

Sustained P450 expression and prodrug activation in bolus cyclophosphamide-treated cultured tumor cells. Impact of prodrug schedule on P450 gene-directed enzyme prodrug therapy

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Cytochrome P450-based gene therapy can substantially increase the sensitivity of tumor cells to P450-activated cancer chemotherapeutic prodrugs such as cyclophosphamide (CPA) without increasing host toxicity. While the role of 4-OH-CPA, the primary active metabolite of CPA, in eliciting tumor cell death is well established, the effect of 4-OH-CPA exposure on the capacity of P450-expressing tumor cells for continued metabolism and activation of CPA has not been investigated. The present study addresses this question and characterizes the impact of CPA dose and treatment schedule on the ability of P450-expressing tumor cells to sustain prodrug activation over time. 9L gliosarcoma cells expressing human P450 2B6 and treated with CPA in a continuous manner exhibited a time- and CPA dose-dependent decrease in P450-catalyzed CPA 4-hydroxylase activity. This decrease reflects a selective, 4-OH-CPA-induced loss of cellular P450 protein content. By contrast, when the P450-expressing tumor cells were treated with CPA as a single 8 hours exposure, cellular CPA 4-hydroxylase activity and P450 protein expression were substantially prolonged when compared to continuous prodrug treatment. This schedule-dependent effect of CPA was influenced by the level of P450 protein expressed in the tumor cells. At high P450 protein and activity levels, which could be achieved by culturing the tumor cells at high cell density, net production and release of 4-OH-CPA into the culture media was increased substantially. This increase fully offset the decline in CPA 4-hydroxylase activity as the tumor cells underwent CPA-induced apoptotic death. These findings demonstrate the impact of CPA dose and treatment schedule on the efficacy of P450 gene-directed enzyme prodrug therapy, with bolus CPA treatment being compatible with sustained expression of P450 protein and maintenance of P450-dependent prodrug activation by the target tumor tissue.

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Cyclophosphamide (CPA) is a widely used anticancer prodrug that is activated by a cytochrome P450-catalyzed 4-hydroxylation reaction. The resulting metabolite, 4-hydroxy-cyclophosphamide (4-OH-CPA), undergoes spontaneous β -elimination to yield phosphoramide mustard and acrolein, which alkylate DNA and protein, respectively.¹ In classic cancer chemotherapy, CPA is activated in the liver by microsomal cytochrome P450 enzymes. This leads to the systemic distribution of 4-OH-CPA and results in the exposure of both normal host cells and tumor tissue to active, cytotoxic drug. In P450 gene-directed enzyme prodrug therapy (P450 GDEPT), tumor cells are transduced with a prodrug-activating P450 gene, which enables the tumor cells to activate the prodrug locally, leading to enhanced antitumor activity without a corresponding increase in host toxicity.²

Preclinical studies using rodent and human tumor xenografts have shown that P450 GDEPT can effect a substantial increase in CPA-induced tumor growth delay and host survival when compared to standard CPA treatment protocols.^{3–7} Delivery and regulated expression of the therapeutic P450 gene can be effected using a variety of gene therapy vectors, including oncolytic viral vectors;^{8,9} human macrophages¹⁰ and encapsulated mammalian cells.¹¹ Initial clinical studies applying the principles of P450 GDEPT in a phase I/phase II clinical trial of inoperable pancreatic carcinoma patients have yielded promising results.¹²

While the mechanism of action of CPA when activated within P450-expressing tumor cells has been well studied,¹³ the impact of CPA activation, and the resultant induction of apoptosis, on the ability of P450-expressing tumor cells to maintain prodrug activation activity is unknown. The present study investigates this question by examining the effects of three distinct *in vitro* CPA treatment regimens, each designed to model a different clinical schedule of CPA administration, on the long-term maintenance of CPA 4-hydroxylase activity in cultured

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tumor cells expressing P450. One *in vitro* regimen was chosen to model high-dose CPA therapy, which is associated with substantial host toxicity and must be implemented in combination with autologous bone marrow transplantation.¹⁴ The second regimen models an alternative clinical protocol involving continuous infusion of CPA at a comparatively low drug concentration.¹⁵ The third *in vitro* regimen mimics the more common clinical protocol of single high-dose (bolus) CPA administration followed by a drug-free period to allow for patient recovery from systemic toxicity.¹⁵ Adult cancer patients given CPA as a bolus injection exhibit plasma half-life values ranging from 6.0 to 12.4 hours for CPA and 5.6 to 12.6 hours for phosphoramidate mustard, the therapeutic metabolite derived from 4-OH-CPA.^{16–18} Accordingly, the single high-dose CPA clinical protocol was modeled in the present study using an 8 hour period of CPA treatment, followed by incubation of the tumor cells in drug-free media.

Cytochrome P450 enzyme activity is dependent on, and can be substantially increased in P450-expressing tumor cells by coexpression of the flavoprotein electron donor NADPH-cytochrome P450 reductase.¹⁹ This, in turn, increases the production of 4-OH-CPA, which diffuses into neighboring tumor cells, thereby extending the cytotoxicity of P450-activated CPA to include the bystander tumor population. This bystander cytotoxicity can greatly amplify the cytotoxic potential of CPA and other P450-activated prodrugs and is essential for the success of all GDEPT strategies of cancer treatment.²⁰ In order to achieve robust bystander cytotoxicity, however, it is necessary to ensure that P450 continues to be expressed once the tumor cells are treated with CPA. The present study investigates the effect of CPA treatment and CPA-induced tumor cell cytotoxicity on P450 expression and activity. The effects of CPA dose and treatment schedule are also investigated to determine their impact on the viability of P450-expressing tumor cells and on the cells' ability to sustain P450 prodrug activation. Finally, growing tumor cells at higher cell density is shown to increase intracellular P450 protein levels. The effect of this increased P450 expression on the ability of tumor cells to activate CPA is examined as a potential mechanism to maximize intratumoral production of activated CPA metabolites.

Materials and methods

Generation of stable cell lines

Rat 9L gliosarcoma cells expressing cytochrome P450 2B6 and human P450 reductase (9L/P450 cells, corresponding to the 9L/2B6/hRed cells described previously)⁶ were kindly provided by Dr Y Jounaidi of this laboratory. Cells were grown as monolayers cultured at 37°C in a humidified atmosphere containing 5% CO₂ in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum. These cells were derived from a retrovirally transduced clonal cell line that expresses human P450 2B6

and was subsequently transduced with a second retrovirus encoding human P450 reductase.⁶

Growth inhibition assays

In a typical experiment, 9L/P450 cells were plated in 12-well dishes at 1.0×10^5 cells per well in 2 ml of media. Cells were treated with CPA beginning 20–24 hours after plating unless indicated otherwise. Cells were either treated with CPA in a continuous manner (0.125 or 1 mM CPA), or were given one or more 8 hour CPA treatments (1 mM), as specified. Cells that were treated with CPA for 8 hours were subsequently incubated in CPA-free media for the duration of the experiment. For experiments with cells incubated in the absence of CPA, CPA 4-hydroxylase activity and cell survival measurements reported in each figure were determined at the indicated times after cell plating. For experiments carried out in CPA-treated cells, the reported CPA 4-hydroxylase activity and cell survival measurements were determined at the indicated times after the initiation of drug exposure, which generally occurred 24 hours after cell plating. For all CPA treatment schedules, cell survival (or cell growth) was measured by staining the cells remaining on the plate with crystal violet at the indicated times after washing each well twice with cold PBS, as described previously.¹³ The stain was eluted from the cells and the absorbance read at 595 nm.⁵

