Multimodal Cancer Treatment Mediated by a Replicating Oncolytic Virus That Delivers the Oxazaphosphorine/Rat Cytochrome P450 2B1 and Ganciclovir/Herpes Simplex Virus Thymidine Kinase Gene Therapies

Manish Aghi, Ting Chao Chou, Kristen Suling, Xandra O. Breakefield, and E. Antonio Chiocca

Abstract
Multimodal therapy is generally more effective than single-agent treatment for cancer. rRp450 is an engineered herpes simplex viral mutant that replicates in and kills tumor cells in a relatively selective fashion. It also expresses, in infected cells, the cyclphosphamide (CYP2B1)-sensitive rat cytochrome P450 2B1 (CYP2B1) and the ganciclovir (GCV)-sensitive herpes simplex virus thymidine kinase (HSV-TK) transgenes. Here, we show that the presence of GCV, suggesting that interactions between the CPA/CYP2B1 and GCV/HSV-TK gene therapies occurred at the level of DNA repair. In vivo, regression of 9L s.c. tumor volumes in athymic mice was achieved only by the multimodal treatment allowed by rRp450 viral oncolysis combined with CPA/CYP2B1 and GCV/HSV-TK gene therapies, whereas all other treatment combinations produced only tumor growth retardation.

Introduction
Recent advances in molecular virology have elicited renewed interest in the use of genetically altered viruses as targeted treatments for cancer (1–3). We have engineered a novel oncolytic virus, rRp450, based on herpes simplex virus type 1 (4). This mutant is characterized by a large deletion of UL39, the gene encoding for ICP6. This peptide provides RR activity, which is essential for viral replication and lysis of quiescent cells. rrP450 selectively replicates in and lyses cells (such as tumor cells) with elevated levels of the endogenous mammalian RR, which provides the deoxynucleotides that are needed for viral DNA synthesis (4, 5). An additional genetic modification was engineered into rrP450’s genome by inserting the CYP2B1 gene, encoding the enzyme responsible for activating the prodrug CPA into its anticancer metabolite, phosphoramidate mustard (6). A therapeutic strategy based on transfer of CYP2B1 by replication-defective viral vectors to endow tumor cells with oxazaphosphorine susceptibility has been shown to be relatively successful (7–9). Naturally, rrP450 also possesses its own endogenous HSV-TK gene, encoding the enzyme responsible for activating the prodrug GCV into its anticancer (and antiviral) metabolites. We have previously shown that the oncolytic virus, rrP450, augmented CPA chemotherapy both in culture and in vivo (4). Because rrP450 retains an intact HSV-TK gene, it is possible that rrP450-mediated oncolysis could also augment GCV chemotherapy (10). The implication is that the single agent, rrP450, might provide multimodal cancer therapy by combining viral oncolysis with the GCV/HSV-TK and CPA/CYP2B1 gene therapies. Here, we show that these two prodrug-activating gene therapies interact in a manner that suggests pharmacological synergism in cell lines infected by rrP450. Supportive evidence for this synergy is provided by the finding that GCV/HSV-TK gene therapy inhibits the repair of cellular DNA cross-links produced by CPA/CYP2B1 gene therapy. On the basis of these findings, we then show that rrP450 treatment of tumors in vitro and in vivo provides multimodal treatment through viral oncolysis and the two synergistic gene therapies.

Materials and Methods
Cell Lines and Viruses. Cells were grown in DMEM (Life Technologies, Inc., Gaithersburg, MD) supplemented with 10% FBS (Sigma Chemical Co., St. Louis, MO), 100 units/ml penicillin, and 100 mg/ml streptomycin (Sigma) in an atmosphere containing 5% carbon dioxide. Human U87MEGFR glioblastoma cells were a generous gift of Dr. J. Cavenee (University of California, San Diego, CA; Ref. 11). Rat 9L gliosarcoma cells have been described previously (4). The hrR3 HSV-1 viral mutant (a generous gift of S. Weller, University of Connecticut Medical School, Farmington, CT) has an insertion of the lacZ gene into the viral ICP6 locus (12). The rrP450 virus was derived from the hrR3 viral mutant by replacement of lacZ with the rat CYP2B1 gene, as described previously (4).

