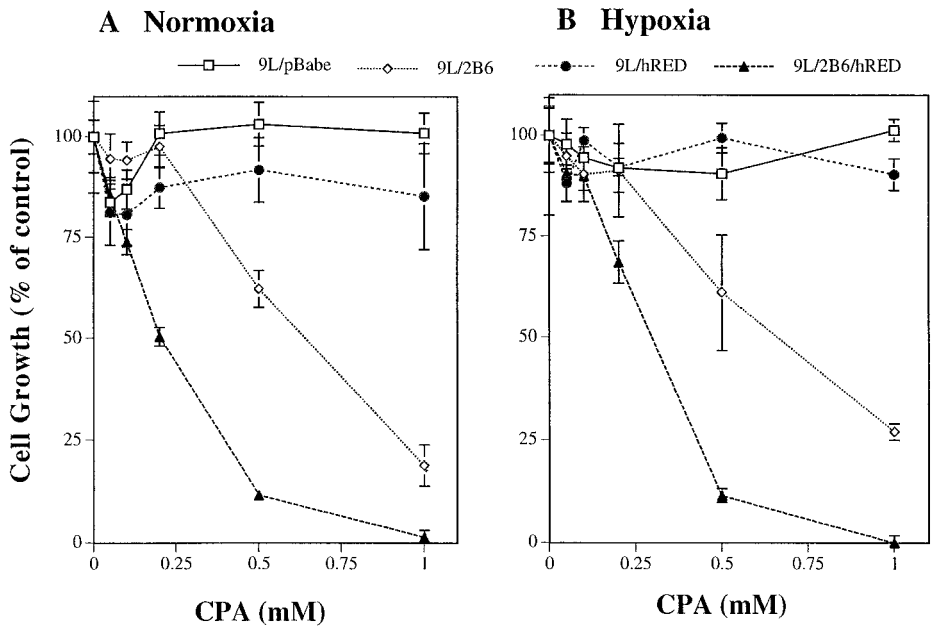

Fig. 1. Retroviral transduction of the *hRED* gene enhances the cytotoxicity of CPA to cultured 9L gliosarcoma cells transduced with the *CYP2B6* gene, both under normoxic culture conditions (*A*) and under hypoxic culture conditions (*B*). Cells were seeded at 4000 cells/well in 48-well plates and treated with increasing concentrations of CPA for 4 days. Cell growth in comparison with drug-free controls was determined by crystal violet staining and is presented as mean ± SD for $n = 3$ replicates.

ditions) or DNA damage via reactive reduced oxygen species formed during the reoxidation of TPZ radical (under aerobic conditions). Whereas an increase in cytotoxic activity might, therefore, result when these two drugs are used in combination for treatment of tumor cells transduced with P450 + P450 reductase, it is alternatively possible that competition between CPA and TPZ for metabolism by the same P450/P450 reductase enzyme couple could result in no increase, or perhaps even an overall decrease, in cytotoxicity. A decrease in cytotoxicity would also be expected if P450 metabolizes TPZ to the inactive two-electron reduced metabolite SR4317 (13) without the intermediacy of the one-electron reduced, cytotoxic TPZ radical.

To distinguish between these possibilities, we examined whether TPZ could be used to augment the sensitivity of the P450/P450 reductase-expressing tumor cells to CPA. The concentration of TPZ used in this study, 5 μM for experiments carried out under normoxia, was chosen to give little or no cytotoxicity on its own toward 9L or 9L/2B6/hRED cells. Fig. 3*A* shows that the combination of 5 μM TPZ with CPA at concentrations ranging from 0.05–0.5 mM led to an increase in antitumor activity compared with CPA alone in the case of 9L/2B6/hRED cells. At 10 μM TPZ, which exhibited significant cytotoxicity in the absence of CPA, an additive increase in cytotoxicity was obtained. In control experiments, TPZ had no enhancing effect on CPA cytotoxicity in cells that did not express P450 2B6 (*i.e.*, 9L/hRED and 9L/pBabe cells; Fig. 3, *B* and *C*). Experiments carried out under hypoxic conditions revealed that TPZ could be used at much lower concentrations (0.5 and 1.5 μM) to enhance cytotoxicity in the case of 9L/2B6/hRED cells treated with CPA (Fig. 4*A*). Under these conditions, TPZ had little or no cytotoxic effect toward 9L/hRED and 9L/pBabe controls (Fig. 4, *B* and *C*). Thus, substantial increases in antitumor effect can be achieved under both hypoxic and normoxic conditions by treating tumor cells with a P450-activated prodrug in combination with a bioreductive drug in the context of P450/P450-reductase-based cancer gene therapy.