Western blotting

Cell extract (20 μ g) prepared from CPA-treated 9L/P450 cells was analyzed on nitrocellulose Western blots of 8% SDS-polyacrylamide gels probed with monoclonal anti-P450 2B6 (1:5000 dilution) (Gentest Corp. Cat. # A326, Woburn, MA) or antipeptide antibody to P450 reductase (1:5000 dilution) (a kind gift of Dr R Edwards, Royal Postgraduate Medical School, London, UK) as described.¹³ The blots were washed with $1 \times$ PBS containing 0.05% Tween 20 and incubated for 1 hour with either goat anti-mouse or goat anti-rabbit HRP-linked secondary antibody (1:10:000 dilution; Pierce, Rockford, IL; cat #31430 and #31460, respectively). The blots were sequentially washed with $1 \times$ PBS containing 0.3% Tween 20 followed by $1 \times$ PBS containing 0.05% Tween.²⁰ Blots were then developed for 1 min in ECL Western blotting detection reagent (Amersham Pharmacia Biotech; Cat# RPN2106) and exposed to Kodak X-OMAT blue film XB-1. Western blot films were scanned with Scan Wizard v.5.02 software and saved as TIFF files. Densitometric values were obtained using Image Quant Mac v1,2 software.

7-Ethoxycoumarin O-deethylase and P450 reductase enzyme assays

9L/P450 cells were plated in 100 mm dishes at 5×10^5 cells per well. Cells were treated with CPA as detailed in each experiment. 9L/P450 cell extracts were collected in $5 \times$ KPi buffer (0.5 M K₂HPO₄, 0.5 mM EDTA, pH 7.4) and lysed by sonication to generate whole-cell extracts.

Whole-cell extract (200 μ g) was incubated with the P450 2B6 substrate 7-ethoxycoumarin (0.7 mM) and NADPH (1.4 mM). Samples were extracted twice with chloroform then back-extracted with 30 mM sodium borate buffer, pH 9.2. Standard curves were generated using 0–400 nM 7-hydroxycoumarin. Fluorescence (excitation at 370 nm and emission at 450 nm) was quantitated using a Shimadzu spectrofluorimeter (RF-1501) and the Shimadzu PC-1501 software package. P450 reductase was assayed using 50 μ g of extract protein diluted into 1 ml of cytochrome *c* buffer (0.526 mg/ml cytochrome *c* in 0.3 M KPi, pH 7.7). Assays were initiated by addition of 25 μ l of 4 mg/ml NADPH. The rate of cytochrome *c* reduction was monitored at 550 nm over a 3 minutes period on a Lamda 35 UV/Visible spectrophotometer (Perkin-Elmer Instruments, Norwalk CT).

Cellular CPA 4-hydroxylase activity assay

9L/P450 cells were plated in 12-well dishes at 1×10^5 cells per well in 2 ml of media unless indicated otherwise. Cells treated with CPA as specified in each experiment were monitored for their ability to release 4-OH-CPA into the culture media. Cells given a single 8 hour CPA treatment were incubated with CPA for 8 hour, followed by incubation in drug-free media for the balance of the experiment. Cellular CPA 4-hydroxylase activity was assayed at the times indicated in each experiment, as follows. Cells were incubated for 4 hours in fresh media containing 1 mM CPA and 5 mM semicarbazide to trap and stabilize the 4-hydroxy metabolite. A 0.5 ml aliquot of the media was then removed from each well and snap-frozen in liquid nitrogen stored at -80°C until used for derivatization with fluorescent reagent (3-aminophenol and hydroxylamine-HCl in 1 M HCl) followed by HPLC analysis.²¹ For the experiment shown in Figure 4a and e, cells were incubated with CPA (0.125 or 1 mM, as specified) in the absence of semicarbazide. The culture medium removed at the times indicated was adjusted to 5 mM semicarbazide by addition of 5 μ l of 0.5 M semicarbazide stock and then frozen at -80°C until used for derivatization and HPLC analysis, as described above. Fluorescent values were converted to nmol 4-hydroxy metabolite/ml of original culture media based on standard curves generated using 4-hydroperoxy-CPA. Background 4-OH-CPA values were typically <0.1 – 0.15 nmol/ml. Cells remaining on the plate were washed with $1 \times$ PBS and stained with crystal violet (A_{595}). Normalized cellular CPA 4-hydroxylase activity values (nmol 4-OH-CPA/ml/ A_{595}) were then calculated. Individual group comparisons were analyzed for statistical significance using the two-tailed Student's *t*-test and GraphPad Prism software ($P < .05$).

Results

Cellular P450 activity declines following CPA treatment

We first examined the effect of CPA treatment, and the associated intracellular metabolism of CPA and CPA-induced cell death, on the ability of 9L/P450 cells to

maintain CPA 4-hydroxylase activity. These experiments were carried out in 9L/P450 cells treated with CPA continuously and at 1 mM CPA, a concentration that approximates the K_m for CPA exhibited by P450 2B6, the CPA-activating P450 enzyme expressed in these cells.^{22,23} 9L/P450 cells showed a dramatic decline in CPA 4-hydroxylase activity as a function of time of CPA treatment. After 72 hours CPA exposure, the rate of 4-OH-CPA production, assayed by the release of metabolite into the culture media, decreased to $\sim 7\%$ of the initial activity of untreated cells (Fig 1a). This decrease preceded the decrease in tumor cell survival associated with CPA-induced apoptosis, measured as the cells remaining on the plate following drug treatment (Fig 1b). Thus, 48 hours after initiation of CPA treatment, the CPA 4-hydroxylase activity of the culture was reduced to $\sim 26\%$ of its initial activity, even though 90% of the initial cell number remained. Overall, a 70% decrease in cellular CPA 4-hydroxylase specific activity (enzyme activity normalized on a per cell protein basis) was observed after 48–72 hours CPA treatment (Fig 1c).

To determine the cause of this decrease in cellular CPA 4-hydroxylase activity, extracts prepared from continuous CPA-treated 9L/P450 cells were assayed for P450 2B6 activity using the P450 substrate 7-ethoxycoumarin. P450 reductase activity was also assayed by monitoring the rate of NADPH-dependent cytochrome *c* reduction. P450 2B6 specific activity measured in the cell extracts decreased by 56–68% after treatment with CPA for 24 and 48 hour (Fig 2a), while P450 reductase specific activity decreased by 40–43% (Fig 2b). Western blot analysis revealed a substantial decrease in P450 2B6 protein content that was both time- and CPA concentration-dependent (Fig 2c). Densitometric analysis indicated this latter decrease was sufficient to account for the decrease in P450 metabolic activity (data not shown).

