Intraneoplastic Polymer-based Delivery of Cyclophosphamide for Intratumoral Bioconversion by a Replicating Oncolytic Viral Vector

Tomotsugu Ichikawa, William P. Petros, Susan M. Ludeman, Jim Fangmeier, Fred H. Hochberg, O. Michael Colvin, and E. Antonio Chiocca

Molecular Neuro-Oncology Laboratory, Neurosurgery Service [T. I., E. A. C.], and Neurology Service [F. H. H.], Massachusetts General Hospital, Harvard Medical School, Boston, Massachusetts 02114; and Duke Comprehensive Cancer Center, Durham, North Carolina 27710 [W. P. P., S. M. L., J. F., O. M. C.]

Abstract

rRp450 is an oncolytic herpesvirus that expresses the CYP2B1 cDNA, responsible for bioconverting cyclophosphamide (CPA) into the active metabolites 4-hydroxyCPA/aldophosphamide (AP). However, formal proof of drug activation is lacking. We report that activation of CPA in cells infected with rRp450 generates a time-dependent increase of diffusible 4-hydroxyCPA/AP. For in vivo applications, a CPA-impregnated polymer was implanted into human tumor xenografts inoculated with rRp450. The area under the curve for 4-hydroxyCPA/AP was 806 µg/g of tumor tissue/h when CPA was administered via intraneoplastic polymer and 3 µg/g of tumor tissue/h when CPA was administered i.p. Therefore, (a) a lytic virus expressing a “suicide” gene can activate a prodrug; and (b) within rRp450-infected tumor, more prolonged and higher concentrations of activated metabolites are generated by intraneoplastic compared with systemic administration of prodrug.

Introduction

Oncolytic viruses are considered a form of “cancer gene therapy,” wherein anticancer function is provided by expression of cytotoxic viral genes expressed during the viral lytic life cycle (1). Oncolytic viruses can generate progeny viruses within tumor, and, theoretically, this should result in a continuous increase of anticancer particles within the growing neoplastic mass. These viruses can also be altered to express additional anticancer cDNAs, such as those that activate prodrugs, thereby further augmenting the direct viral oncolytic effect (2). rRp450 represents one such virus (3). Its deletion of the HSV1 ICP6 gene locus provides its relative selectivity for replication in tumor rather than normal cells. Anticancer efficacy of such ICP6-deleted HSV mutants has been shown in a variety of models (2, 4). To further increase the anticancer effect, the cDNA that encodes for cytochrome P450 2B1 (CYP2B1), a hepatic enzyme that converts the prodrug CPA into its active anticancer metabolites (5) 4-hydroxyCPA and its tautomer AP (6), was inserted into the deleted ICP6 locus. The combination of CPA and rRp450 was shown to provide more effective anticancer action both in vitro and in animal models of tumors compared with either treatment alone. Additional improvements in efficacy were also shown to be possible by combining viral oncolysis with other prodrug-activating gene therapy strategies (2, 3). Despite the above, formal proof of CPA bioconversion in tumor cells infected with a lytic, replicating herpes virus mutant was lacking. Thus, we hypothesized that measurements of the activated CPA metabolites, 4-hydroxyCPA/AP, in cells and their supernatants in vitro and in vivo should provide irrefutable evidence for such conversion. In this study, we show that 4-hydroxyCPA/AP is readily and rapidly found in the supernatant of tumor cells infected with rRp450 and exposed to CPA in vitro. Additionally, we find that in vivo implantation into tumors of polymers impregnated with CPA also results in rapid bioconversion of the prodrug by rRp450. The intratumoral administration of CPA-polymer/rRp450 results in intraneoplastic concentrations of 4-hydroxyCPA/AP that are much higher than those observed after i.p. administration of CPA.

Materials and Methods

Cell Lines and Vectors. Human U87ΔEGFR glioblastoma cells were a generous gift of Dr. H-J. Su Huang (University of California at San Diego, San Diego, CA). Cells were grown at 37°C in DMEM (Life Technologies, Inc.) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 mg/ml of streptomycin in an atmosphere containing 5% CO2 . The oncolytic viral vector used in this study is designated rRp450. It consists of a genetically engineered HSV1 with an almost complete deletion of ICP6 and insertion of the rat cytochrome p450 2B1 gene (CYP2B1; Ref. 3). As a control, hrR3, an HSV1 virus with defective ICP6 function but no CYP2B1 expression, was used (2). Vector stocks were stored at –80°C before their use. Vectors were passaged on African green monkey kidney (Vero) cells, and viral titers were obtained by plaque-formation assays on Vero cell monolayers. Procedures involving viruses were performed in accordance with guidelines issued by the Harvard Office of Biological Safety.