Cell Culture Studies. A stably transfected cell line (9L/TK-450) was used in some studies and was generated by polybrene-promoted infection of 9L/TK cells with a CYP2B1-expressing retrovirus that had been harvested from producer cells, transiently transfected with pBABE-Puro/CYP2B1. Infected cells were then cloned under selection in 5 μg/ml puromycin (Sigma). For viral infection of cultured cells, 3 × 10⁵ cells were plated in 6-cm² dishes and allowed to adhere for 6 h. Virus and/or CPA was added in 10% FBS-DMEM in a total volume of 200 μl. The plates were shaken every 15 min for an h, after which 4 ml of fresh medium (containing CPA for cells infected in the presence of CPA) were added. GCV was added at varying times after infection began. For temperature-shift experiments, plates were transferred to a separate 39.8°C incubator, and GCV was added to some plates.

Analysis of Interaction between Gene Therapies. The multiple drug effect analysis of Chou-Talalay (13) was used to quantify the interaction between the two gene therapies. The Chou-Talalay method determined the expected effect of a given combination if the agents are additive and quantifies synergy or antagonism by determining how much the experimental effect differs from the effect expected with additivity. The stepwise calculations performed using a computer program have been described previously (14).

Ethidium Bromide Fluorescence Assay. For the ethidium bromide fluorescence assay, 2 × 10⁵ cells were plated in 25-cm² flasks. The next day, different treatment groups were set up. Cells were exposed to 9 μg/ml GCV...
and/or 250 μg/ml CPA (or, as a control, medium alone) for 6 h, followed by a variable period of time (6, 12, or 24 h) without CPA (CPA-free period) to allow for repair of DNA cross-links. During the CPA-free period, GCV (9 μg/ml) was added. This dose of GCV was selected because higher doses produced extensive cell death, impeding recovery of genomic DNA, whereas lower doses were not as effective in producing the observed result. For some experiments, cells were preexposed to GCV for 3 h, before the 6-h treatment with CPA and GCV. At each time point, DNA was prepared from harvested cells. The ethidium bromide fluorescence assay was slightly modified (15). The rationale for this assay is that cross-linked DNA will denature less rapidly and renature more rapidly than normal DNA, thus allowing for more ethidium bromide retention. Cells were suspended in 80 μl of PBS, to which 400 μl of lysing solution (4 x NaCl, 50 mM KH$_2$PO$_4$, 10 mM EDTA, and 1% sarkosyl (pH 7.4)) plus 40 μl of heat-inactivated pancreatic RNase (2 mg/ml) were added. Lysis was carried out at 37°C for 16 h. Next, 50 μl of heparin (500 IU/ml) was added to the lysates and incubated for 20 min at 37°C. Then, each sample was divided. The DNA in one half was denatured by boiling 10 min and was then allowed to renature at 4°C. Cell lysates as well as standards containing 0, 5, 10, 20, and 30 μg of salmon sperm DNA dissolved in 570 μl of water were then added to 3 ml of a solution containing 10 μg/ml ethidium bromide, 20 mM KH$_2$PO$_4$, and 0.4 mM EDTA (pH 12). Tubes were stored in the absence of light until the relative amount of cross-linked DNA could be determined by measuring the fluorescence of all samples using an excitation wavelength of 525 nm and an emission wavelength of 580 nm in 4.5-ml polystyrene cuvettes having four clear sides (F-4500 Fluorescence Spectrophotometer; Hitachi Instruments, Danbury, CT). Salmon sperm standards were used to confirm the linear relationship between amount of DNA and fluorescence and the elimination of most fluorescence upon denaturation. For cell samples, the cross-link index of drug-treated cells, $C_i$, an indicator of the percentage of DNA that is cross-linked, was calculated as follows:

$$ C_i = \frac{(f_d - f_e)}{(1 - f_d)} \times 100\% $$

where $f_d$ is the fluorescence of denatured drug-treated cells/fluorescence of nondenatured drug-treated cells and $f_e$ is the fluorescence of denatured non-drug-treated cells/fluorescence of nondenatured non-drug-treated cells.

