

Frequent, Moderate-Dose Cyclophosphamide Administration Improves the Efficacy of Cytochrome P-450/Cytochrome P-450 Reductase-based Cancer Gene Therapy¹

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ABSTRACT

Transduction of tumor cells with a cyclophosphamide (CPA)-activating cytochrome P-450 (P450) gene provides the capacity for localized prodrug activation and greatly sensitizes solid tumors to CPA treatment *in vivo*. The therapeutic impact of this P450-based cancer gene therapy strategy can be substantially enhanced by cotransduction of P450 reductase, a rate-limiting component of P450-dependent intratumoral CPA activation. The present study examined the possibility of further improving P450/P450 reductase-based gene therapy by using a novel schedule of CPA administration, involving repeated CPA injection every 6 days and previously shown to have an antiangiogenic component. 9L gliosarcoma cells transduced with the CPA-activating enzyme couple P450 2B6/P450 reductase and grown s.c. in immunodeficient severe combined immunodeficient (scid) mice were repeatedly challenged with 140 mg/kg CPA every 6 days. Full tumor regression leading to eradication of six of eight tumors was observed when the tumor size at the time of initial drug treatment was $\sim 400 \text{ mm}^3$ ($\sim 1.5\%$ of body weight). Little or no overt toxicity of the repeated CPA treatment regimen was observed. The same CPA schedule was much less effective in inducing regression of 9L tumors that were not transduced with P450/P450 reductase. Repeated CPA treatment of mice bearing large, late-stage P450/P450 reductase-transduced tumors ($\sim 9\text{--}16\%$ of body weight) resulted in major ($\geq 95\%$) regression in 15 of 16 tumors, with tumor eradication observed in 2 cases. Although CPA resistance was found to emerge in the population of P450/P450 reductase-transduced tumors, this resistance primarily involved a loss of expression of the transduced P450 and/or P450 reductase gene, rather than development of intrinsic cellular resistance to the activated form of CPA. These findings demonstrate that repeated CPA treatment on a 6 day schedule can be highly effective when combined with P450/P450 reductase gene therapy and suggest that repeated transduction of tumors with prodrug-activation genes may be necessary to achieve tumor eradication and a sustained therapeutic response.

INTRODUCTION

Several gene therapy-based strategies for cancer treatment have been introduced over the past several years, including prodrug activation strategies designed to generate active drug metabolites directly within the tumor cell target. One such strategy uses CYP³ enzymes in conjunction with NADPH-P450 reductase to activate anticancer prodrugs, such as CPA and its structural isomer, ifosfamide (1, 2). Activation of the alkylating agent prodrug CPA is catalyzed by specific hepatic P450 enzymes (3) and yields 4-OH-CPA, which decomposes to generate phosphoramidate mustard as the proximal cytotoxic metabolite (4). Transduction of tumor cells with the fla-

voenzyme P450 reductase in combination with a CPA-activating P450 gene, such as rat *CYP2B1* or human *CYP2B6*, further enhances the cytotoxicity of CPA both *in vitro* and *in vivo* (5, 6). The efficacy of this chemotherapeutic strategy can be enhanced in several ways, including combination of CPA with the P450/P450 reductase-activated bio-reductive drug tirapazamine (7), combination with antithyroid drugs, which selectively inhibit P450/P450 reductase-catalyzed prodrug activation in the liver (8), or combination with other prodrug activation gene therapies (9). The implementation P450-based cancer gene therapy *in vivo* can be facilitated using various strategies for tumor-selective gene delivery, including the use of tumor-selective oncolytic herpes virus (10), human macrophages transduced with a hypoxia-regulated adenoviral vector (11), and intratumoral injection of P450-expressing cells encapsulated in cellulose sulfate (12).

Another approach to improving the therapeutic efficiency of P450-based cancer gene therapy, investigated in the present study, involves the introduction of novel drug scheduling regimens to enhance the antitumor effect in an *in vivo* setting. The potential importance of drug scheduling for CPA-based cancer gene therapy is suggested by the short plasma elimination half-life values for CPA seen in some cancer patients (*e.g.*, $t_{1/2}$ of ~ 1.5 h in children and young adults; Ref. 4), which may limit the availability of CPA for intratumoral drug activation. The bioavailability of CPA and the isomeric ifosfamide may also be influenced by the ability of these drugs to induce their own metabolism in cancer patients (*e.g.*, Refs. 13, 14) by induction of liver P450s that contribute to prodrug activation (15). Recent preclinical investigations of CPA scheduling reported by Folkman and colleagues demonstrated that the administration of moderate doses of CPA on a schedule of repeated drug treatment every 6 days without interruption is much more effective than conventional high-dose drug schedules, both in the case of drug-sensitive tumors and drug-resistant tumors (16). The improved efficacy of this schedule is a result of the sustained activity of CPA against endothelial cells lining the blood vessels feeding the tumor cells. This sustained antiangiogenic effect is not obtained using classic regimens (16), which use maximally tolerated doses of CPA, necessitating a relatively long drug-free period for recovery of bone marrow and other sensitive host tissues (17).

The present study investigates whether the effectiveness of CPA-based P450 gene therapy can be improved by implementing the 6 day repeated cycle of CPA administration introduced by Browder *et al.* (16). Our findings reveal that this drug scheduling approach leads to long-term suppression of tumor growth, resulting in the eradication of midstage and a portion of late-stage tumors transduced with P450 and P450 reductase. Substantial regression of tumors that are very large at the beginning of drug treatment (up to 16% of the animal's body weight) could also be achieved with remarkably low host toxicity. During the course of these studies, we observed the emergence of a population of P450/P450 reductase-transduced tumor cells that was resistant to CPA but not to activated CPA. We identified, as the mechanism of this resistance, the loss of the transduced P450 or P450 reductase gene, indicating a need for repeated transduction of tumors with P450/P450 reductase for effective gene therapy. Overall, our findings support the conclusion that the every-6 day schedule of CPA treatment (16) can be superior to classical CPA treatment regimens and demonstrate that this therapeutic regimen can be particularly

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³ The abbreviations used are: P450 or CYP, cytochrome P-450; CPA, cyclophosphamide; 4-OH-CPA, 4-hydroxy-CPA; 4OOH-CPA, 4-hydroperoxy-CPA; CPA-6 day, CPA administered repeatedly every 6 days; hRed, human P450 reductase; 9L-P450/Reductase or 9L/2B6/hRed, rat 9L gliosarcoma cells transduced with retrovirus encoding P450 2B6 and with retrovirus encoding hRed; 9L control, 9L cells transduced with the control retroviral vector pBabe-puro; BW, body weight; scid, severe combined immunodeficient.

effective when given in the context of P450/P450 reductase-based gene therapy.

MATERIALS AND METHODS

Chemicals. CPA, Polybrene, chloroquine, collagenase type IV and puromycin hydrochloride were purchased from Sigma Chemical Co. (St. Louis, MO). Blasticidin *S*-hydrochloride was obtained from ICN (Aurora, OH). The chemically activated derivative of CPA, 4OOH-CPA, was obtained from Dr. Ulf Niemeyer, Asta Medica AG, Frankfurt, Germany.