P450 activity is maintained in bolus CPA-treated cells

We next investigated the effect of a bolus, 8 hour CPA treatment on the viability of 9L/P450 cells and on their ability to maintain CPA 4-hydroxylase activity over time. Cells were incubated with CPA (1 mM) for 8 hours followed by incubation in drug-free media to give a total time of 24–72 hours. The 8 hour CPA treatment did not initially lead to a decrease in cell number, nor did it decrease the cell's capacity for CPA 4-hydroxylation (Fig 3a–c), in contrast to the responses seen in continuous CPA-treated cells (c.f., Fig 1). Correspondingly, there was no major decrease in the specific activity of P450 2B6 or P450 reductase, assayed in cell extracts, up to 48 hours after drug treatment (Fig 3d and e). Cellular P450 2B6 protein levels were also stable over the course of at least 72 hours, as shown by Western blotting (Fig 3f). The 8 hour bolus CPA treatment regimen was ultimately cytotoxic, however, as shown by monitoring tumor cell survival at later time points. A single 8 hour CPA treatment substantially killed the 9L/P450 cells by 10 days, while in cells given a second 8 hour CPA treatment on day 3, the time course of cell death was accelerated (Fig 3g).

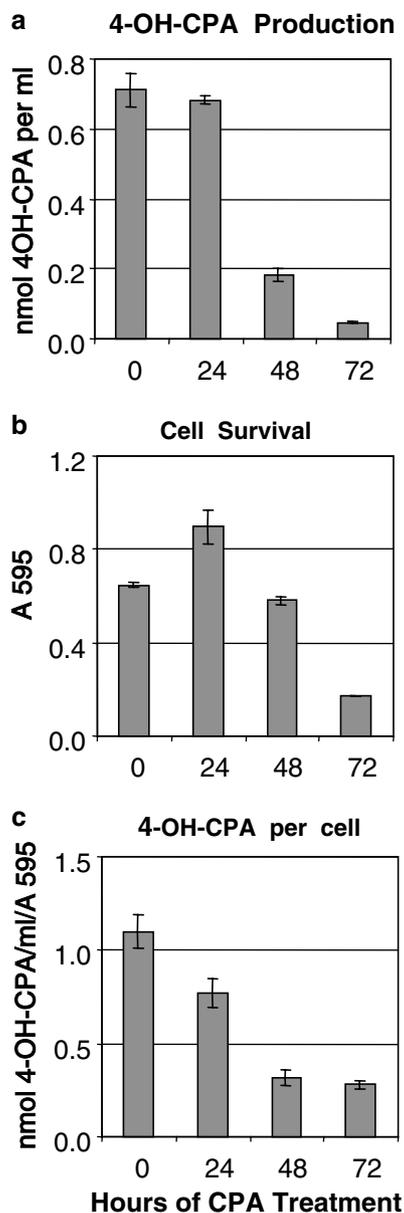


Figure 1 CPA 4-hydroxylase activity of 9L/P450 cells decreases following continuous exposure to CPA. 9L/P450 tumor cells were treated with 1 mM CPA for a total of 72 hours. Cells were monitored for CPA 4-hydroxylase activity at each 24 hours interval (a) by incubation for 4 hours with fresh media containing 1 mM CPA + 5 mM semicarbazide, followed by HPLC analysis of the semicarbazide-trapped 4-OH-CPA released into the culture media (see Materials and methods). Cell survival was assayed by crystal violet staining of the cells remaining on the plates at each time point (A_{595}) (b). Normalized cellular CPA 4-hydroxylase activity (nmol 4-OH-CPA/ml/ A_{595}) was then calculated from the ratio of these values (c). Data shown are from a representative experiment carried out in duplicate (mean values \pm half the range).

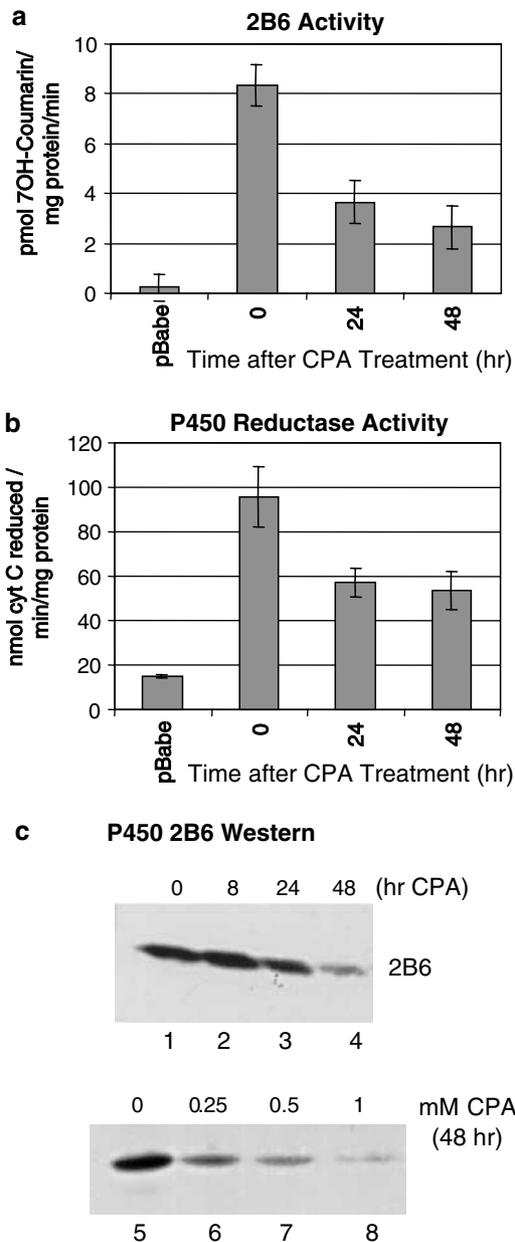


Figure 2 Decrease in P450 2B6 and P450 reductase in 9L/P450 cells treated with CPA by continuous exposure. 9L/P450 cells were treated with 1 mM CPA for a total of 48 hours. Cell extracts prepared 0, 24 and 48 hours after beginning drug treatment were assayed for P450 2B6 activity using the P450 substrate 7-ethoxycoumarin (a). P450 reductase activity was determined by assaying cytochrome c reduction in the same samples (mean \pm SD for $n=3$ independent experiments) (b). The amount of P450 2B6 protein remaining in the cells at each of the indicated time points after treatment with 1 mM CPA (lanes 1–4) was determined by Western blot analysis (c). Lanes 5–8 of panel c show P450 2B6 protein levels in a separate experiment where the 48 hour CPA treatment concentration was varied as shown. 9L cells infected with empty pBabe retroviral vector were assayed in parallel to determine background activities; these cells are termed 'pBabe' (panels a and b).

