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Bluetongue Virus Particles as Nanoreactors for Enzyme Delivery and Cancer Therapy

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Abstract

The side effects of chemotherapy can be reduced by targeting tumor cells with an enzyme (or the corresponding gene) that converts a nontoxic prodrug into a toxic drug inside the tumor cells, also killing the surrounding tumor cells via the bystander effect. Viruses are the most efficient gene delivery vehicles because they have evolved to transfer their own nucleic acids into cells, but their efficiency must be balanced against the risks of infection, the immunogenicity of nucleic acids, and the potential for genomic integration. We therefore tested the effectiveness of genome-free virus-like particles (VLPs) for the delivery of Herpes simplex virus 1 thymidine kinase (HSV1-TK), the most common enzyme used in prodrug conversion therapy. HSV1-TK is typically delivered as a gene, but in the context of VLPs, it must be delivered as a protein. We constructed VLPs and smaller core-like particles (CLPs) based on Bluetongue virus, with HSV1-TK fused to the inner capsid protein VP3. TK-CLPs and TK-VLPs could be produced in large quantities in plants. The TK-VLPs killed human glioblastoma cells efficiently in the presence of ganciclovir,

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Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.molpharmaceut.0c01053.
Sequence of TK:VP3 fusion protein with mass spectrometry analysis; schematic illustration of constructs used in this study; density gradients and SDS-PAGE of TK-CLPs analyzed fresh and after 28 days of storage; sequence of HSV1-TK gene synthesized; protein sequence of HSV1-TK; and comments about deleterious effects of soluble TK on *N. benthamiana* plants (PDF)

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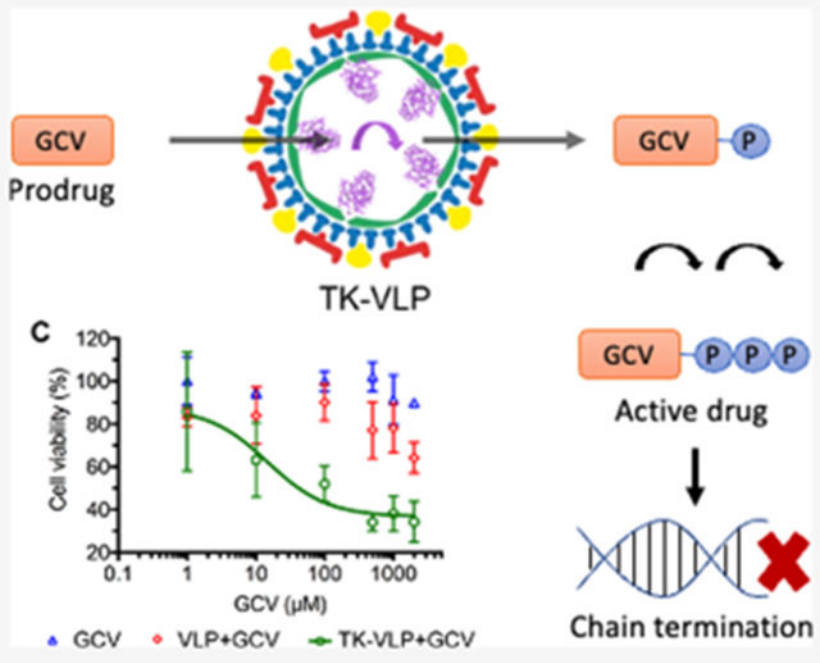
with an IC_{50} value of 14.8 μM. Conversely, CLPs were ineffective because they remained trapped in the endosomal compartment, in common with many synthetic nanoparticles. VLPs are advantageous because they can escape from endosomes and therefore allow HSV1-TK to access the cytosolic adenosine triphosphate (ATP) required for the phosphorylation of ganciclovir. The VLP delivery strategy of TK protein therefore offers a promising new modality for the treatment of cancer with systemic prodrugs such as ganciclovir.