Retroviral Transduction of 9L Gliosarcoma Cells with Human P450 and P450 Reductase cDNAs. Cloning of CYP2B6 cDNA (18) into the retroviral vector pBabe-puro and preparation of P450 2B6-expressing 9L gliosarcoma cells (9L/2B6 cells) by retroviral transduction were described previously (5). cDNA encoding hRed cloned into the *EcoRI* site of pUV1 (19) was obtained from Dr. F. Gonzalez, National Cancer Institute. This cDNA was subcloned into the *EcoRI* site of the retroviral vector pWZL-Blast (obtained from Dr. D. White, Millenium Pharmaceuticals, Cambridge, MA). pWZL-Blast is based on the pBabe plasmid (20) and encodes a blasticidin-resistance gene transcribed from the retroviral 3'-long terminal repeat. The presence of the correct ATG initiation codon in the cloned P450 reductase cDNA was verified by DNA sequencing.

Transfection of the ecotropic packaging cell line Bosc 23 (21) with retroviral plasmid DNA encoding hRed, harvesting of the retroviral supernatant, and infection of 9L gliosarcoma cells transduced with CYP2B6 (9L/2B6 cells; Ref. 5) were carried out as previously described (5). Pools of blasticidin-resistant cells were selected using 2.5 $\mu\text{g/ml}$ blasticidin *S*-hydrochloride for 2 days. The resultant drug-resistant pools of 9L/2B6/hRed cells exhibited a 4- to 5-fold increase in P450 reductase-catalyzed cytochrome C reduction in isolated microsomes ($\Delta A_{550\text{ nm}}$ measured at 30°C) compared with control transfectants. Measured CYP2B6 protein levels in the 9L-P450/Reductase cells were ~ 20 pmol P450 protein/mg microsomal protein by Western blot analysis using a human lymphoblast-expressed CYP2B6 cDNA standard (Gentest, Inc., Woburn, MA). 9L cells transduced with pBabe-puro retrovirus without P450 (9L/pBabe control cells; Ref. 5) were used as a control tumor cell line for direct comparisons to 9L-P450/Reductase cells. 9L/pBabe cells and the corresponding 9L/pBabe tumors used in these experiments are referred to as "9L control" or simply "9L" cells and tumors.

In Vivo Tumor Experiments. 9L gliosarcoma cells used for tumor implantation (9L-P450/Reductase or 9L control cells) were grown in DMEM containing 10% fetal bovine serum on 100-mm tissue culture 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 (28–30 g) male ICR/Fox Chase mice, an outbred scid strain (Ref. 22; Taconic Farms, Germantown, NY), were given injections of 4×10^6 tumor cells 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 (*i.e.*, two tumors/mouse) at each posterior flank. CPA administration every 6 days (see below) or using an episodic schedule (as described in Ref. 5), was initiated between 22 and 49 days after tumor implantation as specified in each experiment. CPA was solubilized in PBS at 4.8 mg/ml and kept at 37°C until injection to maintain drug solubility. Untreated control mice were used to monitor tumor doubling times in the absence of drug treatment, which were similar for 9L control and 9L-P450/Reductase tumors (see Table 1). Autoclaved cages containing sterile food and water were changed twice each week in a laminar flow hood. Mice were housed in the Boston University Laboratory of Animal Care Facility in accordance with approved protocols. Mice were fed Prolab RMH 3000 5P00 diet, which provides ~ 14 , 26, and 60% calories from fat, protein, and carbohydrates, respectively. Dietary additives and medications (*e.g.*, ondansetron and dexamethasone), used to alleviate gastrointestinal dysfunction and ameliorate weight loss (16), were not used in the present study. Tumor growth experiments were generally terminated when tumor volumes exceeded $\sim 11,000\text{ mm}^3$.

In Study A, scid mice with midstage 9L-P450/Reductase or 9L control tumors ($n = 4$ –5 mice per group; mean tumor volumes as shown in Table 2) were treated with CPA by *i.p.* injection at 140 mg/kg BW, repeated every 6 days (CPA-6 day schedule). The first CPA injection was given 22 days after tumor cell implantation. This treatment corresponds to the schedule developed

by Browder *et al.* (16) for C57/BL6/J mice, except that the dose of CPA was decreased from 170 to 140 mg/kg/injection to take into account the greater chemosensitivity of the scid mouse host used in the present study [compare with the maximally tolerated dose of CPA, 265 mg/kg BW, when given as a single *i.p.* injection in the same scid mouse model (22)]. CPA treatments were halted after 11 cycles of CPA injection, at which point none of the 9L-P450/Reductase tumors were detectable. CPA-6 day treatment was resumed 32 days later for all of the mice, at which time regrowth was detected in 4 of 8 tumors. CPA was given to some of the tumor-bearing mice in this study using the conventional schedule of 150 mg CPA/kg body weight/injection \times 2 injections, spaced by a 24-h interval, as described previously (5).

In Study B, scid mice with larger 9L-P450/Reductase or 9L control tumors (~ 2200 – 2400 mm^3 at the time of first drug treatment; 4–5 mice per group) were treated with CPA at 140 mg/kg every 6 days beginning 32–35 days after tumor implantation. Drug treatment was continued for a total of 16 CPA injections. In Study C, the tumors were grown to late-stage (~ 4400 – 5300 mm^3 , corresponding to day 45–49 after tumor implantation) prior to beginning the CPA-6 day injection schedule. Mice in this study ($n = 3$ –4/group) received a total of 14 CPA injections. In Study D, three of the late-stage 9L-P450/Reductase tumor-bearing mice that served as drug-free controls for Study A were treated with CPA at a fixed dose (8.4 mg of CPA/injection) every 6 days beginning on day 44 after tumor implantation when the mean tumor size was $\sim 7200\text{ mm}^3$ ($n = 6$) and the BW was ~ 40 g. This fixed CPA dose was continued for a total of seven CPA injections without adjustment for the subsequent decrease in overall BW that accompanied tumor regression.

Analysis of Tumor Growth Rates. Tumor growth was monitored in measurements taken twice a week using Vernier calipers (Manostat Corp., Switzerland) and tumor areas (length \times width) were calculated. Tumor volumes were calculated from the measured tumor areas using the formula: volume = $\pi/6 (L \times W)^{3/2}$, and in some cases are expressed as a percentage of initial BW (~ 28 g) assuming a tumor density of 1.0 (23). Tumor growth delay data were analyzed as described previously (8), except that tumor volumes were used for the calculations in place of tumor areas. Tumor-doubling time was defined as the time in days required for the tumor volume to double from the time of first drug treatment. Tumor growth delay values were then calculated as the difference in tumor-doubling time between the drug-treated and control groups. Specific growth delay values were calculated as $(T_2 - T_1)/T_1$, where T_1 and T_2 are times in days required for the control and the drug-treated tumors, respectively, to double in volume. The specific growth delay parameter provides an estimate of the number of doubling times by which tumor growth is delayed by drug treatment and enables comparisons of therapeutic responses between tumors that differ in their growth rates (24).

