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Combinatorial targeting of 2 different steps in adenoviral DNA replication by herpes simplex virus thymidine kinase and artificial microRNA expression for the inhibition of virus multiplication in the presence of ganciclovir

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Abstract

Background: Human adenoviruses are a frequent threat to immunocompromised patients, and disseminated disease is associated with severe morbidity and mortality. Current drugs are not capable of preventing all fatalities, thus indicating the need for alternative treatment strategies. Adenoviruses can be rendered susceptible to antiherpetic prodrugs such as ganciclovir (GCV), upon expression of the herpes simplex virus thymidine kinase (HSV-TK) gene in adenovirus-infected cells. Furthermore, adenoviruses are amenable to post-transcriptional gene silencing via small interfering RNAs (siRNAs) or artificial micro RNAs (amiRNAs).

Results: In this study, we combined these 2 approaches by constructing a combinatorial gene expression cassette that comprises the HSV-TK gene and multiple copies of an amiRNA directed against the mRNA encoding the adenoviral preterminal protein (pTP). HSV-TK gene expression was controlled by the adenoviral E4 promoter, which is activated in the presence of the adenoviral E1 gene products (i.e., when adenovirus is present in the cell). When inserted into a replication-deficient (E1-, E3-deleted) adenoviral vector, this cassette effectively inhibited the replication of wild-type adenovirus *in vitro*. The reduction rate mediated by the combinatorial approach was higher compared to that achieved by either of the 2 approaches alone, and these obvious additive effects became most pronounced when the GCV concentration was low.

Conclusions: The concept presented here has the potential to aid in the inhibition of wild-type adenovirus replication. Furthermore, the combinatorial expression cassette may constitute a safeguard to potentially control unintended replication of adenoviral vectors and to prevent immune responses provoked by them.

Keywords: Adenovirus, Vector, RNA interference, MicroRNA, Ganciclovir, HSV-TK

Background

Human adenoviruses are double-stranded (ds) DNA viruses that represent a major risk for immunocompromised patients [1-3], and severe manifestations of adenoviral infections can be life-threatening. Mortality rates as high as 80% have been reported in cases of disseminated disease [4-8]. The incidence of disseminated

disease is highest among hematopoietic stem cell transplant recipients, and adenoviruses belonging to species B and C are the main cause of severe adenovirus infections [3,9-11].

Cidofovir (CDV) is the most commonly used agent for the treatment of adenovirus infections. Although the drug demonstrates clinical efficacy, its activity is not sufficient to prevent fatal outcomes in all instances [12-16], and derivatives of CDV are still being evaluated. Thus, alternative strategies to treat severe adenovirus infections have been developed. Donor lymphocyte infusion

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therapy, and particularly the adoptive transfer of adenovirus-specific T cells represents a promising approach for the treatment of immunocompromised patients [17,18], but its efficacy is still under investigation.

We and others recently investigated the potential of RNA interference (RNAi)-mediated silencing of adenoviral gene expression in the control of the multiplication of adenoviruses *in vitro* [19-22]. RNAi-based approaches to silence viral and non-viral genes employ either the transduction of cells with short interfering RNAs (siRNAs) or the intracellular generation of short hairpin RNAs (shRNAs) and precursors of artificial miRNAs (amiRNAs), respectively, from DNA sequences introduced into those cells [23-28]. In contrast to exogenously added siRNAs, shRNAs and precursor amiRNAs must undergo intracellular processing through the RNAi pathway prior to recognizing their respective target mRNAs and eventually mediating their destruction or causing translational repression. By employing siRNAs directed against a set of adenoviral transcripts required for very different viral processes, genes essential for adenoviral DNA synthesis (i.e., those encoding the pre-terminal protein (pT) and the viral DNA polymerase) emerged as promising targets for the inhibition of virus multiplication [22]. Furthermore, in a modification of the approach, an amiRNA directed against the pTP mRNA was introduced into wild-type (wt) adenovirus-infected cells via adenoviral vectors [21]. In both approaches, the output of infectious virus progeny from infected cells could be decreased by several orders of magnitude, indicating that RNAi-based methods can, in principle, be employed to control adenovirus replication.