Biodegradable CPA Polymer Disc Preparation. The polymer used for the study was the polyanhydride pCPP-SA copolymer (20:80; Ref. 7). The pCPP-SA possesses an average molecular weight of 51,000–74,000 and was a gift from Guilford Pharmaceuticals (Baltimore, MD). CPA hydrate (65.9 mg; Aldrich Chemical Co.) was dissolved in 10 ml of methylene chloride, dried with magnesium sulfate for 10 min, and then filtered. A solution of pCPP-SA (183.4 mg) in methylene chloride (5 ml) was then added to the filtrate. The mixture was stirred for 30 min and then concentrated on a rotary evaporator. The residual material was dried overnight under high vacuum in a desiccator containing Drierite. Using approximately 6 mg at a time, the resultant powder was compressed into discs using a stainless steel press with an inner diameter of 1.9 mm that had been designed by the Biomedical Engineering Mechanical Instrument Shop of The Johns Hopkins Medical Institution. Each disc was weighed and placed in a multiwell plate that was sealed and stored over Drierite at –20°C. The individual discs made by this method had an average weight of 5.0 ± 0.3 mg and measured approximately 1.9 mm (diameter) × 1.3 mm (height). Each disc was composed of 25% CPA by weight. Discs were made as close to experimental use as possible (maximum storage time approximately 2 weeks). Placebo discs were made by compression as described above using drug-free pCPP-SA.

In Vitro Kinetics of CPA Release. To study the temporal kinetics of CPA release in vitro, a CPA-polymer disc was placed in a 5-ml vial and covered with 1.5 ml of 0.2 M BisTris containing 0.9% sodium chloride (pH 7.4). One such vial was allowed to stand at room temperature, and another was kept at 37°C. At intervals of approximately 1 h, the supernatant was removed from
each vial and stored at −20°C. Fresh buffer (1.5 ml) was then added to each vial. Each supernatant was assayed for CPA concentration using HPLC, and the percentage of drug release over each h was determined. To compare the kinetics of CPA release from pCPP:SA with those of a clinically useful BCNU preparation [20% BCNU by weight in pCPP:SA (20:80)], 5% ethanol was used as the medium. The lipophilic BCNU has better solubility and is reasonably stable in ethanolic solutions (at least over the time course of these studies, 1 h at room temperature). As described above, the supernatant was removed hourly and replaced with fresh 5% ethanol. The solutions from the BCNU-release experiments were immediately analyzed by HPLC for BCNU concentration.

The 5-mg discs that were 20% BCNU by weight were prepared as described above for CPA using a powdered mixture of pCPP:SA (20:80) and BCNU (a gift from Guilford Pharmaceuticals, Inc., with purity greater than 99%). Analysis of BCNU concentrations, 25-μl aliquots of the supernatant were injected into an HPLC system consisting of a 25.0 cm × 4.6 mm Supelcosil LC-18-DB column (5-μm particle size; Supelco, Inc., Bellefonte, PA). The mobile phase (55% methanol:45% purified water) was delivered isocratically at a rate of 1.2 ml/min. The Waters Associates HPLC system consisted of a Model 510 pump, a refrigerated Model 715 Ultra WISP sample processor, and a Model 490E programmable detector set at a wavelength of 237 nm. The lower limit of BCNU quantitation for this assay was 0.5 μg/ml. The standard curve was linear over the concentration range 5–80 μg/ml used in this study. Interassay and intraassay variabilities were 4 and 10%, respectively. For CPA analysis, 25-μl aliquots of the supernatants (contained in 5% ethanol, 0.2 μl BisTris buffer with normal saline incubated at 37°C, or 0.2 μl BisTris buffer with normal saline incubated at 37°C, each with a total sample volume of 1.5 ml) were injected immediately into an HPLC system consisting of a 3.9 × 150-mm Waters Novapack C18 analytical column (4-μm particle size; Millipore Inc., Milford, MA). The mobile phase (20% acetonitrile/80% 0.025 M monosodium phosphate) was delivered isocratically at a rate of 1.3 ml/min, and CPA was detected via UV absorbance at 200 nm. The lower limit of CPA quantitation for this assay is 1.0 μg/ml. The standard curve was linear over the concentration range 1–250 μg/ml used in this study. Interassay and intraassay variabilities were 7 and 5%, respectively.