Tumor Excision and in Vitro Analysis of Chemosensitivity to CPA and 4-OOH-CPA. Tumor-bearing mice were killed at the end of each experiment by cervical dislocation. Animals were sprayed with 70% ethanol around the vicinity of the tumor, and individual tumors were then explanted for characterization of the tumor cell population in tissue culture. One-half of each tumor was immediately frozen in liquid nitrogen, whereas the remaining portion was cut into small pieces in 0.5 ml of sterile-filtered DMEM containing collagenase type IV (4500 units/ml). Samples were incubated in sterile tubes for 5 min at room temperature with vigorous pipetting to help digest the tumor. Samples were allowed to stand without pipetting for an additional 3 min to precipitate the undigested tissue and the supernatants were then plated in 100-mm tissue culture dishes with 15 ml of DMEM containing 10% FBS. A total of 14 tumors were explanted using this protocol, each yielding a stable population of 9L tumor cells that could be propagated in cell culture. The efficiency of tumor excision from the CPA-6 day treated mice was ~ 5 - to 10-fold higher for 9L control than for 9L-P450/Reductase tumors, as judged by the yield of 9L cell colonies in cell culture.

To evaluate the chemosensitivity of the explanted 9L-P450/Reductase tumor cells, cells were plated in triplicate at 4000 cells/well of a 48-well plate 18–24 h prior to drug treatment. Cells were then treated with 0–1.5 mM CPA or 0–5 μM 4OOH-CPA, which spontaneously decomposes in aqueous media to yield the cytotoxic metabolite 4-OH-CPA. Cells were incubated for 4 days in a tissue culture incubator maintained under normoxic conditions (19.6% O₂, 5% CO₂, 75.4% N₂). Cells remaining after this time were quantitated using a crystal violet-staining/alcohol-extraction assay (5). Data are presented as cell number relative to drug-free controls, mean \pm SD values for triplicate samples,

unless indicated otherwise. Error bars not visible in the individual figures are too small to be seen.

RESULTS

Effectiveness of the CPA-6 day Schedule on Midstage Tumors.

Tumor cells transduced with P450 + P450 reductase (9L-P450/Reductase tumors) and non-P450-expressing 9L control tumors were grown s.c. in scid mice to a mean size corresponding to 1.5–3% of BW and then treated with CPA. Fig. 1 compares the antitumor effect of CPA administered at a moderate dose, repeated every 6 days, to standard high-dose CPA treatment for both 9L control (Fig. 1A) and 9L-P450/Reductase tumors (Fig. 1B). The conventional high-dose CPA regimen [two injections totaling 300 mg/kg of BW over 24 h (5)] induced a tumor growth delay of ~20 days in the case of 9L-P450/Reductase tumors as compared with only 3 days in the case of 9L control tumors (Table 1). Repeated injection of CPA 3–4 weeks later using the same high dose prolongs the antitumor effect against 9L-P450/Reductase tumors, but does not lead to substantial tumor regression (5)]. In contrast, treatment of the 9L-P450/Reductase tumors with CPA repeatedly each 6 days (140 mg CPA/kg of BW/injection)

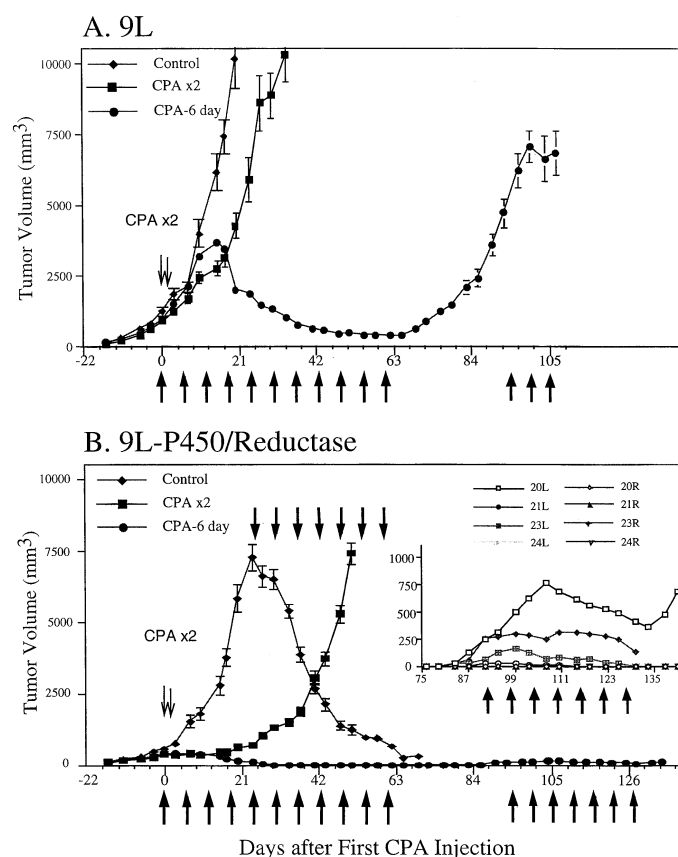


Fig. 1. Comparison of the tumor growth delay effects of the CPA-6 day schedule with conventional CPA treatment. 9L control (A) and 9L-P450/Reductase tumors (B) were grown in scid mice as s.c. solid tumors (Study A). Tumor areas were measured twice a week with Vernier calipers, and tumor volumes were calculated as described under "Materials and Methods." Arrows along the X axis, the days on which CPA-6 day treatment was given by i.p. injection at 140 mg/kg. B, thick arrows pointing down, the period of treatment at a fixed dose (8.4 mg) of CPA every six days (CPA-6 day) given to mice with previously untreated large 9L-P450/Reductase tumors (Study D), beginning on day 44 after tumor implantation. Pair of thin arrows pointing down, the administration of the conventional schedule of CPA, two injections, each at 150 mg/kg, spaced 24 h apart (CPA × 2). The X axis corresponds to days after first CPA injection. Tumors were implanted 22 days earlier. Data points, mean tumor volumes ($n = 8-10$ tumors per treatment group); bars, 0.5 SE. Inset, tumor growth profiles for individual 9L-P450/Reductase tumors in mice numbered 20–24 (L, left-side tumor; R, right-side tumor) after retreatment with the CPA-6 day schedule beginning on day 92.

Table 1 Comparison between CPA-6 day schedule and conventional CPA treatment: effects on tumor doubling time and tumor growth delay in midstage tumors

Data shown are from Study A and are based on data presented in Fig. 1. Tumor doubling time (mean \pm SD), tumor growth delay, and specific growth delay were calculated as described in "Materials and Methods." The number of individual tumors included in each tumor group or mode of treatment is shown in parentheses.