Bystander Killing Effect. Radical metabolites, such as those derived from TPZ, are generally short-lived species that induce cell damage in the local vicinity of their formation. Because the present experiments were carried out under hypoxia rather than under conditions of strict anoxia, some oxygen radical-dependent bystander cell damage could occur on activation of TPZ, particularly in cells that are in close contact with 9L/2B6/hRED cells. To evaluate whether activated TPZ exerts a bystander cytotoxic effect, 9L/2B6/hRED cells

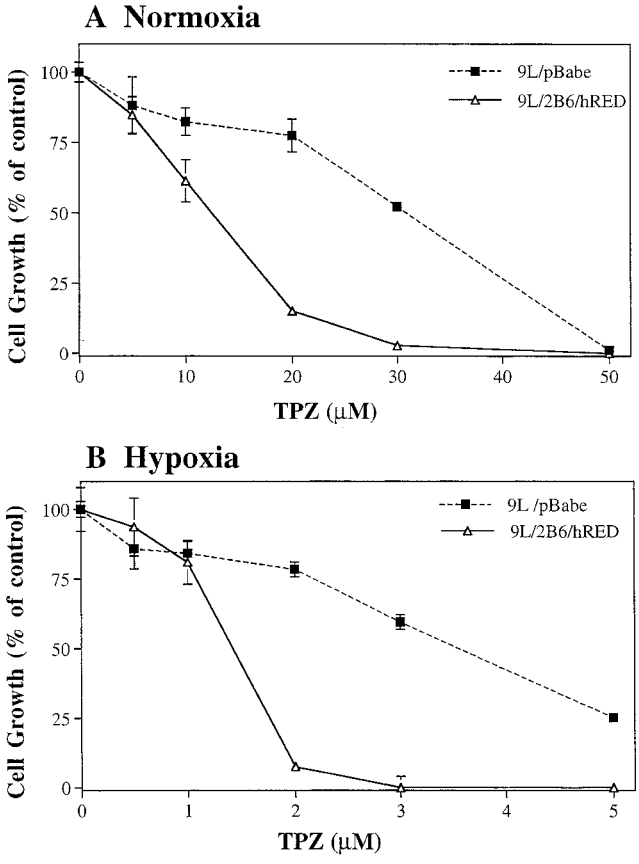

Fig. 2. Growth inhibition assay to assess chemosensitivity of 9L/pBabe and 9L/2B6/hRED cells to TPZ under normoxic (*A*) and hypoxic culture conditions (*B*). Cells were seeded at 4000 cells/well in 48-well plates and treated with the indicated concentrations of TPZ for 4 days. Cell growth in comparison with drug-free controls was determined by crystal violet staining and is presented as mean ± SD for $n = 3$ replicates. Note the 10-fold difference in *x-axis* scale between *A* and *B*.

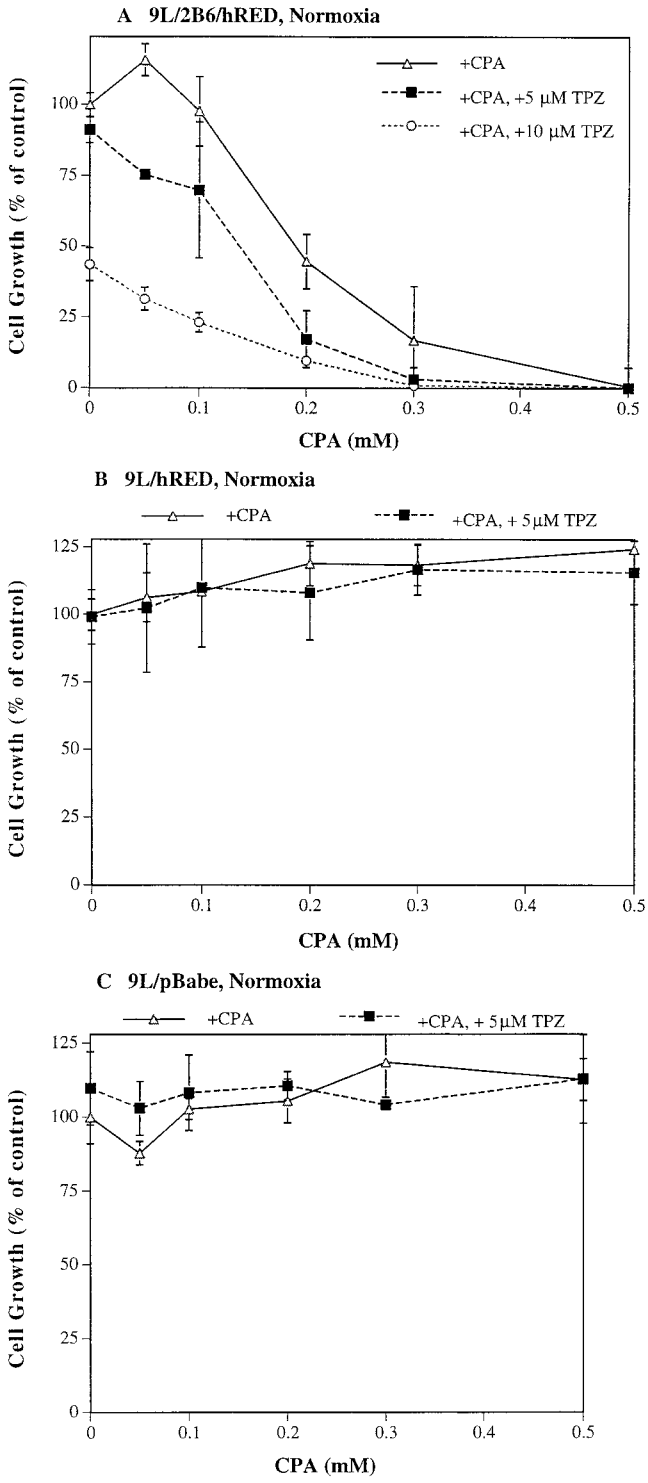

Fig. 3. Chemosensitivity of 9L/2B6/hRED cells to CPA in combination with TPZ under normoxic culture conditions. Cells were seeded at 4000 cells/well in 48-well plates and treated with increasing concentrations of CPA at a fixed concentration of TPZ (5 or 10 μM, as indicated) for 4 days as described in "Materials and Methods." Cell growth in comparison to drug-free controls was determined by crystal violet staining and is presented as mean ± SD for $n = 3$ replicates. *B* and *C*, parallel studies carried out with 9L/hRED and 9L/pBabe cells.