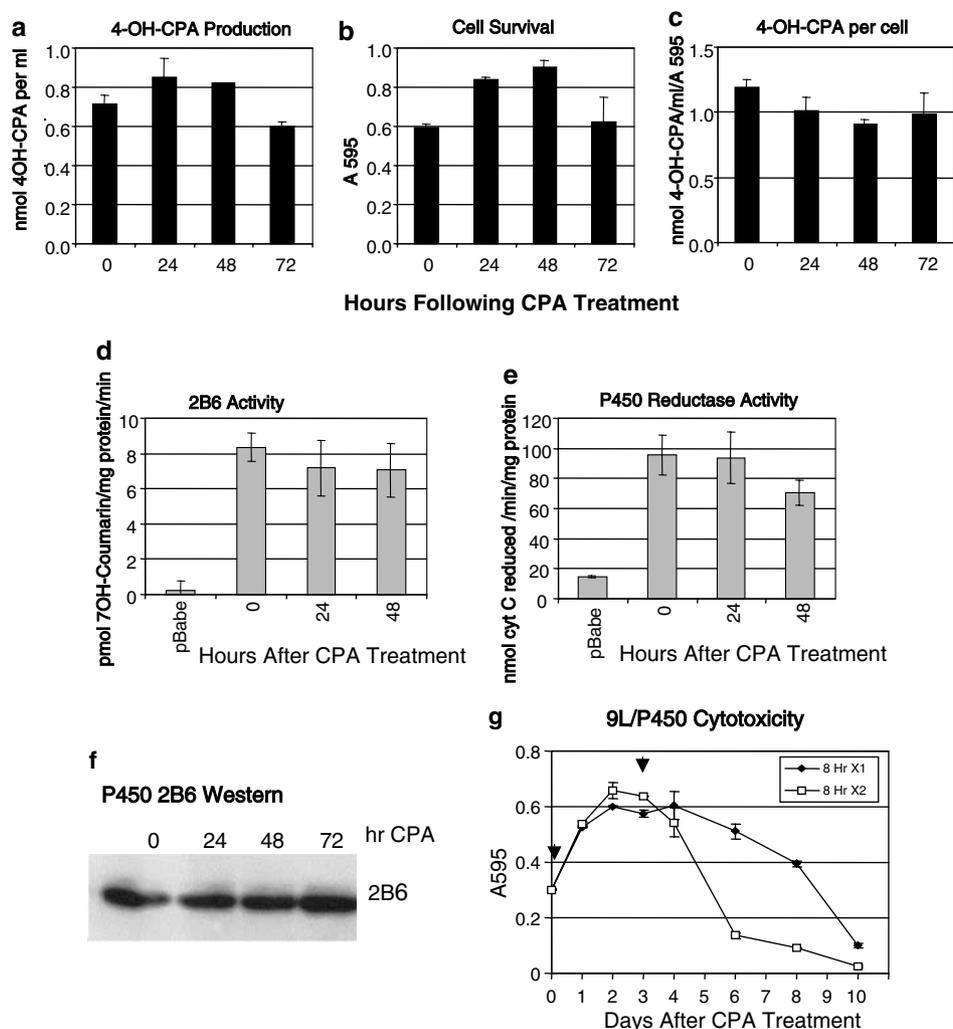


Figure 3 9L/P450 cells treated with CPA for 8 hours retain CPA 4-hydroxylase, P450 2B6 and P450 reductase activities, but ultimately succumb to CPA toxicity. 9L/P450 cells were treated with 1 mM CPA for 8 hours, then incubated in drug-free media for the duration of the experiment. Cellular CPA 4-hydroxylase activity was assayed at each of the indicated time points following initiation of drug treatment by analysis of metabolite released into the culture media as described in Materials and methods (a). Cell survival was measured in the same samples at each time point by crystal violet staining (b). Normalized cellular CPA 4-hydroxylase activity was then calculated (c). Shown is a representative experiment carried out in duplicate (mean values \pm half the range). In a separate series of experiments, cell extracts were prepared 0, 24 and 48 hours after beginning the 8 hours CPA treatment and assayed for P450-dependent 7-ethoxycoumarin *O*-deethylase activity (d) and for P450 reductase activity by cytochrome *c* reduction (e) (mean values \pm SD for $n=3$ independent experiments). Western blot analysis of P450 2B6 protein in cell extracts prepared at each of the indicated times after beginning the 8 hour CPA treatment (f). 9L/P450 cells were exposed either to a single 8 hour CPA treatment or, additionally, to a second 8 hour CPA treatment beginning on day 3 (vertical arrows). Cell survival over 10 days was monitored by crystal violet staining of duplicate plates on each of the days indicated (mean values \pm half the range) (g).

Cellular P450 activity declines following low dose, continuous CPA treatment

We next investigated the impact of CPA treatment schedule – bolus (8 hours) treatment *versus* continuous (72 hours) drug exposure – on the growth rate and on the CPA 4-hydroxylase activity of 9L/P450 cells. The concentration of CPA applied to the continuous drug-treated cells was reduced eight-fold in these experiments, to 0.125 mM, to compensate for the substantially longer period of prodrug exposure. At this lower concentration, CPA was metabolized by 9L/P450 cells at ~four-fold

lower rate than at 1 mM (Fig 4a), as could be predicted from the millimolar K_m (CPA) exhibited by CYP2B6.^{22,23} The continuous 0.125 mM CPA-treated 9L/P450 cells exhibited a somewhat higher initial growth rate than the bolus (8 hours \times 1 mM) CPA-treated cells (Fig 4b; also see Fig 4d) and showed higher intrinsic residual capacity for CPA 4-hydroxylation at 24 hours (assayed at 1 mM CPA and calculated on a per cell basis; Fig 4c). Both findings are consistent with the initial exposure of these cells to a lower concentration of active drug metabolite (Fig 4a). By 72 hours, however, the residual CPA 4-hydroxylase