	Tumor doubling time (days)	Growth delay (days)	Specific growth delay
9L			
Control (6)	5.9 \pm 2.6		
+ CPA × 2 (8)	8.9 \pm 4.1	3	0.51
+ CPA-6 d (10)	6.4 \pm 1.1 ^a	0.5	0.06
9L-P450/Reductase			
Control (6)	5.8 \pm 0.9		
+ CPA × 2 (8)	26.2 \pm 1.3	20.4	3.5
+ CPA-6 day (10)	>140 ^b	>134	>23

^a Tumors doubled in volume by day 6 after the initial CPA injection, reaching a maximum size on days 15–17 (i.e., after the third injection of CPA), and then began to regress.

^b Tumors stopped growing within a few days of the first CPA injection (see Table 2) and never doubled in volume.

resulted in an almost immediate response to drug treatment, as evidenced by the cessation of tumor growth, generally within 3–7 days. This was followed by major tumor regression and an apparent elimination of all eight tumors after ~50–55 days (9–10 CPA injections). Overall, the CPA-6 day treatment schedule effected a specific growth delay of >23 doubling times (>134 days) in the case of the 9L/P450/Reductase tumors (Table 1). By contrast, P450-deficient 9L control tumors treated with the same CPA-6 day schedule continued to grow without regression until after the third CPA injection (day 15–17; Table 2, column 2). During this initial period of CPA-6 day treatment, 9L control tumors increased in volume by an additional 320% and did not exhibit any growth delay compared with drug-free controls (6.4 versus 5.9 days; Table 1). A similar delay in the onset of CPA responsiveness of 9L control compared with 9L-P450/Reductase tumors was seen in two other experiments, where the tumors were up to 10-fold larger in volume at the time of initial drug treatment (Table 2; Study B and Study C; also see Fig. 3A and Fig. 4A, below).

The CPA-6 day schedule was well tolerated and was not associated with major toxicity, as indicated by the stable BW of the mice (Fig. 2). CPA-6 day-treated mice ate normally, in contrast to mice given the conventional high-dose CPA treatment, where a loss of appetite and weight loss frequently occurs for a period of several days following CPA administration (data not shown).

CPA treatment was halted after 11 CPA injections (day 61 after first CPA injection) to ascertain whether any viable tumor cells remained in the case of the 9L-P450/Reductase tumors. No tumor was palpable for the following ~20–25 days. Subsequently, 4 of the 8 9L-P450/Reductase tumors began to regrow, at which time a second series of CPA/6 day injections was initiated (day 92). This CPA retreatment led to either cessation of growth or regression of all 4 tumors, although 1 tumor resumed growth at day ~128 (tumor 20L, Fig. 1B, inset). Overall, 6 of the 8 tumors in this group regressed to the point where they were undetectable (Table 2, last column). By contrast, a rapid resumption of 9L control tumor growth was observed shortly after CPA treatment was halted (day 68). This resulted in tumors 4700 ± 952 mm³ in volume (mean \pm SD, $n = 8$) by the time the CPA-6 day injection regimen was resumed on day 92 (c.f., 93 ± 41 mm³ ($n = 8$) mean volume of 9L-P450/Reductase tumors at that same time point). Moreover, in contrast to mice bearing 9L-P450/Reductase tumors, which tolerated the second series of CPA-6 day injections fairly well, all 4 remaining mice in the 9L control group died after either the second ($n = 1$) or the third CPA injection in the series ($n = 3$).

Table 2 Impact of CPA-6 day schedule on growth of tumors treated at different stages of growth

	Initial tumor size, ^a mm ³	Initial response to CPA			
		Continued tumor growth period ^b (days)	Tumor size increase ^c (%)	Maximal tumor regression ^d (%)	Tumors eradicated ^e
Study A					
9L	954 ± 121 (10)	15–17	321 ± 82	67 ± 24	0/10
9L-P450/Reductase	403 ± 42 (10)	3–10	16 ± 16	100 ± 0	6/8 ^f
Study B					
9L	2247 ± 565 (10)	10–17 (9) ^g	147 ± 67	85 ± 13	0/10
9L-P450/Reductase	2396 ± 329 (8)	4–7	29 ± 16	99 ± 1	2/10 ^h
Study C					
9L	5276 ± 644 (8)	11–18	97 ± 44 (6)	74 ± 11 (4)	0/4
9L-P450/Reductase	4433 ± 866 (6)	0–7	9.5 ± 14	96.5 ± 4	0/6 ⁱ
Study D					
9L-P450/Reductase	7223 ± 910 (6)	0–6	3.8 ± 6.9	93 ± 5	0/6

^a Tumor volume at the time of the first CPA injection, mean ± SD for (n) individual tumors, calculated from measured tumor areas as described in “Materials and Methods.”

^b Time, measured in days after the first CPA injection, until tumor regression was first detected based on tumor size measurements taken twice a week. Data are expressed as a range exhibited by the individual tumors in the group.

^c Percentage of increase in tumor size from the time of the first CPA injection until tumor regression was first observed, mean ± SD.

^d Maximal decrease in tumor size (mean ± SD), which was generally achieved after 10–12 CPA injections. Percentage regression values were calculated based on the volume of each tumor at the time of the first CPA injection (compare column 1).

^e Tumor eradication, which was indicated by a tumor-free period of >100 days (four tumors in Study A) or ~40 days (one tumor in Study A and two tumors in Study B), at which point, the studies were terminated.

^f Two of the 10 tumors initially in this group ultimately developed resistance and regrew (Fig. 1B, inset; also see Fig. 5). In addition, one mouse died on day 75, at which time its two tumors were no longer palpable.

^g Range for 9 of 10 tumors in the group. Tumor regression was first observed after day 10 in the case of the remaining tumor.

^h Tumor-free period of 40 days, seen in a third tumor in this group, was followed by tumor regrowth.

ⁱ Tumor-free periods of 25 and 30 days were respectively seen for two tumors in this group and were followed by tumor regrowth.

Effect of CPA-6 day Schedule on Growth of Large Tumors.

The complete regression of midstage 9L-P450/Reductase tumors treated with the CPA-6 day schedule (Fig. 1B) prompted us to investigate

the effectiveness of this treatment regimen when applied to late-stage tumors. Because the doses of CPA are adjusted to the animal's total weight (BW + tumor weight), we also investigated whether greater host toxicity would be apparent in animals with a larger tumor burden. 9L control and 9L-P450/Reductase tumors were grown to an average size of 2200–2400 mm³ (8–9% of initial body weight; Fig. 3) or 4400–5300 mm³ (late-stage tumors; 16–19% of initial body weight; Fig. 4), at which time CPA-6 day treatment was initiated. In both cases the 9L-P450/Reductase tumors responded to the CPA-6 day schedule more rapidly (within 4–7 days) and more completely than 9L control tumors. Of note, tumor regression was delayed (for up to 18 days) and was less extensive with the 9L control tumors, despite the fact that these mice received more total CPA than mice bearing 9L-P450/Reductase tumors, because of their greater tumor burden and hence larger BW (Table 2). A specific growth delay of >17 tumor doubling times was achieved with the 9L-P450/Reductase tumors but not the 9L control tumors (Table 3). Moreover, the 9L control tumors never disappeared completely (74–85% regression of control tumors versus 96–99% regression of 9L-P450/Reductase tumors; Study B and Study C, Table 2). The dramatic reductions in tumor size seen with the late-stage tumors on the CPA-6 day schedule are reflected in the profiles of body weights, which had increased from ~30 g to as high as ~55 g in parallel to the increase in tumor burden (Fig. 4B). The substantial reduction in body weight in association with CPA-induced tumor regression evidences the dramatic antitumor effect of the CPA-6 day treatment regimen.