Graphical Abstract



Keywords

virus-like particle (VLP); core-like particle (CLP); ganciclovir; thymidine kinase (TK); virus-directed enzyme prodrug therapy (VDEPT)

INTRODUCTION

Traditional chemotherapy is associated with severe off-target effects because the systemic drugs are toxic toward all rapidly dividing cells.[1] This can be addressed by targeted therapy, in which drugs are either delivered specifically to tumor cells[2] or activated specifically within them.[3] Enzymes are often used for targeted cancer therapy, and two major approaches have emerged. The first involves the targeted delivery of enzymes that trigger tumor cell death by modifying a natural intracellular target. Examples include conventional immunotoxins containing *Pseudomonas* exotoxin A or ricin, which catalyzes the ribosylation of elongation factor 2,[4] and cytolytic fusion proteins incorporating human enzymes that trigger apoptosis.[5] The second involves the targeted delivery of enzymes that convert a nontoxic or minimally toxic systemic prodrug into an active drug at the site of the tumor. This antibody-directed enzyme prodrug therapy (ADEPT) can be a more efficient

approach because the enzyme produces high concentrations of the active drug within the tumor, and local diffusion to the surrounding tumor cells causes a lethal bystander effect even if the enzyme itself cannot penetrate fully into the tumor mass and/or if the surrounding cells are negative for the targeted tumor antigen.

Many different enzymes have been tested for ADEPT in preclinical models,[6–8] but only carboxypeptidase G2 has been used in clinical trials;[9,10] a translational barrier is the high immunogenicity of these enzymes. As well as delivering enzymes as proteins, another strategy is to deliver DNA constructs encoding the enzyme, an approach known as gene-directed enzyme prodrug therapy (GDEPT) when a nonviral vector is used or virus-directed enzyme prodrug therapy (VDEPT) when the vector is a virus.[11] The most widely used sequence for GDEPT/VDEPT is the Herpes simplex virus 1 thymidine kinase (HSV1-TK) gene.[12] When expressed in cancer cells, the 41 kDa HSV-TK enzyme converts the prodrug ganciclovir into its monophosphate derivative, which is further converted to the triphosphate by endogenous kinases. Ganciclovir is a nucleoside analogue, and the triphosphate is recognized by DNA polymerase but causes chain termination, thus arresting DNA replication and triggering apoptosis in the cell-expressing HSV1-TK.[13] Furthermore, neighboring cells are killed by the bystander effect facilitated by diffusion of the phosphorylated drug through gap junctions.[14] Although HSV1-TK is the most common sequence for GDEPT/VDEPT strategies, it has not been delivered to cells directly as an enzyme using the original ADEPT approach.

The efficacy of HSV1-TK in part reflects the delivery strategy, which should limit cytotoxicity/immunogenicity and achieve a high efficiency of tumor cell transfection.[15] In a few cases, the HSV1-TK gene has been delivered using autologous stem cells but this approach is generally inefficient and rarely used.[16] Microbial delivery systems have also been explored, as well as polymer-based vectors and liposomes.[17,18] However, the majority of reports have involved viral vectors, with adenovirus most often used in clinical trials because there is only a low risk of unplanned genomic integration in nontumor cells.[19,20] Retroviruses and lentiviruses integrate into the host genome and there is a greater risk of integration in nontumor cells, but they are less immunogenic than adenovirus and allow prolonged *TK* gene expression.[21] Both viral and nonviral delivery vectors have been endowed with additional functional motifs to promote cell targeting, endosome disruption, and the efficacy of the bystander effect.[22]

The risks of viral genome integration can be avoided through the use of virus-like particles (VLPs), which are analogous to natural virions but do not carry any infectious nucleic acids.[23] Encapsulated enzymes within the VLPs could help to overcome the immunogenetic hurdle in ADEPT. Of course, VLPs are also immunogenic; however, stealth coatings can be applied to the VLP to overcome this—the advantage is that the active ingredient (the enzyme) does not need to be modified through coatings, which may reduce or hinder its activity. Furthermore, enzymes could be delivered to target sites via passive or active targeting strategies applied to the VLP carrier system. VLPs therefore integrate the ADEPT approach with VDEPT so that a viral vector is used to deliver the enzyme as a protein rather than an expression construct. To use VLPs in this manner, the enzyme must be contained inside the particle to prevent systemic activation of the prodrug but must be released inside

the cell when its activity is required. The delivery of enzymes without the risk of leaching and systemic dissemination therefore requires modification of the VLP internal surface. We and others have previously shown that VLPs derived from Bluetongue virus (BTV) can accommodate the entire green fluorescent protein (GFP, 27 kDa) genetically fused to the N-terminus of VP3.[24–26] GFP was displayed within the internal cavity of the particles and thus allowed the formation of core-like particles (CLPs) when coexpressed with VP7 and complete VLPs when coexpressed with VP7, VP5, and VP2.