4-HydroxyCPA-AP Derivatization and Analysis. To measure the unstable 4-hydroxyCPA-AP metabolites, samples generated by the in vitro and in vivo experiments were immediately treated with a derivatizing agent. In this derivatization, 4-hydroxyCPA-AP were converted to a pentafluorobenzoyloxime of AP that was stable for extended periods of time (months at low temperature) and that was suitable for quantification by GC-MS. Details of the derivatization and GC/MS analysis have been reported (8, 9). In brief, a known volume or weight of each sample (medium, blood, and tissue) was added to a tube containing the following “trapping” solution: (a) 250 μl of a methanol solution containing the derivatizing agent O-(2,3,4,5,6-pentafluorobenzoyl)hydroxylamine (50 mg/ml); (b) an internal standard of deuterated pentafluorobenzoxime of AP; (c) 2 ml of acetonitrile; (d) 1 ml of methanol; and (e) 1 ml 2 M ammonium phosphate (pH 4.6). The mixtures were shaken and then allowed to sit at room temperature overnight before final storage at −20°C. Samples were then processed for GC-MS analysis. The 4-hydroxyCPA-AP generated during in vitro experiments was stabilized further through the addition of the water-soluble derivatizing agent semicarbazide to the incubation medium to achieve a total concentration of 10 mM. Thus, upon formation, 4-hydroxyCPA-AP was immediately converted to a more stable semicarbazone. After the prescribed incubation period, the reaction medium was added to the trapping tube described above, and the semicarbazone derivative of 4-hydroxyCPA-AP was quantitatively converted to the more stable oxime.

Temperature Shift Assay. To assay activation of CPA into 4-hydroxyCPA-AP in cells infected with rRp450, a temperature-shift experiment was conducted. Human U87AEGFR glioma cells were plated at a density of 1 × 10⁶ cells/6-cm² dishes (Falcon) and allowed to adhere overnight. After washing with Hanks’ buffered saline, rRp450 was added at a multiplicity of infection of 5 in 10% fetal bovine serum/DMEM in a total volume of 500 μl. The plates were shaken every 15 min for 1 h at 37°C, after which 1.5 ml of fresh medium were added. After a total of 4 h of incubation at 37°C, plates were transferred to a separate 39.8°C incubator to block rRp450 replication (2, 3). The next day, supernatant was removed, cells were washed, and fresh medium containing 10 mM semicarbazide plus 1 mM CPA or vehicle was added before reincubation at 39.8°C. Cells and supernatants were then harvested separately at different time periods and lysed by sonication in the “trapping” buffer, described above. Samples were then mailed to the Duke Comprehensive Cancer Center to assay 4-hydroxyCPA-AP generation by a validated GC/MS assay, as described previously (9). The lower limit of quantitation for this technique is 0.1 μg/ml (9). Area under the concentration-time curve analyses was calculated by noncompartmental methods using WinNonlin software (Version 2.1: Pharsight, Mountain View, CA).