(prodrug-activating cells) or 9L/pBabe control cells were cocultured with 9L cells marked with the *lacZ* gene (bystander target cells) and then were treated for 5 days with various concentrations of TPZ under hypoxic conditions. Results obtained with TPZ were compared with parallel studies of cells treated with CPA, which is activated to a 4-hydroxy metabolite that readily diffuses through the culture media and exerts bystander cytotoxicity (18). Increasing concentrations of CPA effected a strong cytotoxic effect on the bystander 9L/lacZ cells, which were stained blue using the β-galactosidase activity substrate X-gal (Fig. 5*B*). In the case of TPZ, bystander cytotoxicity was also

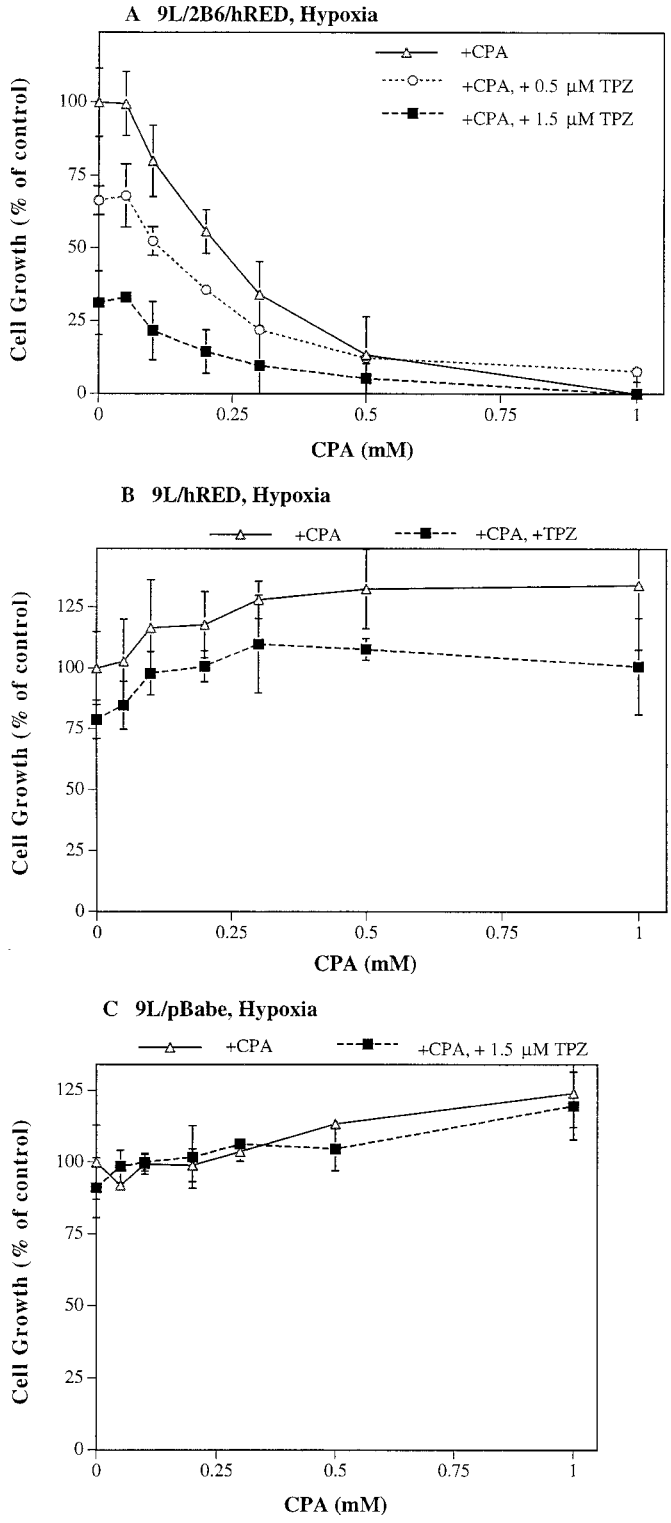

Fig. 4. TPZ enhances chemosensitivity of 9L/2B6/hRED cells to CPA under hypoxia. Experimental design was the same as that described in Fig. 3, except that the experiment used lower concentrations of TPZ (0.5 or 1.5 μM, as indicated), and the cells were cultured under hypoxic conditions.

apparent, as judged by the drug-dependent reduction in the number of 9L/lacZ cells (*blue staining*), in addition to the more striking loss of the 9L/2B6/hRED cells (*unstained cells*; Fig. 5*A*). This bystander cytotoxic response may be associated with cell-to-cell diffusion of reactive oxygen radicals, or perhaps with TPZ radicals, formed by the 9L/2B6/hRED cells. As expected (compare Fig. 2*B*), TPZ at these concentrations showed only moderate toxicity toward the mixed population containing 9L/pBabe and 9L/lacZ cells (Fig. 5*A*, *right*).