BTV is a nonenveloped, icosahedral virus in which four major structural proteins assemble to form three concentric protein shells. The innermost layer is made up of 120 copies of VP3, making this the ideal fusion partner for cargo proteins exposed in the internal cavity. The inner VP3 scaffold is surrounded by a layer of 780 copies of VP7 to form the CLP. The outer layer comprises 180 copies of VP2 and 360 copies of VP5 to form the complete VLP. The role of this outer layer is in cell entry, allowing both cell attachment and membrane fusion to occur. Bluetongue is a veterinary disease of ruminants, and we have previously shown that plant-produced BTV VLPs (but not CLPs) elicit a protective immune response in sheep.[27] Both CLPs and VLPs have been expressed in insect cells using baculovirus vectors,[28,29] but molecular farming in plants provides an inexpensive and virtually limitless supply of the particles.[27,30]

To determine whether the Bluetongue VLPs can accommodate larger proteins with catalytic activity, such as the 41 kDa TK enzyme, and whether that activity is preserved in the context of the VLP allowing the use of BTV as a nanoreactor[31] or for protein–VDEPT, we prepared constructs in which the N-terminus of VP3 was fused to the HSV1-TK gene. We selected TK variant SR39, which has greater sensitivity to ganciclovir in mouse xenograft tumor models and thus achieves greater efficacy.[32] We purified the Bluetongue CLPs and VLPs containing TK and investigated their structure and in vitro toxicity.

MATERIALS AND METHODS

Constructs.

The full-length HSV1-TK gene (Figure S1), including the SR39 mutation,[32] was codon-optimized for *Nicotiana benthamiana* and synthesized by GeneArt (Thermo Fisher Scientific). The gene was inserted upstream of the BTV-8 VP3 sequence at the *Nru*I and *Bsp*EI sites[26] to produce plasmid pEAQ-HSV1-TK:VP3, which was introduced into *Escherichia coli* TOP10 cells (Thermo Fisher Scientific). HSV1-TK on its own was cloned by amplification of the HSV1-TK gene with an end-tailoring primer to introduce a downstream stop codon and *XhoI* site, followed by transfer to the vector pEAQ-*HT*[30] to generate plasmid pEAQ-*HT*-HSV1-TK. Vector integrity was confirmed by sequencing before transforming *Agrobacterium tumefaciens* strain LBA4404. We also used the constructs pEAQ-VP7*HT*, pEAQex-VP7*HT*-VP3wt, and pEAQex-VP5*HT*-VP2*HT* to express the BTV coat proteins.[27] The maps of all constructs in this study are provided in Figure S2.

Production of CLPs and VLPs.

BTV particles were produced in *N. benthamiana* by agroinfiltration.[26,27,30] Leaf tissue was syringe-infiltrated with inoculum (0.2 or 0.3 OD units/mL per construct) in MMA buffer (10 mM MES pH 5.6, 10 mM $MgCl_2$, 100 μM acetosyringone). Co-infiltrations of two or three different *A. tumefaciens* strains therefore reached an overall OD of 0.4 or 0.6. Leaf tissue was harvested 8 days post infiltration and processed immediately.

For the production of CLPs, leaf tissue was blended in three volumes of CLP extraction buffer (50 mM bicine pH 8.4, 140 mM NaCl, 0.1% (w/v) *N*-lauroylsarcosine sodium salt, 1 mM dithiothreitol (DTT), 0.5× Roche EDTA-free Complete Protease Inhibitor Cocktail). Extracts were filtered through two layers of Miracloth (Millipore) and then clarified at 16 000*g* for 10 min at 10 °C. The clarified supernatant was loaded onto iodixanol step gradients (Optiprep, Sigma-Aldrich): 3 mL each of 50, 40, 30, and 20% (w/v) iodixanol in 50 mM Tris–HCl pH 8.4, 140 mM NaCl.

For the production of VLPs, leaf tissue was blended in three volumes of VLP extraction buffer (50 mM bicine pH 8.4, 20 mM NaCl, 0.1% (w/v) *N*-lauroylsarcosine sodium salt, 1 mM DTT, 0.5× Roche EDTA-free Complete Protease Inhibitor Cocktail). Extracts were filtered through two layers of Miracloth and then clarified at 4200*g* for 10 min at 10 °C. The clarified supernatant was loaded onto sucrose step gradients: 3 mL each of 60, 50, 40, and 30% (w/v) sucrose in 20 mM Tris–HCl pH 8.4, 20 mM NaCl.

