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Turning an antiviral into an anticancer drug: Nanoparticle delivery of acyclovir monophosphate

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ABSTRACT

Anti-herpes simplex virus (HSV) drug acyclovir (ACV) is phosphorylated by the viral thymidine kinase (TK), but not the cellular TK. Phosphorylated ACV inhibits cellular DNA synthesis and kills the infected cells. We hypothesize that ACV monophosphate (ACVP), which is an activated metabolite of ACV, should be efficient in killing cells independent of HSV-TK. If so, ACVP should be a cytotoxic agent if properly delivered to the cancer cells. The Lipid/Calcium/Phosphate (LCP) nanoparticles (NPs) with a membrane/core structure were used to encapsulate ACVP to facilitate the targeted delivery of ACVP to the tumor. The LCP NPs showed entrapment efficiency of ~70%, the nano-scaled particle size and positive zeta potential. Moreover, ACVP-loaded LCP NPs (A-LCP NPs) exhibited concentration-dependent cytotoxicity against H460 cells and increased S-phase arrest. More importantly, a significant reduction of the tumor volume over 4 days following administration ($p < 0.05$–0.005) of A-LCP NPs, suggests excellent *in vivo* efficacy. Whereas, two free drugs (ACV and ACVP) and blank LCP NPs showed little or no therapeutic effect. It was also found that the high efficacy of A-LCP NPs was associated with the ability to induce dramatic apoptosis of the tumor cells, as well as significantly inhibit tumor cell proliferation and cell cycle progression. In conclusion, with the help of LCP NPs, monophosphorylation modification of ACV can successfully modify an HSV-TK-dependent antiviral drug into an anti-tumor drug.

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1. Introduction

Acyclovir (ACV), 9-[(2-hydroxyethoxy) methyl] guanine, has been widely used for the treatment of herpes simplex virus (HSV) infection. Its conversion into the corresponding monophosphate (ACVP) is mediated by the viral thymidine kinase (HSV-TK) in the HSV-infected cells. ACV-P was further phosphorylated to ACV diphosphate and triphosphate by cellular kinase in the host cells, which is capable of blocking DNA synthesis through the inhibition of the viral DNA polymerase and terminating the chain elongation of the viral DNA [1,2]. Taking advantage of this characteristic, gene therapy using a combination of ACV or ganciclovir (GCV) and the HSV-TK gene has been developed for the treatment of cancers in previous studies [3]. More interestingly, the bystander effect or metabolic cooperation correlating with the occurrence of gap junctions also contributes to the regression of tumors in these therapy modalities, despite that there was only a small proportion of tumor cells expressing TK [4–6].

Previous studies have demonstrated the potent antitumor effects of HSV-TK/prodrug gene therapy in animal models such as malignant gliomas [7,8]. However, clinical trials of HSV-TK-based suicide gene therapy have generally been disappointing [9]; the low transfection efficiency of the HSV-TK gene, the poor prodrug activation kinetics [10,11], and the poor lipophilicity of ACV or GCV which prevents penetration of the cell membrane may all contribute to the failures. These obstacles impact the extended application of this strategy in the clinic. These studies also indicate that ACV may be more appropriate for clinical application than GCV because it is more lipophilic and less toxic [8]. Targeted delivery of the active ingredient, which is ACVP in this case, should be a major emphasis if we hope to improve the antitumor activity and decrease potential toxicity of these therapies.

The calcium phosphate (CaP)-based nanoparticles have frequently been used to deliver genes because their escape of the endosomes is pH-sensitive, which contributes to the effective drug release, biocompatibility, biodegradability, and minimal toxicity [12,13]. In our previous study, we developed a novel vector, Lipid/Calcium/Phosphate (LCP) nanoparticles (NPs) [14,15]. Compared to the CaP-based formulation, which contains no lipid, the lipid coating of the LCP NPs better prevents the core from aggregation during the preparation of the nanoparticles and facilitates the formation of the outer-leaflet layer