Animal Experiments. Animal studies were performed in accordance with guidelines issued by the Massachusetts General Hospital Subcommittee on Animal Care. Viral inoculation and care of animals treated with rRp450 were performed in approved viral vector rooms. Tumors were established in the flanks of athymic mice (NCY/scid, nuc/nu; MGH breeding colony) by s.c. injection of 10⁶ human U87AEGFR glioma cells in 100 μl of DMEM. Tumors were allowed to grow to a size of 200 mm³, as determined by measuring tumor height, width, and length with external calipers and then multiplying the three values. A direct intratumoral injection with rRp450 (1 × 10⁸ pfu/10 μl of DMEM) was performed using a Hamilton syringe (Hamilton Syringe Company, Reno, NV). Control injections consisted of using a mock-infected Vero cell lysate. For the in vivo kinetics study, a single CPA-polymer (or placebo-polymer) was surgically implanted into the tumor the day after rRp450 injection. For some animals, an i.p. injection of CPA (1.25 mg/animal) was performed. Tumors were then rapidly excised from animals at different time points and dropped into preweighted tubes containing the “trapping” solution described above in order to minimize degradation of the relatively unstable 4-hydroxyCPA-AP metabolites. Tubes with tumors were weighted again, minced into small fragments with a scalpel while making sure that they remained covered with the “trapping” solution, and then homogenized in this solution using a Sonic Disembrator 550 (Fisher Scientific Co.) ultrasonic apparatus. To measure serum concentrations of 4-hydroxyCPA-AP, blood from the same animal was collected by intracardiac puncture and immediately added to the “trapping” solution. Tumor and blood samples were then mailed to the Duke University Comprehensive Cancer for assays of 4-hydroxyCPA-AP levels using GC/MS. To measure growth of tumors in the s.c. flank, single rRp450 inoculations into tumor were performed every other day for 4 days (total dose of rRp450 = 4 × 10⁶ pfu/animal). Biodegradable CPA- or placebo-polymer discs (one/animal) were surgically implanted into the tumor the day after the first injection of rRp450. Tumor volumes were then measured by external caliper measurement.

Results

CPA Is Converted into Freely Diffusible 4-HydroxyCPA-AP upon Oncolytic Virus Infection. We first determined whether bioconversion of CPA into its metabolites, 4-hydroxyCPA-AP, occurred in tumor cells infected with rRp450. Human glioma cells were infected with rRp450 in the presence of CPA. Control experiments consisted of cells treated with rRp450 alone. Other control experiments included treatment with another ICP6-deficient HSV mutant that does not express the CYP2B1 cDNA (hrR3; Ref. 2) in the presence or absence of CPA as well as treatment with mock-infected lysates in the presence or absence of CPA. Supernatant was assayed at different time points for the presence of the CPA metabolites, 4-hydroxyCPA-AP. Fig. 1 shows that there was a time-dependent increase in the concentration of 4-hydroxyCPA-AP in the supernatant of rRp450-infected cells, although these metabolites were not detected in the supernatant of control dishes. In a separate experiment, we also determined if active metabolite was associated with the infected cells rather than with their supernatant. Table 1 shows that, upon intracellular bioconversion of CPA by rRp450, the large majority of the activated metabolites were found in the supernatant of control dishes. These results indicate that rRp450 infection of tumor cells activates CPA into 4-hydroxyCPA-AP, which then freely and rapidly diffuses into the supernatant of the infected cell.

In Vitro Kinetics of CPA Release via Polymer. To maximize local conversion of CPA within tumors infected with rRp450, we sought to determine whether polymers impregnated with the produg would provide a local and sustained delivery method. The release kinetics of CPA from the polymer were studied by placing a disc in a
buffer containing BisTris. The appearance of CPA in the supernatant was then measured at specific time intervals. Fig. 2 shows that after 1 h at 37°C, approximately 25% of the prodrug was released, and an additional 25% was released over the next 2 h. As would be expected, drug release was somewhat slower at room temperature. This data demonstrated that CPA was in fact released from the polymer disc, and this release was gradual; however, the absolute time course of drug release under these conditions did not necessarily correlate with the rate of release in vivo. To provide some preliminary information related to this issue, the rate of CPA release from the polymer was compared with that of BCNU under the same conditions (Fig. 2). BCNU is a chemotherapy agent in current clinical use as a local polymeric formulation for malignant gliomas (Gliadel; Ref. 10). Because the in vitro drug release kinetics for CPA and BCNU were comparable (Fig. 2), the in vivo local and gradual delivery of CPA and BCNU might also be expected to be similar. Thus, these experiments showed that local and gradual delivery of CPA into tumor was feasible using a polymeric formulation.