For both BTV particles, the gradients were centrifuged at 21 500 rpm for 3 h at 10 °C in a Surespin 630 rotor (Thermo Fisher Scientific) before sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis on 4–12% NuPAGE Bis–Tris gels (Thermo Fisher Scientific) stained with InstantBlue (Expedeon). Positive fractions were pooled and buffer was exchanged (20 mM Tris–HCl pH 8.4, 20 mM NaCl) using Amicon 100 kDa MWCO columns (Millipore).

Transmission Electron Microscopy (TEM).

The integrity of CLPs and VLPs was confirmed by TEM using FEI Tecnai 30 (300 kV) and FEI Tecnai 20 (200 kV) instruments, respectively. The samples were spotted onto carbon-coated copper grids, rinsed in water, and stained with 2% (w/v) uranyl acetate. The diameters of 10 randomly selected intact particles were determined using the measure feature in Fiji v2.0.0-rc-69/1.52p and statistical analysis by one-way analysis of variance (ANOVA) in GraphPad Prism v7.0d (significance threshold, $p < 0.05$).

Cell Culture.

The human glioblastoma-derived cell line U-87 MG was purchased from ATCC and cultured in Dulbecco's modified Eagle's medium (DMEM, Cellgro) supplemented with 10% (v/v) fetal bovine serum (Atlanta Biologicals) and 1% (v/v) penicillin/streptomycin (Gibco). Cultures were maintained at 37 °C in a 5% CO_2-humidified incubator.

BTV Particle Labeling with Fluorophores and Cell Imaging.

VLPs at 2.5 mg/mL were labeled using sulfo-Cy5 NHS ester (Lumiprobe) targeting amine groups from solvent-exposed Lys side chains; sulfo-Cy5 NHS ester was added as a 10 000-fold molar excess per VLP. The reaction was carried out for 4 h at room temperature with agitation. Excess Cy5 dye was removed using 10 kDa cutoff spin filters (Millipore, 10 washes with PBS). CLP-Cy5 was produced in a similar manner and kindly provided by Dr. Frank Sainsbury (Griffith University).

Cellular trafficking of Cy5-labeled BTV particles was monitored by using U-87-MG cells; 25 000 cells were seeded on glass coverslips and incubated with 1×10^6 particles for 24 h. Cells were then washed, fixed, and treated with wheat germ agglutinin conjugated to AlexaFluor 555 (WGA555, Invitrogen) for cell membrane staining and with DAPI (Sigma-Aldrich) for nuclei staining. Imaging was conducted using a Leica TCS SPE confocal microscope with a 63× oil immersion objective.

MTT Cytotoxicity Assays.

U-87 MG cells were seeded at 2000 cells per well in a 96-well plate 24 h prior to treatment, with 100 μL of medium per well, and were allowed to grow for 24 h at 37 °C in a 5% CO_2-humidified incubator. The particles (1×10^5 particles per well) were added to triplicate wells followed by different concentrations (1, 10, 25, 50, 100, 250, 500, 1000, and 2000 μM) of the prodrug ganciclovir (Sigma-Aldrich) in triplicate. The cells were incubated for 24 h at 37 °C in a 5% CO_2-humidified incubator, then washed with PBS to remove excess reagents, and allowed to recover for 48 h in fresh medium. Finally, we added 10 μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent (ATCC) and measured the absorbance at 590 nm using a Tecan Infinite M200 PRO plate reader.

RESULTS AND DISCUSSION

Preparation of BTV Particles Containing HSV1-TK Protein.

The molecular mass of the HSV1-TK protein is 41 kDa, so the utilization of BTV particles containing the HSV1-TK SR39 mutant for protein–VDEPT requires efficient particle assembly even when the fusion partner linked to VP3 is more than 50% larger than GFP. We therefore infiltrated *N. benthamiana* plants with the expression constructs for VP3 and VP7 (to generate wild-type CLPs) or VP2, VP3, VP5, and VP7 (to generate wild-type VLPs). We also performed parallel experiments with TK:VP3 in place of wild-type VP3 in an attempt to generate CLPs and VLPs containing HSV1-TK (Figure 1). Finally, we infiltrated plants with the HSV1-TK construct alone to determine the effect of the nonencapsulated enzyme on plant tissues.

Extraction and Structural Analysis of TK-BTV Particles.