In a separate experiment [Fig. 1B; Table 2 (Study D)], 9L-P450/Reductase tumors were grown to a size of ~7200 mm³ (~25% of initial BW), at which time the mice were given a fixed dose of CPA, 8.4 mg CPA/mouse (corresponding to an initial CPA dose of ~210 mg/kg) repeated every 6 days for a total of 7 injections. This drug treatment regimen was highly effective, with major tumor regression (~93% decrease in tumor volume) seen in 6 of 6 tumors. However, the total CPA dose (59 mg/mouse over the course of 36 days) resulted in cumulative toxicity and lethality to all of the treated mice.

CPA-6 day Schedule Does Not Promote Intrinsic Cellular Resistance to Activated CPA. Although initially highly responsive to CPA-6 day treatment, 9L-P450/Reductase tumors from the late-

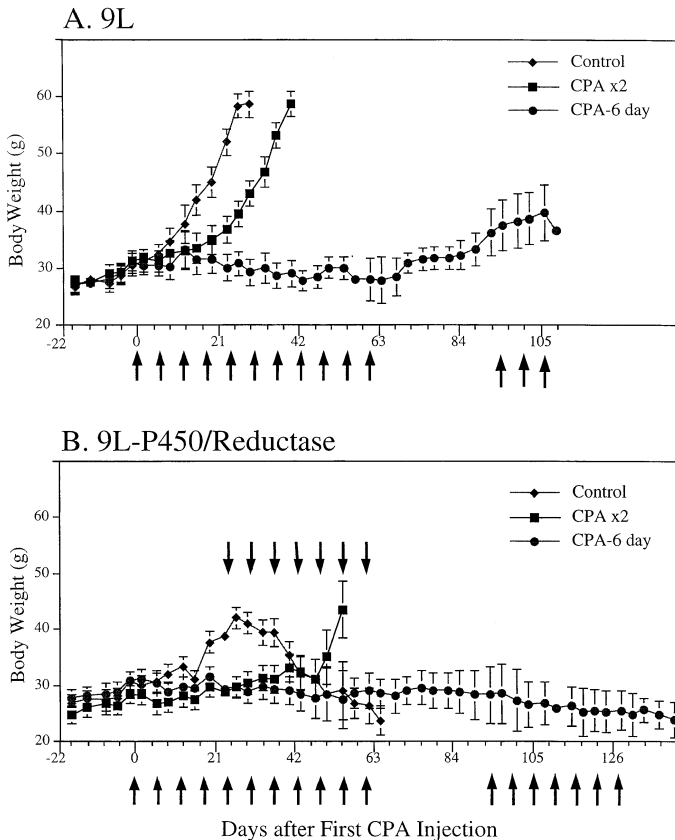


Fig. 2. Effect of CPA-6 day administration on BW profiles in tumor-bearing acid mice. BW profiles are shown for mice bearing 9L control tumors (A) or 9L-P450/Reductase tumors (B), two tumors/mouse. Data shown are for the same animals described in Fig. 1. Animal weights were measured twice a week. No food additives or antiemetics were added to alleviate side effects of CPA. Data points, mean BW ($n = 4-5$ animal per treatment group); bars, SE. Arrows, days on which CPA was administered, as described in Fig. 1.

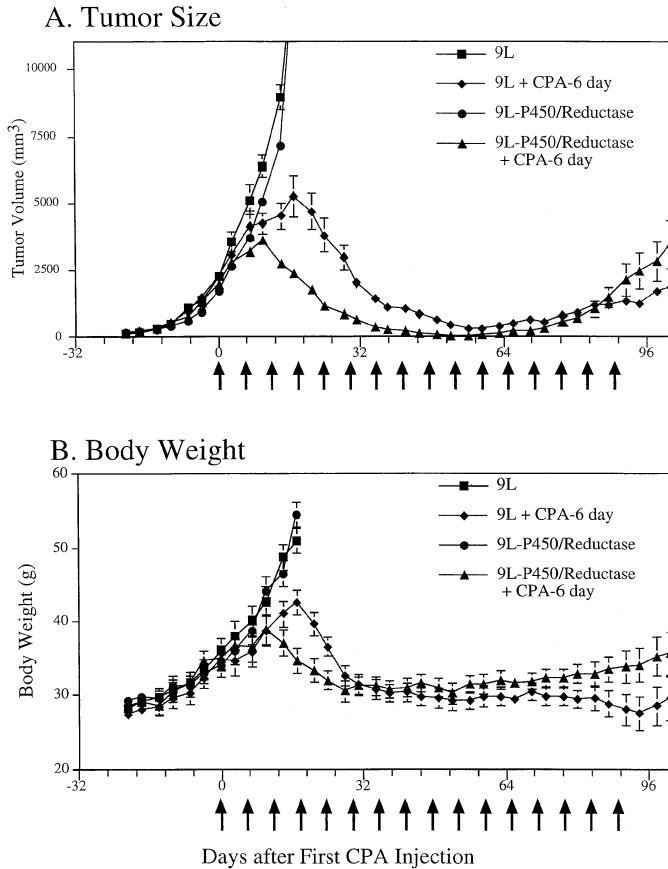


Fig. 3. Effect of CPA-6 day treatment on growth of 9L-P450/Reductase and 9L control tumors in Study B. Experimental design was the same as that described in Fig. 1, except that the mice were treated with a total of 16 i.p. drug injections on the CPA-6 day schedule (140 mg CPA/kg of BW/injection). Tumors were grown to a size of ~2200–2400 mm³ before beginning the CPA-6 day treatment schedule. A, the volumes of 9L-P450/Reductase and 9L control tumors, both with and without CPA-6 day treatment. Tumors were implanted 32 days prior to beginning CPA treatment on day 0. Data points, mean tumor volumes (8–10 tumors per treatment group). B, the corresponding BW changes of the animals during the course of CPA treatment. Bars, 0.5 SE.

stage tumor experiments eventually became resistant to repeated CPA injection (Figs. 3 and 4). Similarly, an apparent resistance to CPA was seen in 1 of 8 midstage tumors (Fig. 1B, inset; tumor 20L). The cause of this apparent resistance to CPA was investigated using tumor cells explanted from 9L-P450/Reductase tumor 20L. Fig. 5 shows that the explanted tumor cell population was rather insensitive to CPA in culture, in contrast to the original 9L-P450/Reductase tumor cell line (panel A). Treatment of the cells with chemically activated CPA (*i.e.*, 4OOH-CPA) induced comparable killing of the tumor 20L explanted cells and the original 9L-P450/Reductase cells (Fig. 5B). Thus, the insensitivity of the explanted tumor cells to CPA is not attributable to an intrinsic resistance to activated CPA, but results from the inability of these tumor cells to activate CPA efficiently. Further investigation revealed that the explanted tumor cells no longer express the transduced P450 reductase gene, as indicated by the decrease in cytochrome C reduction rates back to the level of 9L controls (Fig. 5C) and by the loss of resistance to blasticidin, a selection marker for the transduced P450 reductase gene (panel D). A partial (~60%) loss of resistance to puromycin, selection marker for the transduced P450 2B6 gene, was also seen (Fig. 5D), and correspondingly, there was a detectable decrease in expression of P450 2B6 protein (Fig. 6A; Lane 10 versus Lane 2). These findings are consistent with the reduced CPA sensitivity of the explanted tumor 20L cells shown in Fig. 5A.