Kinetics of in Vivo Intratumoral Conversion of CPA. We sought to study the kinetics of CPA bioconversion within infected tumors in vivo, using the polymeric formulation. Human glioma tumors were grown s.c. in the flank of athymic mice until they reached a volume of approximately 200 mm³. Tumors were then inoculated with rRp450, followed 24 h later by intratumoral implantation of the CPA-polymer pellet. Animal tumors and blood were then rapidly harvested, and the amount of activated CPA metabolites (4-hydroxy-CPA/AP) were determined as a function of time. Fig. 3A shows that 4-hydroxyCPA/AP could be found within tumor tissue with peak concentrations 12 h after polymer implantation. The CPA metabolites persisted within the tumor for at least 72 h. When rRp450 was delivered intratumorally, followed by systemic injection of CPA by an i.p. route, fairly small amounts of the activated metabolites were found within the tumor and then only for a few hs after i.p. injection. The AUC for tumor 4-hydroxyCPA/AP concentration versus time was 806 µg/g of tumor/h with CPA delivery via polymer compared with 3 µg/g/h with i.p. CPA administration. These data thus demonstrate that intratumoral delivery of CPA followed by intratumoral conversion by a replicating, oncolytic virus generates more prolonged and higher concentrations of the activated metabolites within the tumor than when CPA is delivered systemically.

In the same group of animals, we also determined the systemic levels of activated CPA. Fig. 3B shows that peak levels of activated metabolites were found to occur in blood 1 h after i.p. injection of CPA and 6 h after intratumoral delivery via polymer. With intraneoplastic polymeric delivery of CPA, the peak blood levels of 4-hydroxyCPA/AP were 10 times less than the peak tumor levels of 4-hydroxyCPA/AP. There was no difference between the intratumoral and blood concentration of 4-hydroxyCPA/AP when CPA was administered i.p., despite expression of the CYP2B1 transgene in the tumor by the replicating virus. With i.p. CPA injection, the AUCs for 4-hydroxyCPA/AP for the infected and uninfected tumors were 2.7 and 6.2 µg/g of tumor/h, respectively; whereas blood AUCs were 3.2 and 3.0 µg/ml/h, respectively. Taken in conjunction, these results suggest that when CPA is delivered systemically, hepatic conversion provides the bulk of converted metabolites found in blood and tumor with very little contribution by intraneoplastic conversion mediated by transferred CYP2B1.

Antitumor Efficacy of Polymer-delivered CPA and Oncolytic Virus. To show that polymer-based intraneoplastic delivery and virus-based intraneoplastic bioconversion of CPA resulted in a significant anticancer effect, relatively large (200 mm³) s.c. glioma xenografts were treated with the CPA-polymer and rRp450. Fig. 4 shows that this treatment resulted in a significant retardation of tumor growth when compared to treatment with oncolytic virus alone or with CPA-polymer alone. In fact, there was regression of four of eight tumors, although growth retardation was present in the remainder (Table 2). Thus, these findings show that intraneoplastic delivery of CPA through a polymer and its bioconversion by an oncolytic virus expressing P450 2B1 resulted in a significant anticancer effect.
Fig. 3. In vivo kinetics of 4-hydroxyCPA/AP production after intraneoplastic inoculation of rRp450 and intratumoral implantation of CPA-polymer. 4-hydroxyCPA/AP concentrations within s.c. human glioma tumors (Panel A) or serum (Panel B), inoculated with rRp450 followed by CPA-polymer (pCPA), placebo-polymer (C), or i.p. CPA (A).

Fig. 4. Tumor growth after treatment with intraneoplastic rRp450 and CPA-polymer. Implanted s.c. human U87ΔEGFR glioma xenografts were allowed to grow to 200 mm³ before intraneoplastic injection with rRp450 or mock-infected cell lysates (10⁸ pfu/dose) as a single dose every other day for four total doses (black arrows). Between the first and second dose, CPA- or placebo-polymer (white arrow) was surgically implanted into the tumor.

Discussion

In this study, we hypothesized that intratumoral delivery of the prodrug, CPA, and its bioconversion to 4-hydroxyCPA/AP by the oncolytic viral vector, rRp450, would result in elevated levels of these activated metabolites in the tumor when compared with metabolite levels in blood (i.e., systemically). To deliver CPA intratumorally, we used a slow-release polymeric formulation that could be implanted within the neoplasm. We found that: (a) 4-hydroxyCPA/AP concentrations in tumor were 10 times that in blood after CPA-polymer/rRp450 treatment; (b) 4-hydroxyCPA/AP concentrations in tumor and blood were approximately equal when CPA was delivered systemically; (c) tumor levels of 4-hydroxyCPA/AP lasted for at least 72 h after polymer implantation compared with less than 12 h after a single i.p. injection; and (d) an in vitro temperature-shift assay could be used to measure production of activated CPA metabolites by rRp450. Thus, these findings support the use of a polymer-based method to deliver CPA intraneoplastically and convert it into its activated metabolites by use of cDNA transfer. These findings provide supportive evidence to the hypothesis that intratumoral prodrug administration and gene transfer will maximize anticancer effects by achieving elevated local concentrations of activated metabolites.