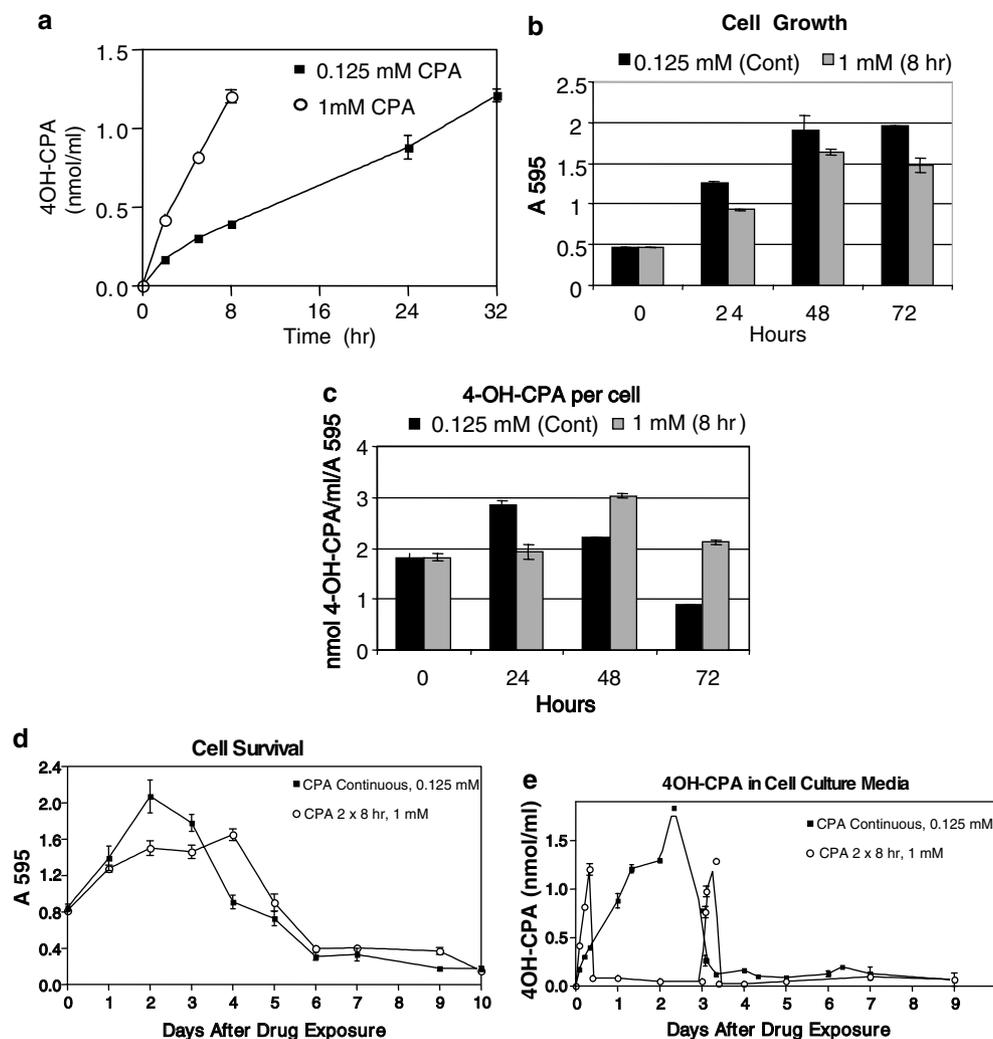


Figure 4 9L/P450 cells treated with 0.125 mM CPA continuously or with 1 mM CPA for an 8 hour period exhibit similar survival rates but different CPA 4-hydroxylase activities. 9L/P450 cells were treated with CPA at 0.125 mM (closed symbols) or with 1 mM CPA (open symbols) and the accumulation of 4-OH-CPA in the culture medium was assayed over the indicated time intervals as described under Materials and methods (mean \pm SD, $n=3$) (a). 9L/P450 cells were treated with 0.125 mM CPA continuously for 3 days or with 1 mM CPA for 8 hours. Cell cultures were assayed at each 24 hour interval for cell growth, by crystal violet staining (b), and for normalized CPA 4-hydroxylase capacity, determined over a 4 hour interval and assayed at 1 mM CPA as described in Figure 1 (c). Data shown are mean \pm half the range values based on duplicate samples for a representative experiment. In a separate experiment, 9L/P450 cells were treated with 0.125 mM CPA continuously (closed symbols) or with 1 mM CPA for two 8 hour exposures, one on day 0 and the second on day 3 (open symbols). Cell survival over 10 days was monitored by staining individual plates of cells with crystal violet on the days indicated. Microscopic examination revealed crystal violet staining of residual cell fragments in the 9- and 10-day samples, with few intact cells present (d). In panel e, 4-OH-CPA was measured in the culture medium of the samples shown in panel D. For panels d and e, data shown are mean \pm SD values for $n=3$ replicates. Cells treated with 0.125 mM CPA were grown in media containing drug for the 10-day duration of the experiment, with the culture medium replaced with medium containing fresh CPA on days 3 and 6. Cells treated with 8 hours \times 1 mM CPA on day 0 and on day 3 were cultured in the absence of CPA at all other times. The 2 \times 8 hours CPA-treated cells were given fresh medium without CPA at the end of each CPA treatment period on days 0 and 3 and then cultured in the absence of CPA.

specific activity of the continuous CPA-treated cells was decreased substantially, to $\sim 70\%$ of its peak level at 24 hours (Fig 4c). This contrasts with the maintenance of cellular CPA 4-hydroxylation by the 8 hour bolus CPA-treated cells (Fig 4c). Long-term survival was similar for the two drug schedules, as evident from the 9L/P450 growth curves after treatment with continuous CPA, 0.125 mM *versus* bolus CPA, applied as two 8

hours \times 1 mM treatments, one on day 0 and the second on day 3 (Fig 4d). Direct comparison of cellular CPA 4-hydroxylase activity under these two conditions of equivalent tumoricidal activity revealed a marked decline in CPA 4-hydroxylase activity on day 3 in continuous CPA-treated 9L/P450 cells but not in bolus high-dose CPA-treated cells (Fig 4e; c.f., low 4-OH-CPA level in the continuous CPA cultures beginning on day 3 *versus*

robust increase in 4-OH-CPA with the second 8 hours bolus CPA treatment on day 3). Overall, however, the net exposure to 4-OH-CPA (AUC) was higher in the continuous CPA-treated cells, where the metabolite was allowed to accumulate over the 3-day interval prior to the first culture medium change. Taken together, these studies demonstrate that the prodrug activation capacity of 9L/P450 cells declines dramatically after continuous exposure to either low (0.125 mM) or high (1 mM) CPA concentrations, but can be maintained for at least 3 days when using an 8 hour bolus CPA (1 mM) treatment protocol.

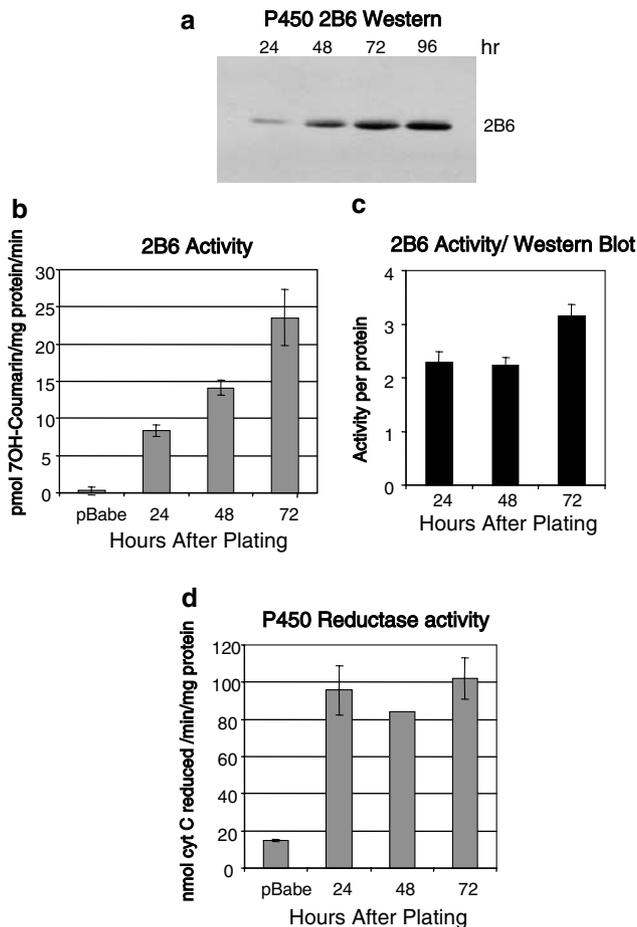


Figure 5 9L/P450 cells exhibit an increase in P450 2B6 protein and activity but not P450 reductase activity over time in culture in the absence of CPA treatment. 9L/P450 cells grown in the absence of CPA treatment were collected and cell extracts prepared at the times indicated (hours after initial cell plating at 1×10^5 cells/well/12-well plate). P450 2B6 protein was visualized by Western blotting (a). Cell extracts were assayed for P450-dependent 7-ethoxycoumarin *O*-deethylase activity (b). P450 2B6 activity values shown in panel b were divided by the P450 2B6 protein levels determined by densitometry of the Western blot to give relative P450 specific activity values, expressed in arbitrary units (c). Cell extracts were also assayed for P450 reductase activity by cytochrome *c* reduction (d). Data shown in panels b and c are mean values \pm SD for $n=3$ replicates.