We extracted wild-type CLPs and TK-CLPs from fresh *N. benthamiana* leaf tissue 8 days post infiltration (dpi, Figure 2). The analysis of gradient fractions by SDS-PAGE revealed the presence of two prominent bands in both preparations, one representing VP7 (39 kDa) and the other representing wild-type VP3 (103 kDa) in the CLP extract or TK:VP3 (144 kDa) in the TK-CLP extract (Figure 3A). A faint third band was observed in the TK-CLP

sample matching the molecular weight of wild-type VP3, and this increased in intensity during prolonged storage at 4 °C, suggesting that it was a TK:VP3 degradation product (Figure S3).

Wild-type VLPs and TK-VLPs were also extracted and purified 8 dpi. SDS-PAGE analysis revealed the same bands present in the CLP extracts (VP7 plus wild-type VP3 or TK:VP3) and additional bands representing VP5 (59 kDa) and VP2 (111 kDa) as expected (Figure 3B). In the TK-VLP preparation, there was initially no band corresponding to wild-type VP3 but this band appeared with prolonged storage at 4 °C, along with a very faint band corresponding to the released TK. These bands are likely degradation products of TK:VP3, as was shown for TK-CLPs (Figure S3). Together, this data indicates that all VP3 present in the assembled TK-VLPs and TK-CLP particles is initially in the form of the intact fusion protein; however, prolonged storage should be avoided as data indicate cleavage over time.

The TK:VP3 band was excised, purified, and digested with trypsin and its identity was confirmed by mass spectrometry (nanoLC-MS/MS), with good coverage of the entire fusion protein (Figure S1). Mass spectrometry was also used to confirm the identity of the degradation products arising during extended storage, and it was confirmed that these were indeed derived from TK:VP3. In previous studies using a GFP-VP3 construct, we also observed longitudinal breakdown of the VP3 fusion proteins.[27] In the case of these fluorescent particles, we found that the majority of fluorescence remained associated with the particles, as evidenced by their sedimentation in density gradients. Similarly, the breakdown products of TK-VP3 cosediment with VP7 (Figure S3) Both GFP:VP3 and TK:VP3 fusion proteins were constructed with a flexible serine–glycine–glycine linker between GFP/TK and VP3. It is likely that this linker region is under some strain in the assembled particles and may be prone to breaking; since the particles remain intact, we would argue that proteolytic cleavage is less likely; however, without further investigation, it cannot be ruled out. A further weak point may be present within VP3, leading to a further breakdown product of ~60 kDa (Figure S3).

Of note, agroinfiltration with the soluble HSV1-TK construct induced severe necrosis in the leaf tissue around the infiltration site, whereas the expression of wild-type CLPs or VLPs, TK-CLPs, and TK-VLPs had no deleterious effect (Figure 2). This indicated that the enzyme sequestered within the CLPs and VLPs did not affect the host plant in the same way as the soluble enzyme (see further discussion in the Supporting Information).

Confirmation of TK-BTV Particle Integrity.

TEM analysis to investigate particle integrity (Figure 3C) revealed that the TK-CLPs were similar in size and morphology to the wild-type CLPs, with prominent spikes as previously reported for BTV core particles with an intact VP7 layer.[33] Most of the particles remained intact after purification (92% for wild-type CLP and 71% for TK-CLP). The diameters of the wild-type CLPs and TK-CLPs were 66.3 ± 2.3 and 67.7 ± 3.5 nm, respectively. As anticipated, the VLPs were generally larger than the CLPs ($p < 0.0001$) The diameters of the VLPs and TK-VLPs were 82.0 ± 2.3 and 81.9 ± 2.3 nm, respectively, consistent with previous reports.[24,25] The variation in VLP size was ~3% compared to >5% for the CLPs, suggesting that the VLPs are more stable than the CLPs.

Cancer Cell Interactions and Cell Killing.