Additional investigations using cells explanted from the late-stage

tumor experiments revealed a 5- to 10-fold higher cloning efficiency for explanted 9L control tumor cells compared with 9L-P450/Reductase tumor cells. Thus, many of the 9L-P450/Reductase tumor cells obtained from the CPA-treated mice were nonviable in the *in vitro* cloning assay, presumably because of cellular damage induced by the *in vivo* CPA treatment. Moreover, tumor cells derived from each of the 9L-P450/Reductase tumors except one (tumor 66L) showed a diminished or total loss of sensitivity to CPA when compared with parental 9L-P450/Reductase cells (Fig. 6B). This loss of CPA sensitivity was associated with the loss of the transduced P450 reductase, as judged by the decrease in cytochrome C reductase activity back to

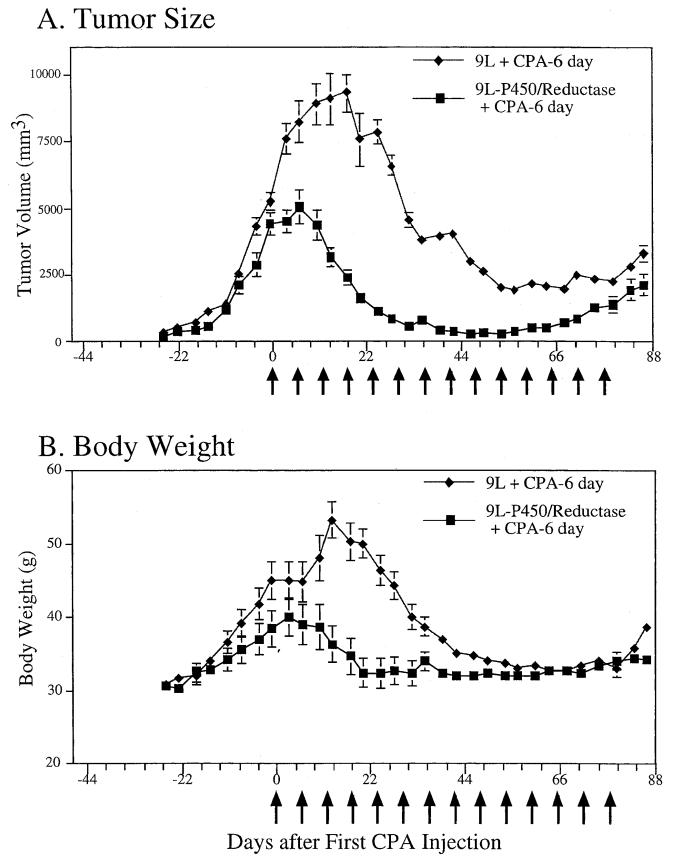


Fig. 4. Effect of CPA-6 day treatment on the growth of large 9L-P450/Reductase and 9L control tumors (Study C). Experimental design was the same as that described in Fig. 3, except that the mice were treated with a total of 14 i.p. CPA injections beginning on day 45 after tumor implantation. Tumor sizes are shown in A and animal weights are shown in B. Data points, mean tumor volumes (8–10 tumors per treatment group). Bars, 0.5 SE.

Table 3 Growth delay effects of the CPA-6 day schedule in late-stage tumors

Data shown are from Study B and are based on data shown in Fig. 3. Tumor doubling time, tumor growth delay, and specific growth delay were calculated as described in "Materials and Methods." The number of tumors included in each group is shown in parentheses. Tumor sizes at the time of the first CPA treatment are shown in Table 2.

Tumor treatment	Tumor doubling time (days)	Growth delay (days)	Specific growth delay
9L			
Control (8)	6.3 ± 2.3		
+ CPA-6 day (10)	12 ± 4.3 ^a	5.7	0.90
9L-P450/Reductase			
Control (8)	5.5 ± 1.5		
+ CPA-6 day (10)	>100 ^b	>94	>17

^a Tumors increased in size for 10–17 days after the initial CPA injection (at which time the mice had received three CPA injections, on days 0, 6, and 12) and then began to regress (see Table 2).

^b No doubling of tumor volume was observed at any point after CPA treatment. Tumors increased in volume by 29 ± 16% (*n* = 10; mean ± SD) during the first 7 days after the first CPA injection and subsequently began to regress.