Abbreviations: ACV, Acyclovir; ACVP, Acyclovir monophosphate; A-LCP NPs, ACVP-loaded LCP nanoparticles; CaP, Calcium phosphate; HSV-TK, Herpes simplex virus thymidine kinase; DOPA, Dioleoylphosphatydic acid; DOTAP, 1, 2-dioleoyl-3-trimethylammonium-propane chloride salt; DSPE–PEG, 1,2-distearoryl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethyleneglycol-2000) ammonium salt; DSPE–PEG–AA, DSPE–PEG–anisamide; EPR, Enhanced permeability and retention effect; GCV, Ganciclovir; HE, Hematoxylin and Eosin; LCP, Lipid/Calcium/Phosphate; NPs, nanoparticles; PCNA, Proliferating cell nuclear antigen; RES, Reticuloendothelial system; TEM, Transmission electron microscope; TUNEL, TdT-mediated dUTP Nick-End Labeling.

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with PEG–lipid derivates. Yang et al. verified the potential of this system for the targeted delivery of siRNA [16]. Treatment with a relatively low dose of therapeutic siRNA in LCP NPs caused a ~70–80% reduction of lung metastases. This system also significantly prolonged the mean survival time of mice without the associated toxicity.

In the present study, ACVP was synthesized, avoiding the limiting step of monophosphorylation that depends on the effective transfection of HSV-TK gene during gene therapy. The phosphorylation also provided an active group to enable ACV to bind with CaP, creating a high encapsulation efficiency. LCP PEGylated with anisamide-containing, PEG–lipid conjugate (DSPE–PEG–AA) encapsulates ACVP and binds to the tumor cells that overexpress the sigma receptor. We hypothesized that LCP would facilitate the targeted delivery of ACVP to the tumor through the synergistic mechanism of ligand-mediated, specific tumor-targeting, the enhanced permeability and retention (EPR) effect, and the reduced reticuloendothelial system (RES) uptake (as shown in Scheme 1). These characteristics should facilitate the transmembrane transport of ACVP, enhance its tumor accumulation, improve the antitumor effect, and also avoid the peripheral toxicity. We investigated the *in vitro* and *in vivo* activity of ACVP-loaded LCP nanoparticles (A-LCP NPs). Moreover, the potential mechanism through which ACVP and A-LCP NPs induce DNA damage was studied by cell cycle, TdT-mediated dUTP Nick-End Labeling (TUNEL), and immunohistochemical and Weston blot assays.

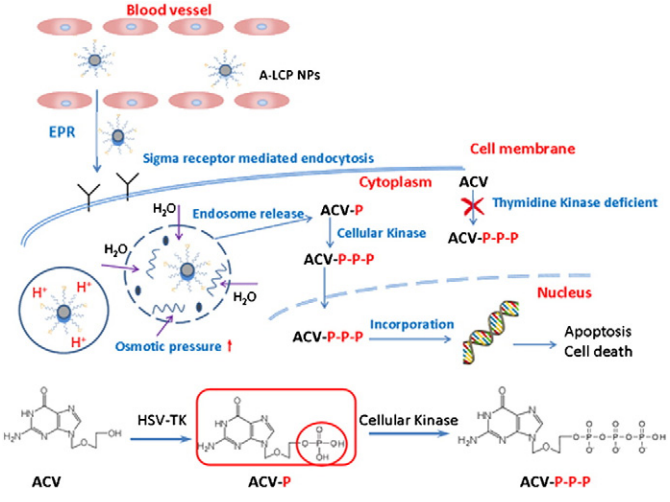
2. Materials and methods

2.1. Materials

ACV was purchased from Carbosynth Limited (Compton Berkshire, UK). 1, 2-dioleoyl-3-trimethylammonium-propane chloride salt (DOTAP), cholesterol, and 1,2-distearoryl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethyleneglycol-2000) ammonium salt (DSPE–PEG) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). DSPE–PE–anisamide (DSPE–PEG–AA) was synthesized in our lab as described [17]. ACVP was also synthesized by following a previously reported procedure [18]. The purity of ACVP was 92.5%. Other chemicals were obtained from Sigma-Aldrich (St. Louis, MO) and were not purified further.