Having established that the TK-VP3 fusion protein supports the assembly of stable CLPs and VLPs that do not have a negative impact on the plant expression host, we next tested the ability of the particles to interact with human glioblastoma cells (cell line U-87 MG) and to kill them in the presence of the prodrug ganciclovir. We first prepared CLP and VLP samples labeled with Cy5 and monitored the fate of the particles in cell culture. Figure 4A indicates that both particles were taken up efficiently in U-87 MG cells; the punctate pattern may indicate uptake and trafficking through vesicles. Interestingly, the TK-CLPs had no effect (at least within the concentration range we tested), but the TK-VLPs showed a ganciclovir-dose-dependent effect with an IC_{50} value of 14.8 μM against U-87 MG cells (Figure 4B,C). As anticipated, the wild-type CLPs and VLPs had no cytotoxic effect on the cells. TK requires cytosolic adenosine triphosphate (ATP) to complete the phosphorylation of ganciclovir, suggesting that the TK-VLPs must escape from endosomes soon after uptake and that perhaps the inactivity of the CLPs reflects their inability to escape, becoming trapped within the endosomal compartment. In a natural BTV infection, the outer capsid proteins function in cell attachment (VP2) and membrane fusion (VP5), allowing the virus core particle (VP3 and VP7 surrounding the virus transcriptional machinery) to be released into the cytoplasm.[34,35] Mammalian viruses have evolved to navigate their host cells successfully, enabling the delivery of their nucleic acid to the cytosol or nucleus (depending on the replication strategy). Intriguingly, our data show that effective trafficking is maintained for the disarmed VLPs, meaning that cellular uptake and the entire intracellular trafficking of the virus are mediated by the capsid proteins without nucleic acids. This is why virus-based vectors remain the predominant vehicle for gene delivery, whereas synthetic vectors, despite their enhanced safety profile, tend to be less effective because they cannot overcome the many biological barriers between them and the appropriate cellular compartment. We found that the VLP behaved like the native virus and was efficiently trafficked to the cytosol due to its “smart” cloak of VP2/VP5. The CLP lacked the requisite functions and behaved more like a “dumb” synthetic particle because the VP3/VP7 proteins could not facilitate escape from the endosome.

CONCLUSIONS

We have shown that VLPs derived from BTV provide an effective platform for the delivery of enzymes, using 41 kDa HSV1-TK as a case study. HSV1-TK is the most common “suicide gene” delivered by GDEPT/VDEPT but it was never considered for the original ADEPT strategy, perhaps due to its size or perhaps just because its origins as a selectable marker ensured that GDEPT/VDEPT was the “path of least resistance”. However, the direct delivery of enzymes rather than genes can be advantageous because nucleic acids are recognized as pathogen-associated molecular patterns (PAMPs) by the immune system and, in the case of DNA, there is also the risk of genomic integration. Furthermore, the delivery of proteins may necessitate more frequent doses to achieve a therapeutic effect, but any adverse effects will be short-lived and easier to address. We therefore developed a protein–VDEPT procedure in which the VDEPT approach was used to deliver the TK enzyme as a protein rather than as a gene. We found that the TK-VLPs killed human glioblastoma cells efficiently in the presence of ganciclovir, with an IC_{50} value of 14.8 μM. Conversely, CLPs

were ineffective vehicles for the delivery of enzymes because they remain trapped in the endosomal compartment, in common with many synthetic nanoparticles. VLPs are advantageous because they can escape from endosomes and therefore allow HSV1-TK to access the cytosolic ATP required for the phosphorylation of ganciclovir. Further in vitro studies will be needed to systematically understand this delivery system. Parameters such as VLP concentrations, VLP toxicity, and interaction with different (cancer) cell types are required to determine how TK-VLPs behave in vitro and in vivo and to develop strategies of targeting of TK-VLPs specifically to tumor tissues. Our new protein–VDEPT strategy with TK protein offers a promising new modality for the treatment of cancer with systemic prodrugs such as ganciclovir.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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The authors declare the following competing financial interest(s): G.P.L. declares that he is a named inventor on granted patent WO 29087391 A1 which describes the HyperTrans expression system and associated pEAQ vectors used in this manuscript.