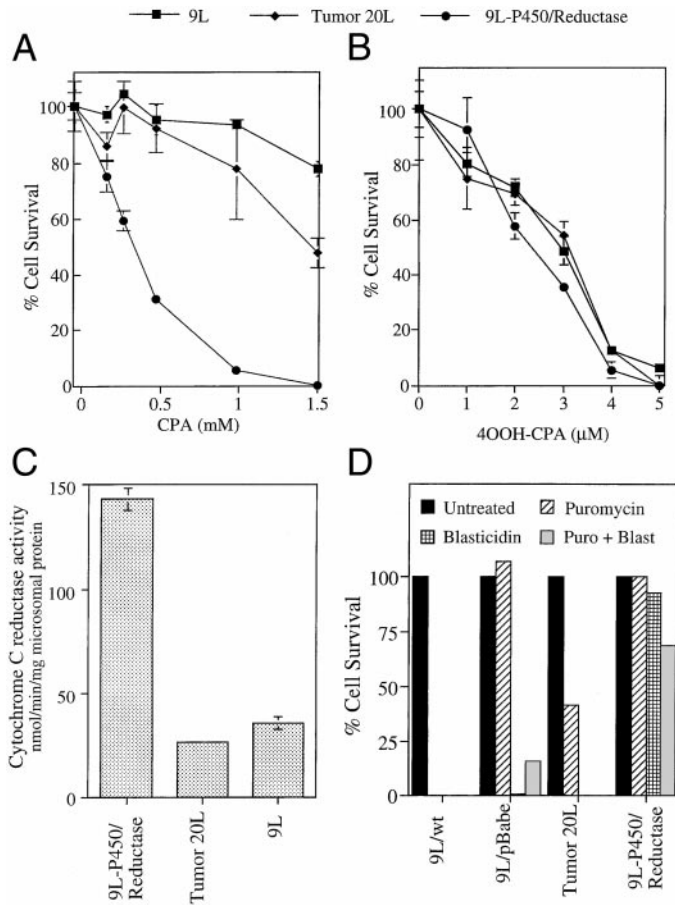


Fig. 5. Cytotoxicity of CPA and 4OOH-CPA to cells explanted from 9L-P450/Reductase tumor 20L. Cells were seeded at 4000 cells/well in 48-well plates and treated with increasing concentrations of CPA (0 to 1.5 mM) or 4OOH-CPA (0 to 5 μ M) for 4 days as described in "Materials and Methods." Cell growth in comparison to drug-free controls was determined by crystal violet staining. Data are presented as means \pm SD values for $n = 3$ replicates. A and B, parallel studies carried out with 9L-P450/Reductase, 9L control cells, and tumor cells explanted from 9L-P450/Reductase tumor 20L after a series of 18 CPA-6 day injections (Study A; see Fig. 1B, inset). C, the P450 reductase enzyme activities of 9L-P450/Reductase parental and the tumor 20L-derived explanted cells (rate of cytochrome C reduction) assayed using 20 μ g of microsomal protein. D, the sensitivity of the explanted tumor 20L-derived cells to puromycin (2.5 μ g/ml) or blasticidin (2.5 μ g/ml), or to a combination of both drugs (1.5 μ g/ml each) in comparison to the wild-type 9L cells and the parental cell lines 9L-P450/Reductase and 9L control (9L/pBabe). Cells survival was determined by crystal violet staining.

the level of the 9L cell controls (Fig. 6C). Whereas a loss of P450 2B6 protein was apparent in cells derived from two tumors (43R and 64R; Fig. 6A), all of the other excised tumor cells retained a level of P450 2B6 protein comparable with that found in the original 9L-P450/Reductase cells. One exception was tumor 66L, in which P450 2B6 protein levels were inexplicably elevated several-fold. When challenged with chemically activated CPA, all of the tumor cells except those derived from tumors 42R and 66L showed strong intrinsic drug sensitivity (Fig. 6B), excluding intrinsic cellular resistance to the activated CPA metabolite as the mechanism of CPA resistance *in vivo*.

DISCUSSION

CPA is a widely used anticancer alkylating agent prodrug that requires liver P450 metabolism for anticancer activity. CPA is typically administered at high doses associated with acute host toxicities, necessitating drug-free recovery periods that often lead to tumor regrowth and patient relapse. In an effort to circumvent this problem, a novel schedule of CPA administration, involving repeated injection of moderate CPA doses every 6 days, was introduced and shown to

induce substantial tumor regression with minimal host toxicity (16). This CPA-6 day schedule has been characterized as antiangiogenic (16) insofar as it stimulates repetitive waves of apoptosis of the vascular endothelial cells lining the tumor bed and can sustain and thereby maximize the antiangiogenic effect by inhibiting the regrowth of blood vessels feeding the tumor during the drug-free host recovery period that generally accompanies conventional cancer chemotherapeutic regimens (17). The present study extends this earlier work and demonstrates that dramatically enhanced therapeutic effects leading to long-term suppression and tumor eradication with reduced host toxicity can be achieved by combining the CPA-6 day schedule with a P450 prodrug activation-based cancer gene therapy strategy (6).

When using conventional CPA scheduling, which comprises an initial treatment with high doses of CPA, a tumor growth delay of \sim 25 days is observed in mice using the present P450/P450 reductase gene therapy model (5, 7). Repeated CPA injection after a 3–4 week recovery period prolongs the period of tumor growth delay but does not eliminate the tumors (5). Some improvement can be obtained by cotreatment with the antithyroid drug methimazole, which selectively inhibits hepatic P450-catalyzed prodrug activation (8), or by combination of CPA with the bioreductive drug tirapazamine (7); however, large-scale tumor regression leading to tumor eradication has not been achieved. By contrast, combination of the CPA-6 day scheduling approach of Browder *et al.* (16) with P450/P450 reductase gene therapy induced major tumor regression, even in the case of tumors that were very large (up to 16% of BW) at the time of initial CPA treatment (Table 2). The antitumor effects of this CPA-6 day schedule

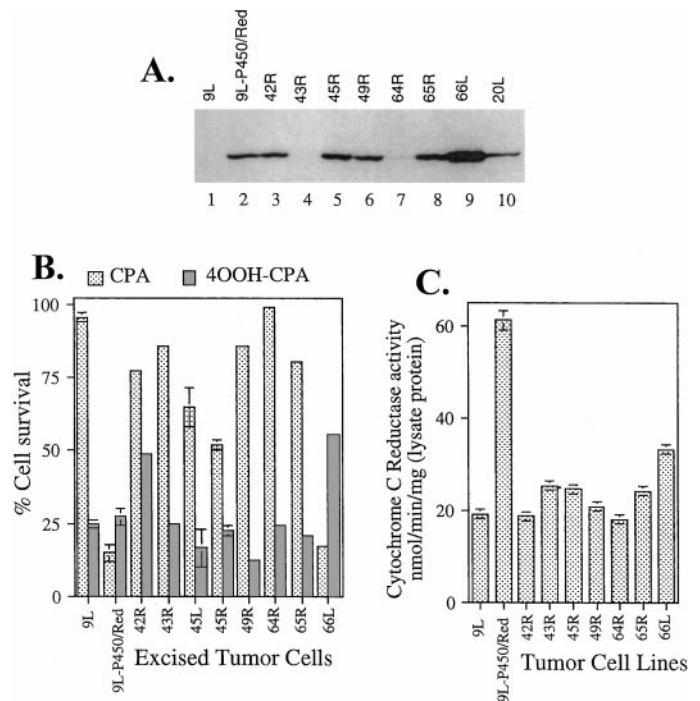


Fig. 6. *In vitro* analysis of explanted tumors. A, Western blot analysis of P450 2B6 protein in various 9L-P450/Reductase explanted tumor cell lines. Shown are cell extracts prepared from 9L control (Lane 1) and 9L-P450/Reductase cells (\sim 1 pmol P450; Lane 2) and 9 different individual explanted, 9L-P450/Reductase tumor-derived cell lines (Lanes 3–10). Cell lysates (60 μ g/lane) were analyzed on a Western blot probed with an anti-P450 2B6 COOH-terminal antipeptide antibody (anti-IDTYLLRMEKEK-), which recognizes both rat CYP2B1 and human CYP2B6 protein. B, the effects of CPA and 4OOH-CPA on the chemosensitivity of 9L-P450/Reductase explanted tumor cell lines in comparison with the parental 9L cell lines. Cells were seeded at 4000 cells/well in 48-well plates and treated with 1 mM CPA or 5 μ M 4OOH-CPA 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 \pm SD for $n = 3$ replicates. C, P450 reductase enzymatic activities of the 9L-P450/Reductase parental and explanted tumor-derived cell lines (rate of cytochrome C reduction), assayed using 20 μ g of cell lysate.

were much less extensive in the case of 9L tumors that were not transduced with P450 and P450 reductase. Moreover, whereas P450/P450 reductase-transduced tumors generally responded within a few days to the CPA-6 day treatment regimen, as indicated by the onset of tumor regression, control 9L cells required up to 18 days to respond to the drug, during which time a doubling or tripling of tumor volumes occurred. Moreover, the subsequent regression of the P450-deficient tumors that ultimately did result was not nearly as dramatic and complete as that of the 9L-P450/Reductase tumors (Table 2). Although some of the P450-expressing tumors did begin to regrow when CPA treatment was halted, the majority of these tumors were still intrinsically responsive to a second series of CPA-6 day injections. The potential clinical utility of the CPA-6 day schedule is supported by the apparent absence of drug toxicity during the overall period of CPA treatment (up to ~130 days), as judged by monitoring food consumption and by the absence of substantial weight loss during the course of the study.