Polymers have been used in both an experimental and a clinical setting to deliver active chemotherapy agents into tumors in the brain (11, 12). Clinically, polymer-based BCNU delivery (Gliadel) into tumors has been approved for clinical use against recurrent malignant glioma (10). In experimental animal tumor models, systemic delivery of a preactivated analogue of CPA (4-hydroperoxyCPA) has shown evidence of significant anticancer effects, but the elevated local doses of the drug also were shown to produce undesirable side effects to tissues adjacent to the tumor (13, 14). Herein lies the advantage for polymeric delivery of the prodrug: conversion into activated metabolites would occur only in tumor cells infected by the viral vector. Even the rapid diffusion of the activated metabolite from the tumor cell would be expected to primarily affect neighboring tumor cells rather than more distant normal tissues. In fact, although the peak concentration of 4-hydroxyCPA/AP was about 40 μg/g of tumor, it was only 3 μg/ml of blood, thereby showing that local prodrug activation can provide elevated doses of activated anticancer agent to tumor cells while minimizing exposure to more distant normal cells.

For these studies, an oncolytic viral vector (rRp450) was used. By itself, an oncolytic virus can provide a significant anticancer effect, but several factors (presence of cellular receptors for the virus, tumor cell complementation of the viral defect, and host immune responses) may inhibit effective anticancer action. Therefore, combining the viral oncolytic effect with other therapeutic modalities represents an avenue for augmenting anticancer action. Oncolytic viruses provide direct cytotoxicity to tumor cells through their replicative functions, allow for production of viral progeny that can itself infect additional tumor cells, and can also be used as vectors to deliver additional anticancer functions such as prodrug-activating or cytokine cDNAs (2, 15).

Table 2 Regression of s.c. tumors treated with rRp450 and CPA-polymer

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a S.C. human U87ΔEGFR glioma tumors (measuring 200 mm³) were inoculated with lysate from mock-infected cells or with rRp450 (10⁸ plus in 100 μl) every other day for a total of four doses. Placebo polymer or CPA-polymer were implanted into the tumors between the first and second viral injection.

b Regression is defined as a tumor volume less than 100 mm³ for the time course of the experiment (28 days).
Several different types of oncolytic viruses have completed Phase I clinical trials for a variety of cancers, and their basic safety has been shown even in an organ such as the brain (16, 17). Recently, one oncolytic adenovirus has shown evidence of efficacy against recalcitrant head and neck cancer when combined with cisplatin or 5-fluorouracil chemotherapy (18). The ability to anatomically spread through a neoplastic mass by successive rounds of replication in infected tumor cells accounts for the ability of these viruses to achieve more extensive tumor infection than that achieved by replication-defective vectors, where most infected cells remain localized around the injecting needle tract. The additional “arming” of oncolytic viruses with anticancer cDNAs can further augment their antitumor effects, particularly if such anticancer cDNAs do not affect viral replication.

Another advantage of CPA and its bioconversion by rRp450 as compared with other prodrug-activating gene therapies is that 4-hydroxyCPA/AP are freely diffusible metabolites (19). Our measurements show that little of the activated metabolites remain cell-associated upon activation, and they rapidly diffuse into the extracellular environment. Thus, the CPA/CYP2B1 gene therapy does not depend on a “bystander effect” to exert its anticancer action on noninfected cells (20). With other prodrug-activating or “suicide” gene therapies, such bystander effects would occur only in close proximity to the transduced tumor cells, and distant areas of tumor would be unlikely targets for anticancer effects. Instead, the presence of elevated concentrations of a freely permeable metabolite within a tumor mass would be more likely to affect tumor cells even if they were at a distance from an infected cell. The relatively short half-life of 4-hydroxyCPA/AP provides further rationale for strategies that will maximize its generation in the tumor in situ rather than within liver.

The findings in this study thus provide preclinical support for the use of CPA-polymer combined with the CPA-activating, oncolytic rRp450 as a local intraneoplastic treatment for tumors. Additional safety data in appropriate subhuman primate species should provide justification for testing in humans.

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References

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