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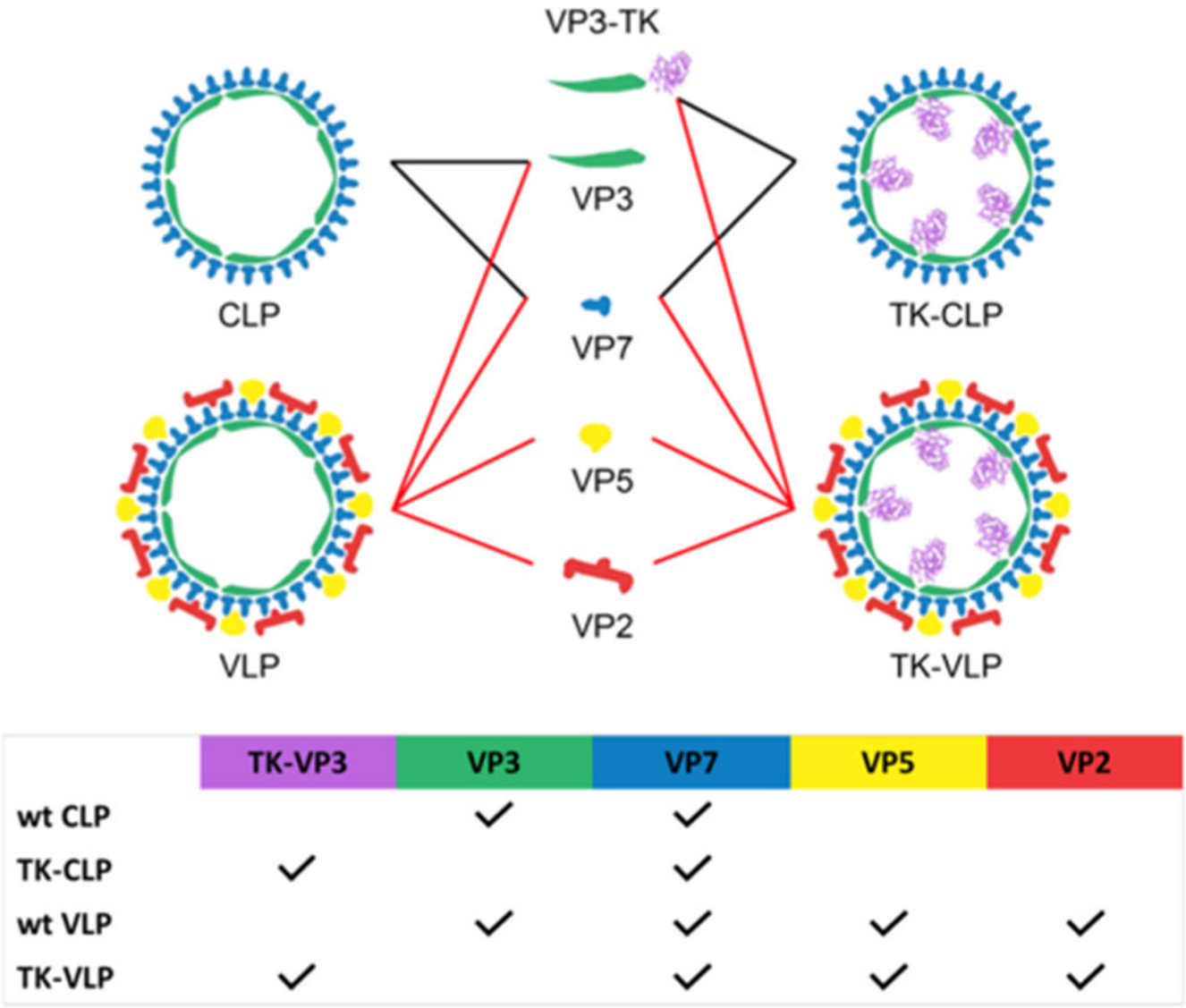

	TK-VP3	VP3	VP7	VP5	VP2
wt CLP		✓	✓		
TK-CLP	✓		✓		
wt VLP		✓	✓	✓	✓
TK-VLP	✓		✓	✓	✓

Figure 1.

Schematic illustration of the assembly of wild-type core-like particles (CLPs) from coat proteins VP3 and VP7 and virus-like particles (VLPs) from VP2, VP3, VP5, and VP7, as well as analogues incorporating the TK:VP3 fusion construct (HSV1-TK shown in purple). The table shows the protein composition (top) of the four different particle types (left).

Figure 2.

HSV1-TK causes necrosis in leaf tissues when expressed on its own, but not when it is encased in BTV CLP or VLP. Plants were infiltrated with constructs as follows (each at OD = 0.3) and imaged at 7 dpi. HSV1-TK: pEAQ-*HT*-HSV1-TK; empty vector: pEAQ-*HT*; TK-CLP: pEAQ-HSV1-TK:VP3, pEAQ-VP7*HT* (overall OD = 0.6); TK-VLP: pEAQ-HSV1-TK:VP3, pEAQ-VP7*HT*, pEAQex-VP5*HT*-VP2*HT* (overall OD = 0.9).