Continued CPA-6 day treatment was associated with an apparent development of resistance to CPA and significant regrowth of a portion of the P450/P450 reductase-containing tumors examined in this study. This resistance resulted from a loss of the transduced P450 and/or P450 reductase gene and, hence, the capacity to activate CPA, rather than *de novo* acquisition of intrinsic resistance to activated CPA. Presumably, this reflects the selection and regrowth of a population of tumor cells that has spontaneously lost the drug-sensitivity P450/P450 reductase genes under selective pressure of CPA treatment. Loss of either P450 or P450 reductase gene expression may occur with greater frequency in large tumors and would be expected to lead to a decrease in CPA sensitivity, in the latter instance because the endogenous level of P450 reductase, which is found in 9L and many other tumor cells, is insufficient to fully support efficient metabolism by the transduced P450 gene (6). In the present study, the P450 reductase-expressing 9L tumor cells were derived from a pool of retrovirally transduced cells; and, accordingly, P450 reductase-negative tumor cells were apparently selected under CPA selection pressure. Accordingly, repeated transduction of tumors with a gene therapy vector may be required to achieve tumor eradication using this and probably other prodrug activation-based cancer gene therapies.

The mechanism for the apparent development of CPA resistance seen in the case of 9L control tumors is uncertain. *In vitro* culture of 9L cells excised from the CPA-6 day-treated mice indicated that the majority of these tumor cells retained a sensitivity to activated CPA similar to that of the initial 9L tumor cell line (data not shown). Conceivably, these cells could exhibit *in vivo* CPA resistance by a mechanism analogous to the acquired multicellular resistance/tumor compaction mechanism described elsewhere, which is manifest only in an *in vivo* setting, in which the tumor adapts and becomes less responsive to activated CPA produced in the liver (25–27). Alternatively, although not seen in other studies (28), endothelial cells lining the tumor vasculature could potentially develop intrinsic resistance to CPA, *e.g.*, by overexpression of aldehyde dehydrogenase, which inactivates the active CPA metabolite, 4-OH-CPA (4). Our inability to eradicate nontransduced 9L gliosarcoma tumors in scid mice contrasts with the findings of Browder *et al.* (16), who found that CPA-6 day scheduling led to the eradication of Lewis lung carcinomas grown as solid tumors in C57Bl6/J mice (16). This difference may be explained by contributions of the immune system to tumor surveillance and its active participation in the elimination of apoptotic Lewis lung carcinoma cells in the earlier studies, performed using immunocompetent C57/Bl6/J mice (compare Ref. 29). This immune surveillance mechanism would not be operative in our study, in which immunodeficient scid mice were used. Other possibilities include the use of a lower CPA dose in our experiments (140 mg/kg of BW/injection in the

present study *versus* 170 mg/kg of BW/injection by Browder *et al.*) and the fact that the Lewis lung tumors in the prior study were very-early-stage tumors (~0.1 cm³) and presumably more sensitive to antiangiogenic CPA scheduling at the time of the first CPA treatment (compare initial 9L control tumor sizes ranging from ~0.9 cm³ to ~5 cm³ in the present study). Intrinsic differences in the CPA sensitivity of 9L gliosarcoma as compared with Lewis lung carcinoma, which is known to secrete angiostatin (30), could also play a role.

The precise mechanism whereby P450-expressing tumors dramatically regress in response to the CPA-6 day schedule is not known. In the case of Lewis lung tumors (which were not transduced with a P450 gene), CPA-6 day treatment induces apoptosis of endothelial cells lining the tumor vasculature as a primary response, followed by tumor cell apoptosis after an ~3.5-day delay (16). Although we did not measure apoptosis directly in the present study, a substantial delay in the apoptosis of P450-deficient 9L tumor cells is strongly indicated by the continued growth of 9L control tumors for up to 18 days after the initiation of CPA-6 day treatment (Table 2). This delay suggests that the antiangiogenic mechanism described by Browder *et al.* (16) may also be operative in our P450-deficient 9L control tumors. This supposition is further supported by the rapid regrowth of the 9L control tumors after the cessation of CPA-6 day treatment (Fig. 1), which is consistent with the residual tumor cells being intrinsically viable but restricted from rapid growth by the antiangiogenic effects of the CPA-6 day schedule. By contrast, 9L-P450/Reductase tumors typically began to regress prior to the second CPA injection on day 6, strongly suggesting a direct cytotoxic response to CPA that was activated locally within the tumor. This direct cytotoxic effect of the CPA-6 day treatment could conceivably be magnified by an endothelial cell-directed, antiangiogenic mechanism analogous to that documented by Browder *et al.* in their studies of Lewis lung tumors using the same drug schedule (16). Although antiangiogenic CPA therapy could potentially compromise a prodrug activation-based gene therapy strategy by inhibiting the efficient entry of prodrug into the solid-tumor mass, the repeated generation of even low levels of activated drug within the tumor by P450 metabolism might be sufficient to kill tumor cells weakened as a consequence of an antiangiogenic drug regimen. We have not, however, directly examined the occurrence of such an antiangiogenic response in the present study, in which CPA-induced apoptosis of the P450-expressing tumor cells is likely to obscure any enhanced apoptosis that may occur in the endothelial cells lining the tumor vasculature as a consequence of localized CPA activation by adjacent P450-containing tumor cells. Indeed, Browder *et al.* (16) were only able to visualize and characterize CPA-6 day-induced endothelial cell apoptosis by studying CPA-resistant tumors, for which the background level of drug-induced tumor cell apoptosis is low. Further study will be required to clarify this point, as well as to determine whether enhanced antiangiogenic and antitumor responses can be achieved by targeting CPA-activating P450 genes directly to tumor endothelial cells. Other strategies to be investigated include combination of CPA with antiangiogenesis inhibitors (16, 31, 32), development of antiangiogenic schedules for other anticancer prodrugs or prodrug combinations (*c.f.*, Ref. 33), and the design of antiangiogenic schedules for use with CPA in the clinic, where CPA has a substantially longer plasma half-life (up to 12 h in adults) than in the mouse ($t_{1/2}$, ~30 min; Ref. 4).

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