Evaluation of Human P450-based Gene Therapy in a scid Mouse Model. The impact of TPZ treatment alone, or the combination of TPZ and CPA, on the chemosensitivity of P450/P450 reductase-transduced 9L gliosarcoma cells was evaluated *in vivo* in an immunodeficient scid mouse solid tumor model. The scid mouse model (32) is free of the immunological contributions that can confer an apparent increase in cytotoxicity (34). This mouse model is also devoid of the immunogenic responses that can result in inefficient tumor implantation, as is seen when 9L tumors expressing human P450 genes are grown in Fischer 344 rats (28). scid mice were implanted with either 9L/pBabe or 9L/2B6/hRED tumors (two s.c. tumors per mouse). In the absence of drug treatment, 9L/pBabe and 9L/2B6/hRED tumors exhibited similar growth rates, as indicated by the slopes of the tumor growth curves (Fig. 6 and Fig. 7*A*, *open symbols*) and their similar tumor surface area doubling times (Table 1). In one experiment, mice were treated with TPZ at 40 mg/kg body weight, i.p., 19 days after tumor implantation, at which time the tumor size was ~100 mm^2. Little or no therapeutic effect was observed after this first round of treatment, or even after a second TPZ treatment given 4 weeks later (Fig. 6, *arrows*, *filled symbols*; Table 1, Experiment 1).

In a separate experiment, tumor-bearing mice were treated with TPZ (40 mg/kg) and CPA (150 mg/kg × 2 injections) in combination. The schedule used, TPZ at $t = 0$ h followed by CPA at $t = 2$ and 26 h (see "Materials and Methods") is based on the report that TPZ + CPA combinations are most effective when TPZ is given either 1–3 h before CPA or 24 h before CPA (35). This drug combination resulted in a detectable tumor delay in 9L/pBabe tumors (Fig. 7*A*), an effect that was associated with some host toxicity, as evidenced by body weight loss during a 7-day period after drug administration (Fig. 7*B*). The rapid weight gain seen in untreated mice bearing the 9L/pBabe and 9L/2B6/hRED tumors after day ~35 largely reflects the rapid growth in tumor size during this period. In contrast to the modest tumor growth delay (~4 days) seen in response to TPZ + CPA treatment of 9L/pBabe tumors, the combination of TPZ + CPA conferred a growth delay of 35 days for the 9L/2B6/hRED tumors. Treatment of these tumors with CPA alone was associated with a 23-day growth delay. These antitumor activities were associated with a SGD of 3.9 for TPZ + CPA, in the case of 9L/2B6/hRED tumors, compared with an SGD of only 0.48 with 9L/pBabe tumors (Table 1). The more pronounced decrease in body weight seen in this combination drug treatment group (Fig. 7*B*) is indicative of toxicity associated with the drug doses used, which is greater than that observed after treatment with CPA alone.

DISCUSSION

The antitumor activity of CPA, an alkylating agent prodrug that is activated by hepatic P450 metabolism, can be significantly increased