In a very distinct approach, we rendered adenovirus susceptible to treatment with the antiherpetic compound, ganciclovir (GCV), through the targeted expression of the herpes simplex virus thymidine kinase (HSV-TK) gene in wt Ad5-infected cells [29]. GCV is a prodrug that requires phosphorylation by herpes virus-encoded thymidine kinases to be efficiently converted into its active form [30]. Once activated (i.e., when present in its tri-phosphorylated form), GCV functions as a nucleoside analog, blocking both cellular and viral DNA synthesis by competing with dGTP for incorporation into nascent DNA strands [31,32]. The first phosphorylation step, which is efficiently carried out only by thymidine kinases encoded by herpes viruses (and not by adenoviruses or other viruses), explains the selectivity of GCV for herpes virus-infected cells [33]. Targeted expression of HSV-TK in wt Ad5-infected cells was accomplished by inserting the HSV-TK open reading frame downstream of the Ad5 E4 promoter, whose activity is strongly increased in the presence of the Ad5 E1A gene products (i.e., in cells infected with wt adenovirus) [34,35]. Introducing the HSV-TK expression cassette into wt Ad5-infected cells via

a replication-deficient adenoviral vector lacking the E1A region strongly inhibited wt Ad5 DNA replication upon treatment of the cells with low concentrations of GCV, while no obvious effects on viability were observed in cells not infected with wt Ad5.

In the study presented here, we integrated the 2 approaches by generating adenoviral vectors that express both the HSV-TK gene from the adenoviral E4 promoter and, from a distinct expression unit, multiple copies of an amiRNA directed against the wt Ad5 pTP mRNA. We present data indicating that, upon treatment with GCV, the simultaneous expression of both cassettes in wt Ad5-infected cells results in additive anti-adenoviral effects *in vitro*. Moreover, we demonstrate that the additional expression of amiRNAs directed against viral pTP transcripts allows for lower levels of GCV treatment without a loss of antiadenoviral activity. Finally, we discuss how this combinatorial gene expression cassette may be used as a safeguard to potentially control unintended replication of adenoviral vectors and to prevent immune responses provoked by them.

Methods

Cell culture, virus amplification, and titer determination

HEK 293 (human embryonic kidney; ATCC CRL-1573), A549 (human epithelial lung carcinoma; ATCC CCL-185), and T-REx-293 cells (Life Technologies Austria, Vienna, Austria) were cultivated in Dulbecco's Modified Eagle's Medium (DMEM) with stabilized glutamine (PAA Laboratories, Pasching, Austria) and supplemented with 10% fetal bovine serum (FBS; PAA Laboratories) in a humidified 5% CO₂ atmosphere at 37°C. Recombinant adenoviral vectors expressing Ad5-directed amiRNAs alone or in combination with the HSV-TK gene were amplified in T-REx-293 cells. Titers of recombinant adenoviruses expressing amiRNAs were determined on T-REx-293 cells by 50% tissue culture infective dose (TCID₅₀) assays. Titers of wt Ad5 present in mixed virus suspensions containing both wt and recombinant virus as obtained in co-infection experiments were determined on A549 cells using the same method. All other TCID₅₀ assays were performed with HEK 293 cells. Crude virus suspensions for titer determination were obtained by freeze-thawing the samples thrice and removing cell debris by centrifugation at 2800 rpm for 15 min.

Vector construction

Adenoviral vectors for the combinatorial expression of amiRNAs and HSV-TK were generated by first constructing plasmid vector versions thereof. These "entry" vectors are based on Life Technologie's Gateway system for recombination-mediated cloning. From these entry vectors, the expression cassettes were eventually transferred into the adenoviral vector backbones via site-