The H460 (H460-TK$^-$) cells originally obtained from American Type Culture Collection (ATCC) were cultured in an RPMI 1640 cell culture medium with 10% fetal bovine serum (Invitrogen, Carlsbad, CA), 100 U/mL penicillin, and 100 μg/mL streptomycin (Invitrogen, Carlsbad, CA). The HSV-TK-expressing H460 (H460-TK$^+$) cells were obtained by transfection with pCDNA3.1-HSV1-TK using lipofectamine2000. Cells were seeded at 1×10^5 cells in a 10-cm plate and grown overnight in the growth medium (to reach 90% confluence at the time of transfection). The primary growth medium was removed and replaced with Opti-MEM®I. The transfection complex was added and allowed to incubate for 4 h. The medium was then removed and replaced with RPMI 1640 cell culture medium. After 24 h, the cells were seeded for further study. Cells were cultivated in a humidified incubator at 37 °C and 5% CO_2.

Mice were purchased from the National Cancer Institute (Bethesda, MD). All experiments performed on animals were in accordance with and approved by the Institutional Animal Care and Use Committees at the University of North Carolina at Chapel Hill.



Scheme 1. Illustration of the structure, and uptake by tumor cells and intracellular delivery of A-LCP NPs.

2.2. Preparation and characterization of A-LCP NPs

The LCP NPs were prepared using the methods described in our previous studies with some additional improvement [14]. Three hundred microliters of P phase including 3.125 mM $NaHPO_4$ (pH =9.0) and 24 mM ACVP were dispersed in 10 mL cyclohexane/Igepal CO-520 (71/29, v/v). The Ca phase was prepared by adding 300 μL $CaCl_2$ (2.5 mM) into a separate oil phase. Four hundred microliter (20 mM) dioleoylphosphatydic acid (DOPA) in chloroform was added to the P phase. After mixing the two microemulsions for 20 min, 20 mL of absolute ethanol was added to the mixture and centrifuged at 10,000 *g* for 20 min to pellet the LCP cores. After being extensively washed by ethanol twice and dried under N_2, the LCP core pellets were dissolved in 1 mL chloroform and stored at −20 °C for further use.

A-LCP NPs were prepared by mixing 250 μL of the cores with 145 μL of 4 mM cholesterol, 4 mM DOTAP, 2.7 mM DSPE–PEG-2000 and 0.6 mM DSPE–PEG–AA. After evaporating the chloroform, the residual lipid was dissolved in 80 μL of THF–ethanol solution (3:5, v/v). One hundred and sixty microliters of distilled water was added to form the NPs, and then dialyzed against water for 1 h (dialysis membrane MWCO 20,000). The amount of ACVP in the nanoparticles was measured at 254 nm using a DU 800 spectrophotometer (Beckman Coulter Inc.). The solvent was composed of THF-1 M hydrochloric acid solution (7:3, v/v). The zeta potential of A-LCP nanoparticles was determined through dynamic light scattering measurements (Malvern ZetaSizer Nano series, Westborough, MA). The shape and surface morphology of the nanoparticles were observed by using a JEOL 100CX II transmission electron microscope (TEM) (Tokyo, Japan).

2.3. In vitro cytotoxicity studies

The cytotoxicity of the A-LCP NPs was assessed using the MTT assay. The H460-TK$^-$ and H460-TK$^+$ cells were seeded at a density of 1×10^5 cells per well in 96-well microtiter plates, respectively and incubated for 24 h. The cells were treated with different formulations for 48 h. After incubation, MTT solution (15 μL, 5 mg/mL in PBS) was then added to each well and the cells were incubated further for 4 h at 37 °C. The media were removed and the cells were dissolved in DMSO. Absorbance at 570 nm was measured with a microplate reader. Cell viability (%) was calculated as (OD of test group / OD of control group) × 100.

2.4. Cell cycle arrest

H460 cells growing exponentially were seeded at 1×10^5 cells/mL in 6-well plates. Cells were treated with free ACV, free ACVP and A-LCP NPs for 48 h. The PBS solution served as the control. Ice-cold, 70% ethanol was used to fix the cells at 4 °C overnight. After centrifuging to remove the supernatant, the cells were re-suspended with 1 mL staining buffer and washed once. After re-suspension, the cells were incubated with 10 μL of RNase A (10 mg/mL) at 37 °C for 30 min, and were stained with 5 μL of propidium iodide (1 mg/mL) at room temperature for 30 min. The cell-cycle analysis was performed