P450 2B6 activity increases as 9L/P450 cells become more confluent

During the course of these studies, we made the unexpected observation that the P450 2B6 protein content of 9L/P450 cells increases substantially over the course of 2–4 days in culture in the absence of CPA treatment (Fig 5a). Analysis of cell extracts from untreated 9L/P450 cells

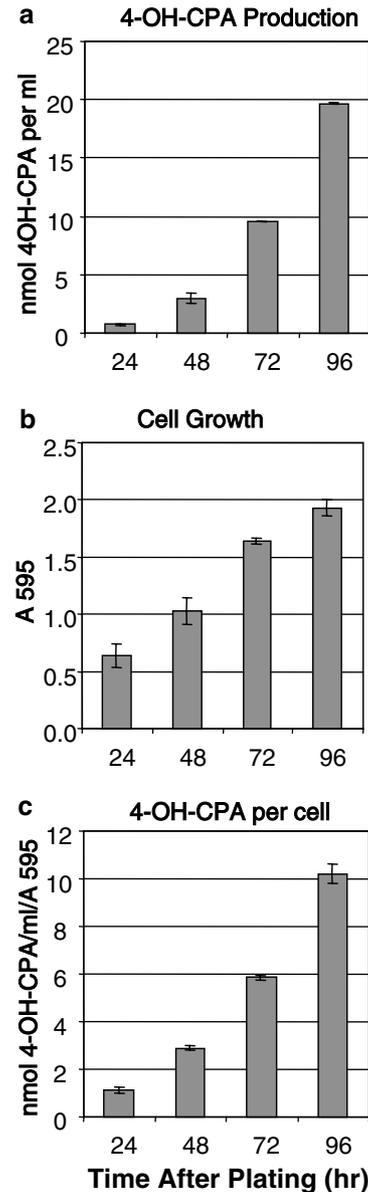


Figure 6 9L/P450 cells exhibit an increase in CPA 4-hydroxylase activity when cultured in the absence of CPA. 9L/P450 cells were plated at 1×10^5 cells/well/12-well plate and then cultured in the absence of CPA for times up to 96 hours, as in Figure 5. Cellular CPA 4-hydroxylase activity was monitored as described in Figure 1 (a). Relative cell protein levels were determined by crystal violet staining (b) and normalized CPA 4-hydroxylase activity was calculated (nmol 4-OH-CPA/ml/A₅₉₅) (c). Data shown are based on a representative experiment with duplicate tissue culture wells analyzed in parallel (mean values \pm half the range).

using the P450 2B6 substrate 7-ethoxycoumarin revealed that cellular P450 2B6 specific activity also increased, by ~ 2.5 to 3-fold, over the course of 72 hours following cell plating (Fig 5b). This increase in P450 activity could largely be explained by the increase in P450 2B6 protein content (Fig 5c). By contrast, cellular P450 reductase activity was nearly unchanged over the same time period (Fig 5d). This indicates that the increase in P450 expression is a specific response, and is not a general effect of cell plating at moderately low densities. Analysis of the CPA 4-hydroxylase activity of the 9L/P450 cells revealed a large increase in prodrug activation (Fig 6a), which reached 10-fold when calculated on the basis of total cell mass (crystal violet staining intensity) after 96 hours in culture as compared to the 24 hours time point (Fig 6c). These findings were confirmed in separate experiments using a 9L/P450 cell line prepared with a different retroviral vector, in which the cDNAs encoding P450 2B6 and P450 reductase were linked by an internal ribosome entry sequence, allowing for the transcription of both genes from the same promoter (data not shown). The time-dependent increase in P450 2B6 activity and protein, but not P450 reductase activity is thus likely to result from effects on the translational efficiency or protein stability of P450 2B6, rather than from changes in the rate of transcription of the P450 cDNA or in the stability of its mRNA.

Initial cell density and duration of culture both influence cellular 4-OH-CPA production

We next investigated whether the increase in P450 protein and P450 activity content over time in 9L/P450 cells was a cell density-dependent response. To test this possibility, cells were plated at three different densities (1×10^5 , 1.5×10^5 and 2×10^5 cells/well in 12-well tissue culture plates) and cellular CPA 4-hydroxylase activity (metabolite released into the culture media) was assayed 24 or 72 hours after plating. As shown in Fig 7, the CPA 4-hydroxylase activity of cultures assayed 24 hours after cell plating was not proportional to the initial number of cells seeded, insofar as a ~ 20 fold increase in 4-OH-CPA production was obtained with only a two-fold increase in initial cell number (Fig 7a). By contrast, 72 hours after cell plating the CPA 4-hydroxylase activity of each sample was nearly proportional to the number of cells initially plated (Fig 7b). Overall, a \sim six-fold increase in the culture's CPA 4-hydroxylase activity occurred from 24 to 72 hours when 2×10^5 cells were seeded, whereas a 60-fold increase was obtained over the same time period when 1×10^5 cells/well were seeded (Fig 7c). The 4OH-CPA produced at the lowest cell plating density (1×10^5) is comparable to that seen in cells given high-dose continuous CPA treatment (Fig 1a). Under the conditions of high P450 expression achieved in these cells, 4-OH-CPA concentrations as high as 40–50 μ M could be generated in the culture media (Fig 7b) as compared to 0.7–5 μ M in the experiments shown in 1a, 3a and 4a. The increase in cellular P450 2B6 activity with increasing cell density raises the possibility that the high cell density conditions

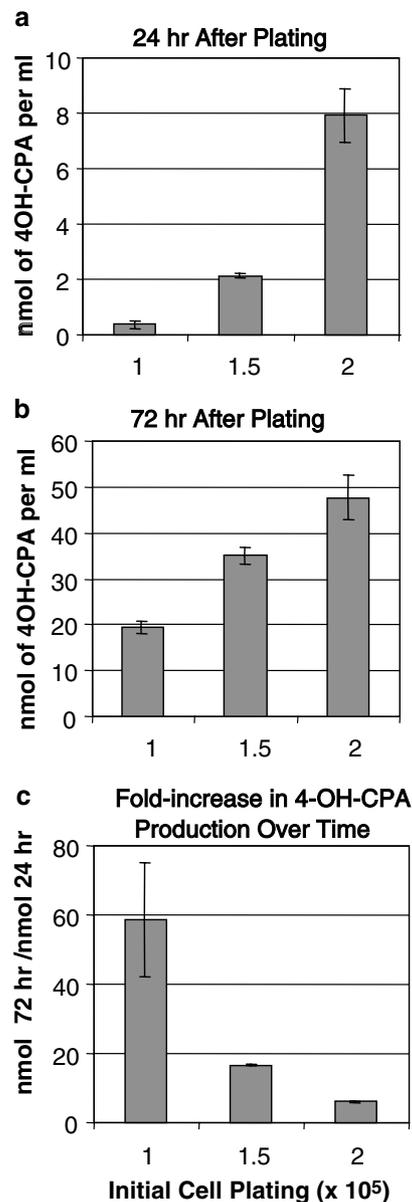


Figure 7 Cellular 9L/P450 CPA 4-hydroxylase is cell density dependent. 9L/P450 cells were plated at 1, 1.5 or 2×10^5 cells/well/12-well plate, as indicated. Cellular CPA 4-hydroxylase activity was assayed over a 4 hours time period as described in Figure 1, either 24 hours (a) or 72 hours (b) after cell plating. The fold-increase in CPA 4-hydroxylase activity from 24 to 72 hours after cell plating was calculated for each of the indicated cell plating densities by dividing the values shown in panel b by the values in panel a (c). Data shown are mean values \pm half the range for duplicate samples.

that are intrinsic to solid tumors may be conducive to high-level expression of this P450 protein.