**In Vivo Experiments.** 9L cells (10$^5$) in 200 μl of DMEM (without FBS) were injected s.c. into the flanks of 6-week-old female nude mice (Ncr/Sed, nu/nu, 20 g; Massachusetts General Hospital breeding colony). After 14 days, when the tumors had reached an average volume of 73 mm$^3$ (range, 30 –141 mm$^3$), the mice were randomly divided into treatment groups with five mice per group. Intratumoral injection of virus (2.5 × 10$^8$ pfu) and/or 100 mg CPA/kg body weight in a total volume of 60 μl of lysing solution [4 M NaCl, 50 mM KH$_2$PO$_4$, 10 mM EDTA, and ethidium bromide, 20 mM KH$_2$PO$_4$, and 0.4 mM EDTA (pH 12)] was then allowed to renature at 37°C.

**Results**

**Combined Prodrug Treatment of 9L Cells Infected with the rRp450 Replication-conditional Virus.** We sought to determine whether rRp450 infection of tumor cells provided both CPA and GCV susceptibility to infected tumor cells and whether the combination of the two prodrug-activating gene therapies resulted in antagonistic or nonantagonistic (additive or supra-additive) interactions. Because addition of rRp450 to cells will result in relatively rapid oncolysis, we were interested in determining whether the combination of CPA and GCV treated cells/fluorescence of nondenatured non-drug-treated cells.

**Combined prodrug treatment of 9L cells infected with rRp450** was then performed using the same temperature shift and prodrug schedule (Fig. 1B). With this combination, the ED$_{50}$ for both prodrugs decreased. Chou-Talalay analysis revealed that combination indices of susceptibility to infected tumor cells and whether the combination of CPA and GCV treated cells/fluorescence of nondenatured non-drug-treated cells.

**Combined prodrug treatment of 9L cells infected with rRp450** was then performed using the same temperature shift and prodrug schedule (Fig. 1B). With this combination, the ED$_{50}$ for both prodrugs decreased. Chou-Talalay analysis revealed that combination indices of susceptibility to infected tumor cells and whether the combination of CPA and GCV treated cells/fluorescence of nondenatured non-drug-treated cells.

**Combined prodrug treatment of 9L cells infected with rRp450** was then performed using the same temperature shift and prodrug schedule (Fig. 1B). With this combination, the ED$_{50}$ for both prodrugs decreased. Chou-Talalay analysis revealed that combination indices of susceptibility to infected tumor cells and whether the combination of CPA and GCV treated cells/fluorescence of nondenatured non-drug-treated cells.

![Graph A](image1.png)