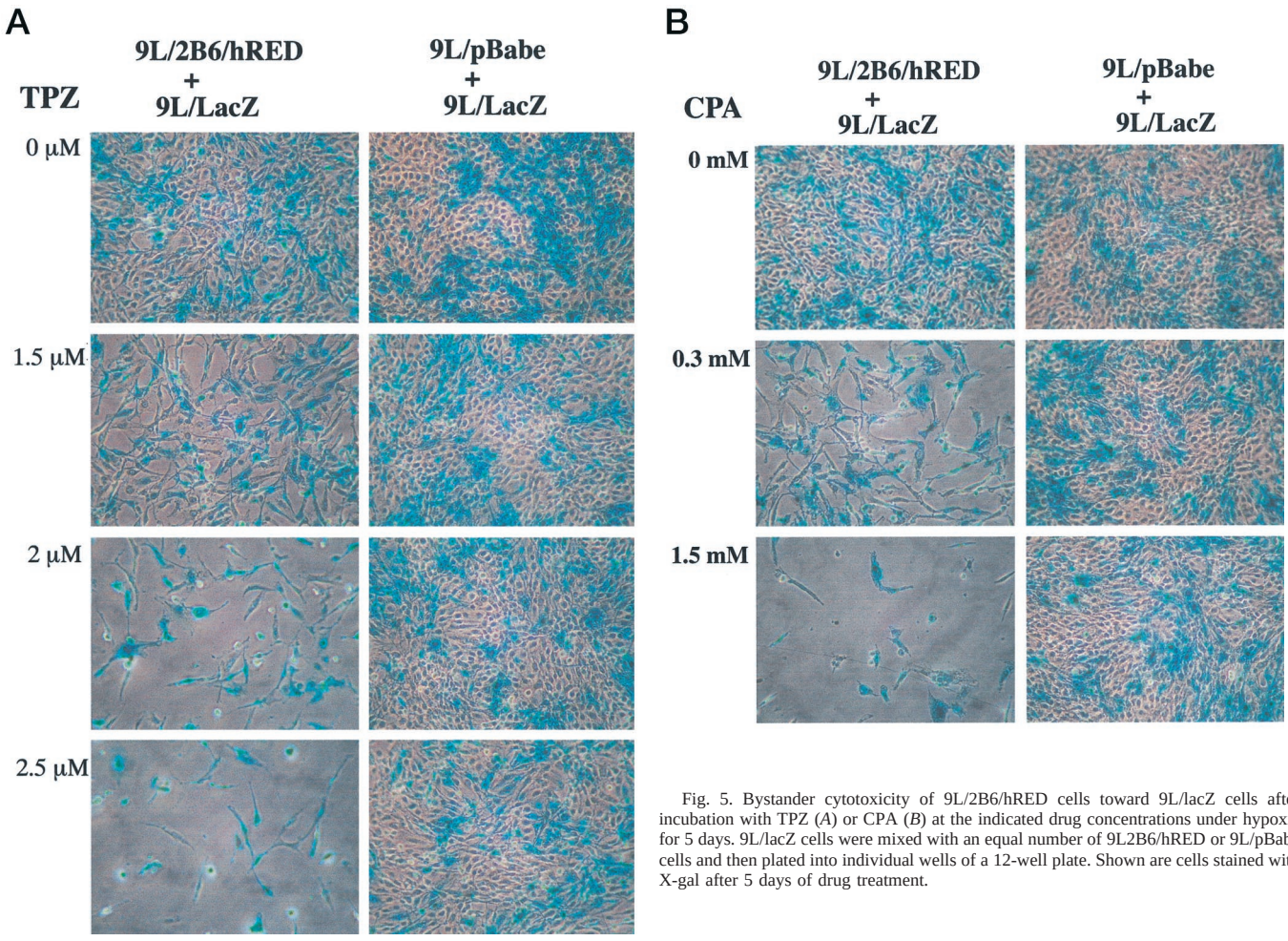

Fig. 5. Bystander cytotoxicity of 9L/2B6/hRED cells toward 9L/lacZ cells after incubation with TPZ (*A*) or CPA (*B*) at the indicated drug concentrations under hypoxia for 5 days. 9L/lacZ cells were mixed with an equal number of 9L2B6/hRED or 9L/pBabe cells and then plated into individual wells of a 12-well plate. Shown are cells stained with X-gal after 5 days of drug treatment.

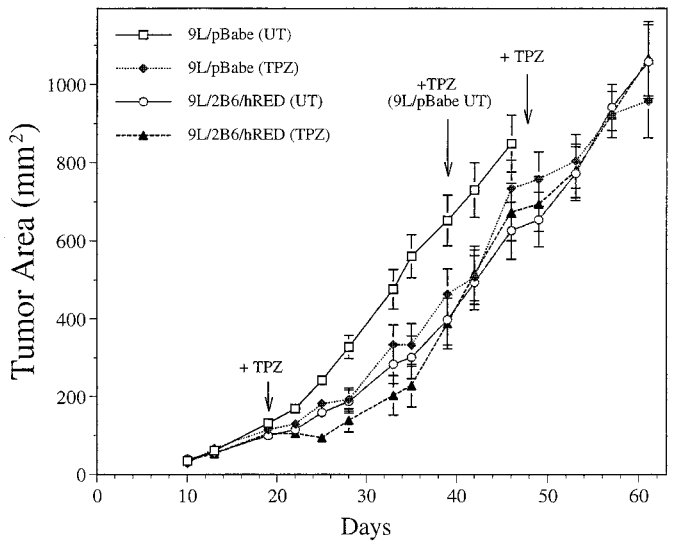

Fig. 6. Effect of TPZ on 9L/pBabe and 9L/2B6/hRED solid tumors grown in scid mice. Shown are the effects of TPZ treatment on the growth of 9L/pBabe and 9L/2B6/hRED tumors implanted in scid mice. Tumor areas were measured twice a week with Vernier calipers. *Arrows* (*X* axis), the days on which TPZ was given by i.p. injection at 40 mg/kg, as described under "Materials and Methods." Days shown on the *X* axis correspond to days after tumor implantation. *Data points*, mean tumor areas (mm^2; $n = 6$ tumors per treatment group); *bars*, SE. A second injection of TPZ (40 mg/kg) was given to all of the groups except the untreated 9L/pBabe controls on day 47. Data shown were analyzed as presented in Table 1, Experiment 1.

by intratumoral expression of cytochrome P450 in combination with P450 reductase, which provides for localized activation of CPA at its site of action (18). The present study was designed to evaluate (*a*) whether this P450/P450 reductase-based gene therapy strategy for cancer treatment is applicable to hypoxic tumor cells; and (*b*) whether antitumor activity can be enhanced by incorporation of a bioreductive drug that is activated by P450 reductase. The bioreductive drug examined, TPZ, is a lead compound of a series of second generation bioreductive agents with enhanced specificity for hypoxic tumor cells (3, 4), which are often resistant to conventional chemotherapy and radiation treatment (1). Our findings demonstrate that P450/P450 reductase-based cancer gene therapy is effective under both hypoxic and normoxic conditions, and that an increase in antitumor activity can be achieved by combining the P450-activated prodrug CPA with the bioreductive drug TPZ.