9L/P450 cell density influences the cytotoxic effects of CPA

To determine the impact that cell density has on the cytotoxic effects of CPA treatment, 9L/P450 cells were

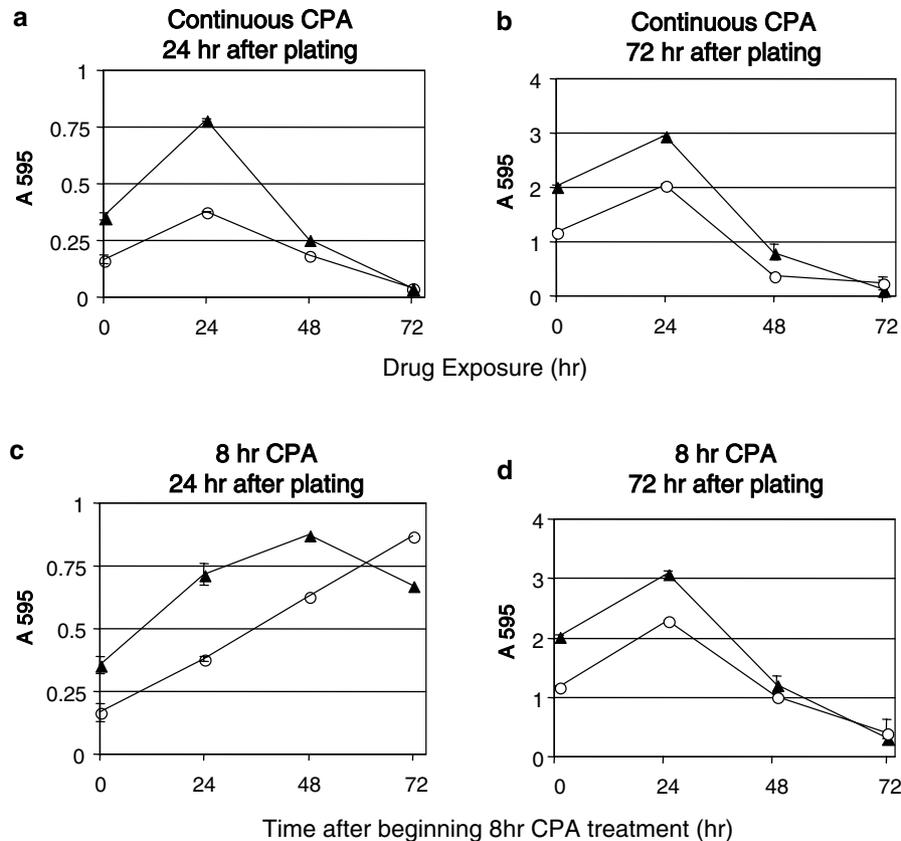


Figure 8 Enhancement of the cytotoxic effects of an 8 hour treatment with 1 mM CPA in 9L/P450 cells grown for 72 hours prior to drug treatment. 9L/P450 cells were plated at two cell densities, either 1×10^5 (filled triangles) or 0.5×10^5 (open circles) cells per well/12 well plate. Cells were treated with 1 mM CPA either continuously (**a** and **b**) or for 8 hours (**c** and **d**) beginning either 24 hours (panels **a**, **c**) or 72 hours after the cells were plated (panels **b**, **d**). The cytotoxic effects of CPA treatment were determined by crystal violet staining of the cells remaining on the plate. Data shown are mean values \pm half the range for duplicate samples.

plated at two different densities (0.5×10^5 and 1×10^5 cells/well in 12-well tissue culture dishes) and treated with 1 mM CPA beginning either 24 or 72 hours after cell plating. Effective killing of 9L/P450 cells was induced by continuous CPA treatment, independent of the initial cell density or the time the cells were in culture prior to drug treatment (Fig 8a and b). In contrast, the pattern of cell survival in cells given an 8 hour CPA treatment was dependent on the initial cell density and the time the cells were in culture prior to beginning drug treatment. When 9L/P450 cells were seeded at the lower density and then treated with CPA 24 hours later, the 8 hours drug treatment was not sufficient to kill the tumor cells. Rather, the cells continued to grow over the course of the experiment (Fig 8c, open symbols). When twice as many 9L/P450 cells were seeded, the 8 hour CPA treatment rendered the cells cytostatic, with the total cell number remaining at ~ 2 times the number of cells initially seeded from 24 to 72 hours (Fig 8c, closed symbols). By contrast, when the tumor cells were cultured for 72 hours before beginning the 8 hour CPA treatment, the substantially higher cellular P450 level and P450-dependent prodrug activity achieved in these higher density cultures (c.f.,

Fig 7) led to efficient killing of almost all of the tumor cells, regardless of the initial cell density (Fig 8d). These findings highlight the importance of both the level of P450 expression and the schedule of CPA treatment for achieving an effective prodrug-induced cytotoxic response.

Discussion

The present study investigated the effects of three CPA treatment schedules, designed to model three distinct clinical CPA treatment protocols, on the activation of CPA catalyzed by 9L tumor cells that express the CPA-activating enzyme P450 2B6. Continuous treatment of 9L/P450 tumor cells with CPA at either a high (1 mM) (Fig 1) or a low drug concentration (0.125 mM) (Fig 4) led to a time- and dose-dependent decrease in the tumor cell's capacity for CPA activation, assayed by the release of 4-OH-CPA into the culture media. This decrease was associated with the loss of P450 2B6 protein and may be mechanistically related to the inactivation of the

orthologous rat P450 2B1 by the CPA metabolite acrolein,²⁴ which may facilitate P450 protein degradation. By contrast, when P450-expressing tumor cells were subject to bolus CPA treatment over an 8 hour period, followed by a drug-free interval prior to further CPA treatment on day 3, the decrease in P450 protein and cellular CPA 4-hydroxylase activity was not observed and the tumor cell's capacity to activate CPA was maintained for at least 72 hours. These findings suggest that continuous infusion CPA treatment protocols that use either high-dose CPA¹⁴ or standard dose CPA¹⁵ may not be optimal in conjunction with P450 GDEPT, insofar as the tumor's capacity for ongoing CPA activation, required to ensure a strong bystander cytotoxic response, may be short-lived. By contrast, the prolonged maintenance of P450-dependent CPA 4-hydroxylation by tumor cells given an 8 hour CPA treatment, designed to model bolus CPA administration *in vivo*, suggests that continued production of bystander cytotoxic metabolites may be achieved, and that re-infection with the P450 gene delivery vector might not be necessary when using this schedule. Further increases in the capacity of bolus CPA-treated 9L/P450 cells for 4-OH-CPA production, and an associated increase in bystander cytotoxic response, may be obtained by delaying the death of P450-transduced tumor cells treated with CPA, as we have recently demonstrated in experiments using retrovirus to deliver the antiapoptotic factor p35.²⁵