**Prodrug Concentration (units = 0.0625 μg/ml GCV; 7.5 μg/ml CPA)**

**A**. Time (hrs) at 37°C prior to temperature shift

**B**. Percent survival

**C**. Percent survival

**D**. Percent survival

**E**. Percent survival

**F**. Percent survival

**G**. Percent survival

**H**. Percent survival

**I**. Percent survival

**J**. Percent survival

**K**. Percent survival

**L**. Percent survival

**M**. Percent survival

**N**. Percent survival

**O**. Percent survival

**P**. Percent survival

**Q**. Percent survival

**R**. Percent survival

**S**. Percent survival

**T**. Percent survival

**U**. Percent survival

**V**. Percent survival

**W**. Percent survival

**X**. Percent survival

**Y**. Percent survival

**Z**. Percent survival

---

**Fig. 1.** A: **(A and B)** and **U87ΔEGFR** cells (C) infected with rRp450 at 37°C followed by temperature shift to 39.8°C for 5 days. A, percentage survival as a function of time at 37°C prior to temperature shift for 9L cells infected with rRp450 at MOIs of 0.5 (○) and 1 (△). Data points, means of triplicate evaluations; bars, SEs (<8.4% survival). B, dose-response curves for CPA (△), GCV (○), and CPA plus GCV (○) treatment of 9L cells infected with rRp450 at a MOI of 0.5 for 4 h at 37°C, followed by temperature shift to 39.8°C. Prodrug concentration on the X axis is in units of 0.0625 μg/ml GCV (added upon temperature shift) and/or 7.5 μg/ml CPA (present from the start). Data points, mean concentrations, evaluated in triplicate; bars, SEs (<6.8% survival). Chou-Talalay analysis of these data (data not shown) revealed synergism. C, percentage survival as a function of MOI for U87ΔEGFR cells infected with rRp450 for 4 h at 37°C followed by temperature shift to 39.8°C. Untreated cells (□) and cells treated with 70 μg/ml CPA (○), 1 μg/ml GCV (△), and 70 μg/ml CPA plus 1 μg/ml GCV (○) are shown. Data points, means of triplicate evaluations; bars, SEs (<7.3% survival).
were <1 for most effect levels, suggestive of no antagonism and of pharmacological synergism between the GCV/HSV-TK and CPA/CYP2B1 gene therapies in rat 9L tumor cells infected with the oncolytic virus, rRp450. Similar findings were also observed with stably transfected 9L/TK-450 cells, thus providing additional confirmation for the observed synergy (data not shown).

To show that the observed synergism was not exclusive to rat 9L gliosarcoma cells, we performed similar experiments using human U87/EGFR glioma cells. Fig. 1C reveals that, to have no oncosynthesis for these cells, temperature shift after a 4-h infection at 37°C and an MOI of 0.1 (or 2 h at an MOI of 0.5; data not shown) was necessary. Longer periods of infection or higher MOIs produced gradual increases in oncosynthesis. Single and combined prodrug treatment of cells followed by temperature shift produced significant enhancements in oncosynthesis (Fig. 1C). Again, combination indices of <1 were calculated for most doses. These results indicated that prodrug augmentation of rRp450’s oncosynthesis and the observed synergism between the two gene therapies delivered by rRp450 were operative and reproducible in a second cell line of human origin.

**GCV/HSV-TK and CPA/CYP2B1 Interactions Are Associated with Inhibition of DNA Repair.** The active metabolite of CPA, phosphoramide mustard, produces inter-and intranucleotide DNA cross-links (15). Cross-links are repaired by excision of damaged DNA, followed by DNA synthesis mediated by polymerases δ and ε (16). Because GCV-triphosphate has a strong inhibitory effect against DNA polymerase δ (17), we reasoned that observed pharmacological enhancement may result from an interaction between the two gene therapies at the level of DNA repair. We thus used the ethidium bromide fluorescence assay to measure the amount of cross-linked DNA in 9L/TK-450 cells pulsed with CPA for 6 h and then allowed to repair their DNA in the absence of CPA for 24 h. Fig. 2 shows that the cross-link index (Ci) of DNA was 47% at the end of the CPA pulse and then gradually decreased over the next 24 h to 3%, suggestive of relatively rapid DNA repair. Addition of GCV to the CPA pulse did not affect the initial amount of DNA cross-linking, but it did significantly inhibit DNA repair at all times during the recovery phase. Preexposure of cells to GCV before the CPA pulse or addition of GCV during the CPA pulse did not produce more inhibition of DNA repair (data not shown). GCV alone did not induce significant cross-linking (data not shown). These findings suggest that the observed pharmacological enhancement between the GCV/HSV-TK and CPA/CYP2B1 gene therapies were mechanistically related at the level of DNA cross-linking.