Transduction of the rat gliosarcoma cell line 9L with replication-defective retrovirus encoding any one of several human P450s from gene subfamilies CYP2B, CYP2C, or CYP3A chemosensitizes the tumor cells to the cytotoxic effects of CPA and its isomer ifosfamide (28). In the case of CPA, human P450 form 2B6 provides the greatest chemosensitization, and this effect is significantly increased by co-transduction of the P450 reductase gene. In the present study, we sought to further enhance this P450/P450 reductase-dependent gene therapy strategy by combining CPA and TPZ, chemotherapeutic prodrugs with distinct mechanisms of action. To evaluate this possibility, we first investigated whether P450-dependent prodrug activation and cytotoxicity are manifest in a hypoxic environment in which bioreductive drugs such as TPZ have enhanced activity. Comparisons of the cytotoxicity of CPA toward 9L/2B6/hRED tumor cells grown under hypoxic *versus* normoxic conditions demonstrated good antitumor activity under both of the culture conditions. Moreover, the bystander cytotoxic potential of P450-activated CPA (18) is retained under condition of hypoxia (Fig. 5*B*). This finding indicates that the intracellular O_2 concentrations in hypoxic tumor cells are apparently sufficiently high in relation to the concentration of P450 2B6 and its affinity for O_2 to support the modest P450 2B6 turnover, ~20 nmol/min/nmol P450 with CPA as substrate (22). Furthermore, other chemotherapeutic prodrugs subject to P450 activation (36, 37), which are typically metabolized at rates similar to CPA, are also likely to be activated within P450-expressing hypoxic tumor cells, thus broadening the range of chemotherapeutic regimens that may be used with P450 gene therapy.

P450 reductase activates TPZ by a one-electron reduction reaction

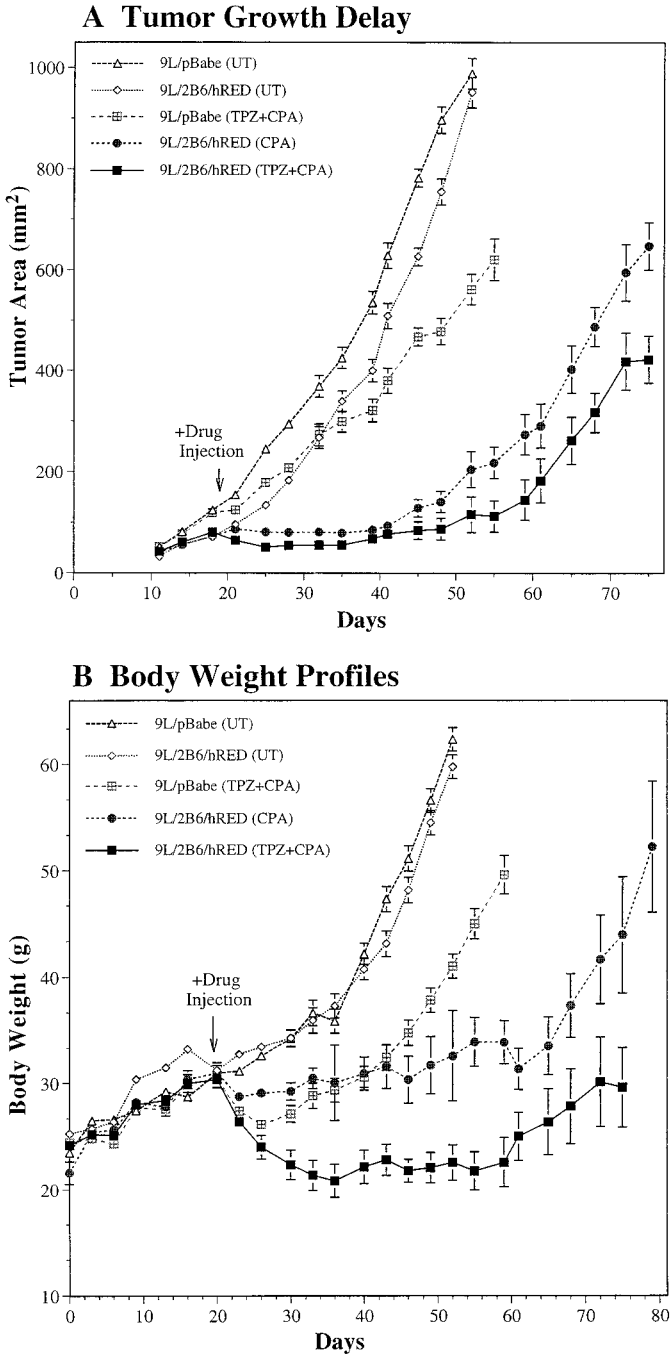

Fig. 7. Antitumor effect of TPZ in combination with CPA evaluated in scid mice. The experiment was carried out as described in Fig. 6, except that the mice were treated with TPZ (40 mg/kg) in combination with CPA (150 mg/kg × 2 injections) using the schedule described under "Materials and Methods." A group consisting of CPA treatment alone for 9L/2B6/hRED tumors was included for comparison. *Data*, mean ± SE values for $n = 8$ to 10 tumors per group. Data analysis is presented in Table 1, Experiment 2. *B*, body weight profiles for the animals in *A*. The weight of the mice at the time of initial drug treatment, 30 ± 2 g, corresponds to the normal adult weight of this mouse strain.