Area-under-curve (AUC) of concentration \times time pharmacokinetic studies have established that treatment with a high dose of alkylating agent administered over a short period of time, as in the case of bolus CPA treatment, yields the same overall amount of active metabolite, and hence equivalent cytotoxic activity, as when the alkylating agent is administered at a correspondingly lower concentration for a longer period of time.²⁶ In the present *in vitro* studies, a decrease in cellular capacity for CPA 4-hydroxylation was seen after 72 hours of continuous low-dose CPA treatment, but was not seen when the cells were exposed to bolus high-dose CPA (Fig 4). This difference may, in part, reflect the higher overall drug exposure of the continuous low-dose CPA-treated cells during the initial 3 day period (Fig 4e), as well as the drug-free recovery period available to the bolus high-dose CPA-treated cells but not the continuous low-dose CPA-treated cells. Net cytotoxicity evaluated after 6 days was, however, similar for both treatments, despite the higher cumulative exposure of the continuous CPA-treated cells to active drug metabolite. Continuous low-dose CPA treatment can lead to enhanced tumor regression *in vivo*, by inhibition of tumor angiogenesis.^{27,28} Bolus CPA administered intermittently on a repeated 6 day schedule can also induce a strong antiangiogenic response,²⁹ one that confers an additional advantage when applied in the context of P450 gene therapy.⁶

P450-expressing 9L tumor cells exposed to CPA continuously maintained full 4-OH-CPA production activity only during the initial 24 hours period of drug exposure, whereas in cells where CPA treatment was limited to an initial 8 hours exposure, the capacity for 4-

OH-CPA production was maintained for at least 72 hours. It is important to note, however, that while the 8 hour CPA-treated tumor cells maintained their capacity for CPA 4-hydroxylation, multiple 8 hour CPA treatments would be required to produce the same net 4-OH-CPA production over time as the continuous CPA-treated cells (Fig 4e). Net production of 4-OH-CPA can be enhanced, however, in 8 hour CPA-treated tumor cells by increasing cellular P450 specific activity. This may be achieved by coexpression of P450 reductase¹⁹ or, as shown in the present study, by culturing the tumor cells at higher cell densities. In this latter instance, the increased level of active metabolite was able to induce the same overall cytotoxicity as could be achieved by continuous CPA treatment of either low-density or high-density tumor cell cultures. Preclinical solid tumor model studies have shown that an intermittent bolus CPA treatment schedule can lend additional therapeutic advantages to P450 GDEPT, in particular when the repeated drug treatment is administered on an antiangiogenic, 6 day repeated schedule.⁶

An important goal of P450 GDEPT is to generate a high intratumoral concentration of 4-OH-CPA without significantly increasing the circulating level of active drug and its associated host toxicity. In patients given high-dose CPA as a continuous infusion, in the absence of P450 GDEPT, plasma levels of CPA during the infusion are 500–600 μM , while those of 4-OH-CPA are 9–10 μM , with an AUC for the active metabolite of $\sim 90 \mu\text{M} \times \text{hour}$.^{1,30} Patients given the more common bolus CPA treatment typically have plasma 4-OH-CPA AUC values of $\sim 30 \mu\text{M} \times \text{hour}$.³¹ These drug treatment regimens are associated with host toxicity owing to the high circulating concentrations of active CPA metabolite. The very high level of 4-OH-CPA that accumulates over a 4 hour period in culture media of 9L/P450 cells grown at higher cell density (up to $\sim 45 \mu\text{M}$; Fig 7b) suggests that a high intratumoral P450 level can provide for the desired selective increase in intratumoral concentrations of active CPA metabolites. This preferential increase in 4-OH-CPA levels within tumor cells may be enhanced even further by localized delivery of the prodrug, as shown in a recent study combining P450 GDEPT with intraneoplastic CPA delivery using a biodegradable polymer.⁸

9L/P450 cells cultured for 4 days prior to the initiation of CPA treatment exhibited a striking ~ 10 -fold increase in cellular CPA 4-hydroxylase specific activity. Treatment of these P450-expressing tumor cells with 1 mM CPA for a single 8 hour period yielded a very substantial cell culture AUC (4-OH-CPA) of ~ 50 – $100 \mu\text{M} \times \text{hour}$ (Fig 6). This cell culture AUC is comparable to the plasma AUC (4-OH-CPA) of $\sim 90 \mu\text{M}$ measured in high-dose continuous CPA-treated patients, for which autologous stem cell support is required to assure patient survival,³⁰ suggesting that P450-based gene therapies have the potential to generate a very high level of active drug within the tumor. Moreover, preclinical tumor model studies have shown that the increase in the tumor's capacity for CPA 4-hydroxylation that is obtained with

P450 GDEPT is associated with little or no added host toxicity.² The strikingly high AUC (4-OH-CPA) value of 50–100 $\mu\text{M} \times \text{hour}$ observed in the P450 GDEPT cell culture model was achieved in experiments using a single 8 hour CPA treatment schedule, suggesting that such a high, therapeutic intratumoral 4-OH-CPA concentration could be generated under conditions where the systemic AUC (4-OH-CPA) would not need to exceed $\sim 30 \mu\text{M} \times \text{hour}$, as is seen in bolus CPA-treated patients.³² While the ability of tumor cells, *in vivo*, to generate such a high concentration of active CPA metabolite would be counteracted by the efflux of active metabolite from the tumor, it seems reasonable to propose that a substantial cytotoxic response may nevertheless be anticipated for tumor cells that express high P450 levels, with perhaps little or no significant increase in systemic toxicity.

Our observation that P450 2B6 protein levels increase in a cell density-dependent manner is consistent with the observed increase in P450 2B1 protein levels seen in primary rat hepatocytes grown as multicellular spheroids compared to hepatocytes grown in monolayers.³³ The mechanism for the increased P450 expression in spheroids is unknown, however, it is of interest that the hepatocytes in the core of the spheroid exhibit higher P450 activity than those at the surface.³⁴ Further support for the cell density-dependent expression of P450 2B6 reported here is provided by the observation that another human P450, P450 3A4, also undergoes cell density-dependent expression, in that case in human primary hepatocytes, where the constitutive and the inducible levels of P450 3A4 are both decreased at lower cell densities in a cell–cell contact-dependent process.³⁵ While it is difficult to extrapolate the present *in vitro* finding of a tumor cell density-dependent increase in P450 2B6 protein content and CPA 4-hydroxylase activity to P450 GDEPT applications *in vivo*, P450 protein levels may conceivably undergo a corresponding increase in tumor cells as a function of time following P450 gene delivery *in vivo* under the high cell density growth conditions that characterize solid tumors. Further investigation of the optimal time interval between P450 gene delivery and the initiation of prodrug treatment thus seems warranted, as does an investigation of other approaches to achieving increased P450 expression and enhanced prodrug activation with the tumor target.

Abbreviations

P450, cytochrome P450; CPA, cyclophosphamide; 4-OH-CPA, 4-hydroxy-cyclophosphamide; GDEPT, gene-directed enzyme prodrug therapy; 9L/P450 cells, 9L gliosarcoma cells that stably express human P450 2B6 and human P450 reductase; AUC, area under the curve.

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