**Treatment with the Single Agent, rRp450, Provides Three Modes of Therapy against Tumors in Vivo.** To confirm the finding of therapeutic efficacy of the three modes of therapy offered by rRp450 in vivo, we established rat 9L tumors in the s.c. flanks of athymic mice and were then treated with rRp450 and/or prodrug(s). Additional groups were treated with a control virus (hrR3, an ICP6 mutant with an intact HSV-TK, but lacking CYP2B1 expression; Refs. 5 and 10), alone or combined with prodrug(s). Fig. 3, A and B, compares the gross effects of treatment with saline alone, prodrug alone, or virus. In general, rRp450 alone or in combination with GCV, CPA, or GCV plus CPA significantly inhibited tumor growth when compared to the saline or CPA alone controls. As expected, hrR3 possessed a similar effect. It is evident that hepatic metabolism of CPA also produced inhibition of tumor growth (Fig. 3, A and B). In Fig. 3C, a more detailed comparison did not show differences in the growth of tumors infected with rRp450 or hrR3 in the presence or absence of GCV, presumably because, under these conditions, this prodrug metabolites’ antiviral effects and anticaner effects were equal. As reported previously, rRp450 plus CPA significantly inhibited tumor growth (4), whereas rRp450 combined with CPA and GCV was even more effective and was the only treatment that achieved tumor regression (compare the growth curves for rRp450 plus CPA and rRp450 plus CPA plus GCV in Fig. 3C). Because GCV alone did not have an effect on augmenting or inhibiting rRp450 oncosynthesis (see Fig. 3C), its mode of action must have involved potentiation of CPA’s activity, thus providing further suggestive evidence for GCV/HSV-TK enhancing CPA/CYP2B1 gene therapy. Similar considerations apply to the comparisons between the growth of tumors treated with hrR3 and GCV versus hrR3, GCV, and CPA. Although hepatic activation of CPA increased hrR3 oncosynthesis, intratumoral CPA activation by rRp450 was clearly more effective, as evidenced by the growth curves of hrR3 plus CPA compared to rRp450 plus CPA (Fig. 3C). At the conclusion of the experiment, treatment with rRp450, CPA, and GCV caused 0.3-fold growth, the only experimental group that displayed tumor regression. Therefore, rRp450 provided three distinct modes of therapy against a tumor, and in treating s.c. 9L tumors, all three modes had to be used to achieve consistent tumor regression.

**Discussion**

The objective of this study was to determine the anticancer effect of rRp450 oncosynthesis combined with GCV/HSV-TK and CPA/CYP2B1 gene therapies. Several novel findings were brought about by this study. (a) These two gene therapies exhibited evidence of pharmacological synergism in rat and human tumor cells infected with rRp450. (b) The observed decrease in DNA repair in cells treated with GCV and CPA, compared to cells treated with CPA alone, suggests that GCV inhibits enzymes needed to repair the DNA cross-links produced by CPA metabolites. (c) The infection time and MOI required to transform rRp450 from an oncolytic virus into a gene delivery vector were characterized. (d) The combination of rRp450 oncosynthesis and its two synergistic gene therapies produced regression of established tumors in animals.

The finding of synergism between different prodrug-activating gene therapies can provide an insight into mechanisms of action. In fact, we and others have previously shown that the GCV/HSV-TK and cytosine deaminase/5-fluorocytosine gene therapies interact synergistically, probably through the ability of 5-fluorocytosine metabolites to modify nucleotide pools in a manner that enhances GCV phosphoryl-
biochemical studies using DNA polymerase agent synergisms observed with traditional chemotherapy. Detailed same mechanism that gives rise to the nucleoside analogue-alkylating viral inactivation through temperature shift abrogated viral oncolysis. We showed that a pulse of viral infection followed by CYP2B1 ected cells, expressing the gene of interest such as HSV-TK and CPA, and rRp450, hrR3, and all prodrug combinations (close-up graph to permit comparison between rRp450 and hrR3; C. Data points, fold growth, represented as the ratio of tumor volume at indicated time and tumor volume at the beginning of treatment; bars, SEs [<7-fold growth (treatment groups) or <24-fold growth (saline-treated)]. The difference in the fold growth between rRp450 plus CPA and rRp450 plus CPA plus GCV was statistically significant at the 15- and 20-day time points (P < 0.05; Student’s t test), and the difference in the fold growth between hrR3 plus CPA and hrR3 plus CPA plus GCV was statistically significant at the 15- and 20-day time points (P < 0.05; Student’s t test). The difference in the fold growth between either hrR3 plus GCV and hrR3 alone or rRp450 plus GCV and rRp450 alone were not significant at any time point, whereas the differences between either hrR3 plus CPA and hrR3 alone or rRp450 plus CPA or rRp450 alone were statistically significant at the 15- and 20-day time points (P < 0.05; Student’s t test). Finally, the difference in the fold growth between hrR3 plus CPA and rRp450 plus CPA was statistically significant at the 15- and 20-day time points (P < 0.05; Student’s t test).