Table 1 *Impact of CPA in combination with TPZ on tumor growth delay*

Data shown are based on Fig. 7. Tumor doubling time, tumor growth delay, and SGD were calculated as described in "Materials and Methods." The number of tumors included in each group is shown in parenthesis.

Tumor	Treatment	Tumor doubling time (days)	Growth delay (days)	SGD
Experiment 1				
9L/pBabe	Control (6)	7.4 ± 1.8		
	TPZ (6)	10.6 ± 1.5	3.2	0.43
9L/2B6/RED	Control (6)	10.5 ± 2.1		
	TPZ (6)	15.3 ± 4.8	4.8	0.46
Experiment 2				
9L/pBabe	Control (8)	8.3 ± 2.0		
	TPZ + CPA (10)	12.3 ± 1.8	4.0	0.48
9L/2B6/RED	Control (8)	9 ± 2		
	CPA (10)	31.6 ± 6.3	22.6	2.5
	TPZ + CPA (10)	44.1 ± 4.3	35.1	3.9

that yields the TPZ nitroxide radical (6, 7), which causes DNA single and double-strand breaks and has been implicated in the cytotoxicity of TPZ under hypoxic conditions (9, 10). Under aerobic conditions, TPZ radical is rapidly reoxidized concomitant with the conversion of molecular oxygen to superoxide radical and other reactive reduced oxygen species that mediate the cytotoxic effects of TPZ in normoxia (12). The potential role of P450 enzymes in TPZ activation has not been directly examined; however, earlier studies did demonstrate that P450 enzymes can contribute to the two-electron metabolism of TPZ to its noncytotoxic mono-*N*-oxide SR 4317 (13, 14), albeit not in all systems (8, 38). These findings raised the possibility that intratumoral P450 expression, in the context of P450 prodrug activation-based cancer therapy (18–20), might lead to a decrease in the antitumor effect of TPZ. Moreover, the potential for TPZ to compete with CPA for P450/P450 reductase-catalyzed prodrug activation could perhaps also lead to a decrease in the antitumor effect of CPA when the two drugs are combined. Nevertheless, significant increases in tumor cell toxicity were achieved in the present study when the P450-activated prodrug CPA was combined with the bioreductive drug TPZ under hypoxic conditions and in the context of P450/P450 reductase gene transfer. This finding may reflect the fact that CPA and TPZ kill tumor cells by distinct mechanisms: DNA cross-linking, in the case of phosphoramide mustard derived from CPA; and DNA strand scission caused by TPZ radical under hypoxic conditions. The enhanced cytotoxic response to TPZ + CPA in tumor cells transduced with both P450 and P450 reductase indicates that competition between TPZ and CPA for metabolism by the same P450/P450 reductase enzyme couple either does not occur or, if it does occur, is outweighed by the intrinsic enhanced activity of this drug combination.

P450 enzymes can catalyze both one-electron and two-electron reductions with xenobiotic substrates under hypoxic conditions (15). One-electron reductions yielding semiquinone radicals have been reported for the rat enzyme P450 2B1 with the substituted *p*-benzoquinone anticancer drugs Adriamycin and mitomycin C (39, 40). Further investigation will be required to ascertain whether the net two-electron reduction of TPZ, previously described for liver P450 enzymes (13, 14), is the result of a direct two-electron reduction, or alternatively, whether the two-electron reduced metabolite is formed by disproportionation of a one-electron reduced TPZ radical formed via a P450 catalyzed reaction. This latter possibility would suggest a direct role for P450 in TPZ bioactivation under hypoxic conditions.

A bystander cytotoxic response is an essential component of any prodrug activation-based cancer gene therapy strategy that, in part, helps compensate for the relative inefficiency of gene transfer to the tumor cell target (41). P450 activation of CPA is associated with bystander cytotoxicity, which is manifest both in conventional chemotherapy, in which the prodrug is primarily activated in the liver, and in the context of intratumoral drug activation associated with P450 gene therapy (Fig. 5*B*; 18). Interestingly, TPZ, when activated by 9L/2B6/hRED cells, also exerts bystander cytotoxicity, as demonstrated by the killing of 9L/lacZ cells in a coculture study (Fig. 5*A*). The primary activated species, TPZ radical, is short-lived and highly reactive, which suggests that it may not be the direct mediator of this bystander effect. Reactive oxygen species formed via redox recycling of TPZ are not formed under anoxic conditions but are formed under the low oxygen concentrations (1%) of these experiments and could serve as mediators of the observed bystander response. Alternatively, apoptotic factors transferred from dying 9L/2B6/hRED cells may be involved. Independent of the precise mechanism, the occurrence of a TPZ bystander cytotoxic effect under conditions of hypoxia that are likely to be relevant for tumors *in vivo* (1) is an important finding and strengthens the proposed incorporation of TPZ into P450/P450 reductase-based gene therapy regimens.