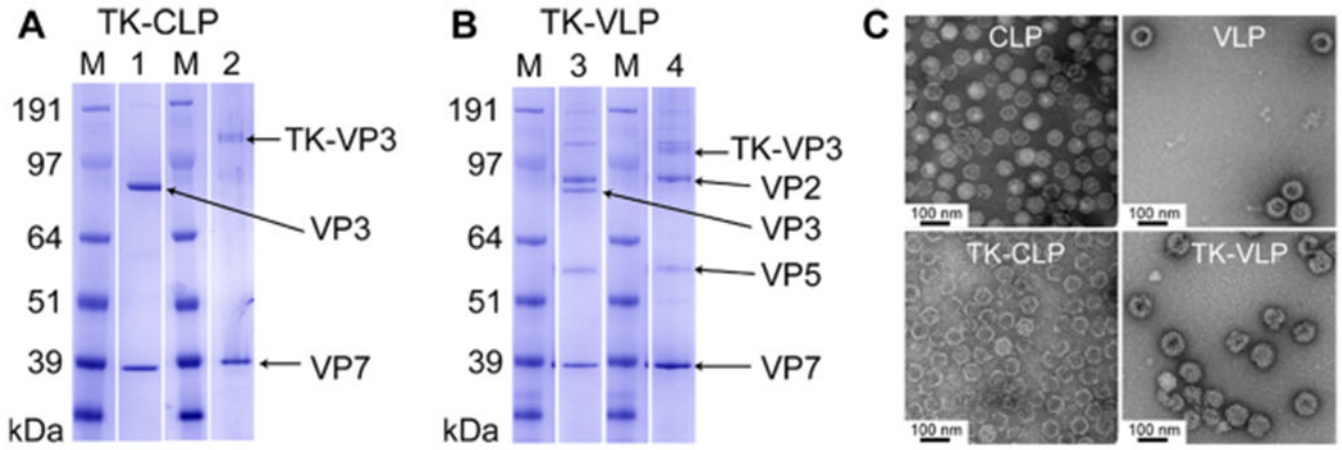

Figure 3.

Characterization of VLPs and CLPs. (A) SDS-PAGE analysis of purified wild-type CLPs (lane 1) and TK-CLPs (lane 2). (B) SDS-PAGE analysis of purified wild-type VLPs (lane 3) and TK-VLPs (lane 4). SDS-PAGE was carried out under denaturing conditions, and the gels were stained with InstantBlue. M = size marker. (C) TEM images of assembled CLPs and VLPs.

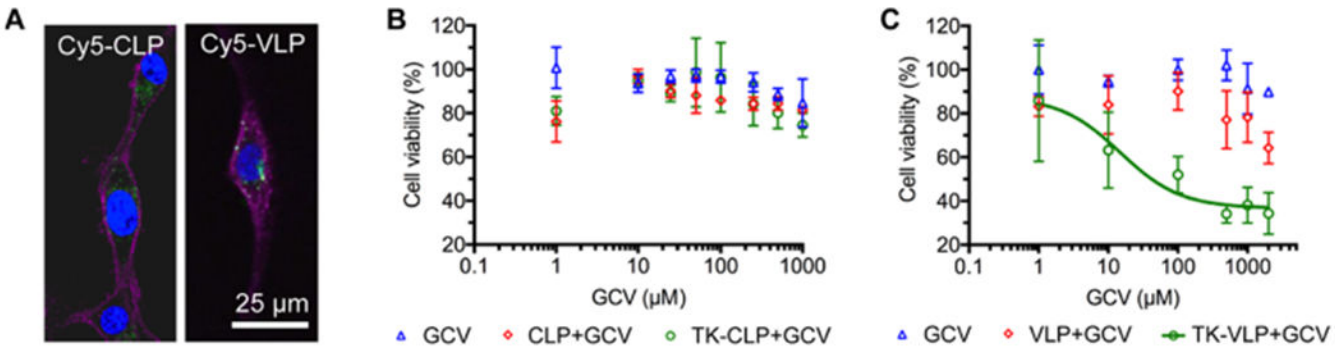

Figure 4.

Intracellular localization and cytotoxicity of BTV particles in the context of prodrug activation therapy. (A) Uptake of Cy5-VLPs and Cy5-CLPs by U-87 MG cells, with the particles shown in green (Cy5), nuclei shown in blue (DAPI), and membranes in magenta (WGA555). (B, C) MTT assays to determine the viability of U-87 MG cells exposed for 24 h to various concentrations of ganciclovir (GCV, *x*-axis), in combination with (B) wild-type CLPs or TK-CLPs, and (C) wild-type VLPs or TK-VLPs. Cells were also exposed to ganciclovir alone as an additional control.