Fig. 3. Treatment of s.c. 9L tumors with virus and/or prodrug(s). Fold growth versus days of treatment (tumor growth curve) is shown for tumors treated with saline, CPA, and rRp450 with all prodrug combinations (A); tumors treated with saline, CPA, and hrR3 with all prodrug combinations (B); and tumors treated with rRp450, hrR3, and all prodrug combinations (close-up graph to permit comparison between rRp450 and hrR3; C). Data points, fold growth, represented as the ratio of tumor volume at indicated time and tumor volume at the beginning of treatment; bars, SEs [<7-fold growth (treatment groups) or <24-fold growth (saline-treated)].

An additional question may relate to the effect of each of the prodrugs and their metabolites on the replication of the oncolytic rRp450 virus at the permissive temperature (37°C) in vitro and in vivo. In published experiments, we have shown that CPA treatment does not inhibit rRp450 replication either in vitro or in vivo at the doses used in the present report (4), and it actually augments viral replication by inhibition of both innate and elicited immune antiviral responses (20). Conversely, GCV’s metabolites can display both strong anticancer and antiviral effects. With the described cell lines and under the described culture conditions, GCV’s anticancer effect always predominated over its antiviral effect, if GCV was administered after the occurrence of viral replication and this type of result was previously observed with rat 9L tumor cells (10). Instead, published...
experiments using human colon carcinoma cells in which GCV was also administered 3 days into a 5-day infection showed that GCV’s antiviral effects predominated over and antagonized its anticancer effects. The difference may stem from the infectivity of the cells or their levels of gap junctions; gap junctions may mediate a “bystander effect,” in which toxic phosphorylated GCV is transferred from infected to uninfected cells. Antiviral effects of GCV may predominate over anticancer effects in tumor cell lines that allow for vigorous viral replication (such as most human tumor cell lines) and that have low levels of gap junctions (such as human colon carcinoma cells; Ref. 21), whereas the opposite may be true under conditions that may not be as favorable to viral replication (such as most rat tumor cell lines) or in cells with high levels of gap junctions (such as the rat 9L glioma cell line).

Comparisons of growth curves from the in vivo study led us to several conclusions. (a) GCV activation by hrR3 or rRp450 did not appear to significantly enhance or inhibit the oncolytic effect (Fig. 3C), suggesting a balance between the prodrug metabolites’ antiviral and anticancer action under these particular experimental conditions. We added GCV 4 days after the last administration of virus and CPA. It is likely that simultaneous oncolytic virus and GCV administration would have resulted in antiviral activity predominating over anticancer activity (i.e., before HSV-TK expression), whereas the effect of further delay in GCV administration may not be beneficial because there could be less CPA available for synergistic interactions. (b) CPA could be added simultaneously because it appears to possess minimal antiviral activity when compared to its anticancer effects (4). (c) There must have been hepatic metabolism of CPA, administered intratumorally, thus enhancing the anticancer action of both hrR3 and rRp450. However, the anticancer action of rRp450 was enhanced more than that of hrR3, presumably because of additional intratumoral conversion of prodrg by the CYP2B1 transgene delivered by the former virus. (d) GCV, which by itself did not augment hrR3 or rRp450 oncolysis, did enhance the oncolysis by rRp450 or hrR3 when CPA was present, providing further evidence for pharmacological synergy by these two therapies, even in vivo. In conclusion, rRp450 provides three distinct modes of anticancer therapy. Addition of other anticancer genes within rRp450’s genome that might potentiate CPA/CYP2B1 action could further expand the versatility of oncolytic viral delivery of therapeutic genes.

References
Multimodal Cancer Treatment Mediated by a Replicating Oncolytic Virus That Delivers the Oxazaphosphorine/Rat Cytochrome P450 2B1 and Ganciclovir/Herpes Simplex Virus Thymidine Kinase Gene Therapies

Manish Aghi, Ting Chao Chou, Kristen Suling, et al.