Tumor growth delay experiments were carried out to determine whether the substantial activity of TPZ observed *in vitro* translates into a corresponding therapeutic effect *in vivo*. Chemotherapeutic responses to several anticancer drugs in mouse and human tumor xenographs models can be enhanced by coadministration of TPZ under conditions in which TPZ on its own exhibits little or no antitumor activity (33, 35, 42, 43). Administration of TPZ to mice bearing 9L/2B6/hRED tumors resulted in little or no significant antitumor effect, a finding that is consistent with the low activity of TPZ seen in several other preclinical tumor models. By contrast, TPZ treatment increased the growth delay of 9L/2B6/hRED tumors substantially, from ~23 days in mice treated with CPA alone to ~35 days in mice administered the two-drug combination. This improved antitumor effect was associated with a SGD of ~4 tumor doubling times (Table 1, Experiment 2). Some increase in host toxicity was observed, as seen previously for TPZ in combination with other chemotherapeutic agents (33, 35). The basis for this enhanced toxicity, evident from an increase in body weight loss, is not established but could in part reflect decreases in feeding and water consumption that we observed after administration of the TPZ + CPA drug combination. As we did not use any antiemetics in these studies, the enhanced weight loss could in part result from drug-induced nausea. Further study is required to clarify this point.

The failure of TPZ alone to exert significant antitumor activity *in vivo* is in striking contrast to the potent cytotoxic effects of TPZ, both in our studies (Figs. 2 and 5) and in several earlier reports in cultured tumor cells (33, 35, 42, 43). TPZ treatment *in vivo* is associated with transient decreases in tumor blood flow (44), which may decrease the access of the tumor to the drug. Extensive metabolism of TPZ *in vivo* to the inactive, two-electron reduced metabolite (45), and the likelihood that the hypoxic tumor fraction is low in the small s.c. tumor xenograft models used in this and other studies could also contribute to the relative inactivity of TPZ *in vivo*. The local availability of prodrug within the heterogeneous tumor microenvironment may also be an important factor in determining the efficiency with which TPZ, and CPA, are activated within P450/P450 reductase-expressing tumor cells *in vivo*. It is unclear, however, how TPZ would be able to give rise to the observed potentiation of CPA antitumor activity if it is indeed present at such low concentrations *in vivo*. Large discrepancies between *in vitro* and *in vivo* drug toxicities have been observed with several other anticancer drugs and, in some cases, have been ascribed to poor drug penetration from the vascular compartment through the multiple layers of cells required to reach distant tumor cells (46–48). Although bioreductive drugs such as TPZ kill tumor cells under hypoxic conditions *in vitro* with up to a 200-fold selectivity compared with normoxia (2, 11), these drugs exhibit a significantly lower degree of selectivity *in vivo* (44). The importance of scheduling of drug

administration when TPZ is combined with CPA or other anticancer drugs (35, 43) may also be an important determinant of the physiological changes that occur after TPZ administration (44). Further investigation should help establish the optimal doses and scheduling for TPZ and CPA to maximize the antitumor effect while minimizing host toxicity.

Given the clear advantages of chemosensitizing tumor cells by transduction of P450 reductase in combination with cytochrome P450 (18), bioreductive prodrugs that are activated by cytochrome P450 and/or by P450 reductase under hypoxic conditions may be added to a combination chemotherapy/gene therapy regimen that includes a P450-activated prodrug, such as CPA. Cancer chemotherapeutic drugs known to be bioactivated through reductive metabolism carried out by cytochrome P450 enzymes include quinone-containing molecules, such as Adriamycin, mitomycin C, tetramethylbenzoquinone, and the anthroquinone di-*N*-oxide prodrug AQ4N (15, 39, 49). Because these same drugs can also be bioactivated through reduction reactions catalyzed by P450 reductase (50–52), an enhanced cytotoxic response can be expected from the combination of P450 with P450 reductase gene transfer for these and other bioreductive prodrugs, including various quinones, nitroimidazoles, heterocyclic *N*-oxides and bioreducible DNA alkylators (53). The full expression of P450-dependent prodrug activity and the retention of bystander cytotoxicity in P450-transduced tumor cells under hypoxic conditions, discussed above, is strongly supportive of the proposed use of bioreductive drugs in the context of P450/P450 reductase-based cancer gene therapy. Tumor-specific expression of the prodrug-activating genes may be facilitated by a number of approaches, including the use of hypoxia response elements (54, 55) for transcriptional targeting of P450 and P450 reductase to the localized hypoxic environment that is characteristic of solid tumors (56, 57).

Bioreductive drugs constitute an important class of cancer chemotherapeutic agents with particularly strong activity against hypoxic tumor cells, which are often resistant to traditional radiation and chemotherapy treatments. The present demonstration that an enhanced antitumor effect is achieved by combining the P450-activated prodrug CPA with the bioreductive drug TPZ further extends the potential of P450-based gene therapy to include combinations of these important two classes of agents. Combinations of cancer chemotherapeutic drugs are invariably required for effective and durable clinical responses in the cancer patient. TPZ has previously been shown to enhance the activity of CPA and other cancer chemotherapeutic in both rodent and human tumor xenograph models. The present demonstration that such a combination is effective in the context of P450/P450 reductase-based gene transfer provides the opportunity to better exploit the intrinsic benefits of this combination through localized prodrug activation while potentially minimizing host toxic responses.

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