specific recombination. All entry vectors for combinatorial amiRNA/HSV-TK expression are based on pEE4_TK [29] and carry the herpes simplex virus 1 thymidine kinase gene downstream of the Ad5 E4 promoter. To generate the combinatorial vectors, the amiRNA expression cassettes were inserted into the *XmnI* site located downstream of the HSV-TK expression unit. The amiRNA expression cassettes comprise a CMV promoter/enhancer followed by a 2xTetO2 tetracyclin repressor binding site, and end with a BGH poly(A) site. This fragment was obtained by PCR amplification from pcDNA4/TO (Life Technologies Austria, Vienna, Austria) by using primers CMV-TO-f1(5'-TTGCATTTCTGAATCTGCTTAGGGT TAGG-3') and BGHpA-r2 (5'-CCCAGCGAATTCT TTCCGCCTCAGAAG-3'). The *BclI* site, located downstream of the promoter/operator region, was used for insertion of the EGFP/amiRNA cassettes, which comprise an open reading frame for EGFP and 1 or 6 copies of either the pTP-mi5 amiRNA or the universal, non-targeting amiRNA inserted into the EGFP 3'UTR. These cassettes were obtained by PCR amplification from vectors pcDNA6.2-GW/EmGFP-miR-neg (Life Technologies; carrying 1 copy of the non-targeting negative control amiRNA), pmiREx6 ([21]; carrying 6 copies of the non-targeting negative control amiRNA), pmiRE-pTP-mi5 ([21]; carrying 1 copy of pTP-mi5), and pmiRE-pTP-mi5x6 ([21]; carrying 6 copies of pTP-mi5) using primers pmiRE-f2 (5'-CAAAAATGATCACTTT AAAACCATGGTGAGC-3') and pmiRE-r2 (5'-AA GCTGTGATCAGATATCTCGAGTGCGGC-3'). In all amiRNA expression cassettes, the sequences giving rise to pre-amiRNA hairpins are flanked by sequences derived from the murine Mmu-miR-155 pri-miRNA. The final entry vectors were designated pTO-TK-mi- and pTO-TK-mi-x6 (carrying 1 or 6 copies of the non-targeting negative control amiRNA, respectively), and pTO-TK-pTP-mi5 and pTO-TK-pTP-mi5x6 (carrying 1 or 6 copies of pTP-mi5, respectively).

Eventually, the expression cassettes present in the entry vectors were cloned into the deleted E1 region of the adenoviral vector pAd/PL-DEST (Life Technologies Austria, Vienna, Austria), giving rise to the combinatorial adenoviral vectors AdTO-TK-mi-, AdTO-TK-mi-x6, AdTO-pTP-pTP-mi5, and AdTO-pTP-pTP-mi5x6 (Figure 1). This final cloning step was mediated by Life Technologies' Gateway technology (i.e., by site-specific recombination between sequences flanking the expression cassettes in the entry vectors and the corresponding sequences located in the adenoviral vector). The recombination reaction was performed according to the instructions of the manufacturer (Life Technologies Austria, Vienna, Austria). The construction of the adenoviral vectors AdEE4, AdEE4-TK, Ad-mi-, AdTO-mi-x6, and AdTO-pTP-mi5x6 has been described [21,29].

Restriction enzymes and DNA-modifying enzymes were purchased from Fermentas (St. Leon-Rot, Germany) or New England Biolabs (Frankfurt am Main, Germany). PCR was performed with Pwo DNA polymerase obtained from Roche Diagnostics (Vienna, Austria) or PEQLAB (Erlangen, Germany).

Nucleic acid extraction

For the extraction of circular plasmid DNA, an EasyPrep Pro Plasmid Miniprep Kit (Biozym, Oldendorf, Germany) or a HiSpeed Plasmid Midi Kit (QIAGEN, Hilden, Germany) was used. PCR products were purified using a QIAquick PCR Purification Kit (QIAGEN, Hilden, Germany), and adenoviral DNA was isolated with a QIAamp DNA Blood Mini Kit (QIAGEN, Hilden, Germany).

Virus replication experiments

For inhibition of adenovirus replication by siRNA-mediated gene silencing and concomitant HSV-TK expression/GCV treatment, 3×10^4 A549 cells were seeded into the wells of a 96-well plate and transfected with 30 nM siRNA specific for transcripts of the viral DNA polymerase (Pol-si2; sense 5'-CAACGUCUCCAGCGUC CAACCAUA-3', antisense 5'-UAUGGUUGGACGCU GGAAGACGUUG-3'), preterminal protein (pTP-si8; sense 5'-GAAAUUGAUUCUGUCGAACUCUCUU-3', antisense 5'-AAGAGAGUUCGACAGAAUCAUUUC-3'), IVa2 (IVa2-si2; sense 5'-AAAUACAGUCCAAGAU GCAUCUCAU-3', antisense 5'-AUGAGAUGCAUCUU CGGACUGUAUUU-3'), hexon (Hex-si2; sense 5'-GAGAACUAAUGGGCCAACAAUCUAU-3', antisense 5'-AUAGAUUGUUGGCCCAUUAGUUCUC-3'), or protease (Prot si-1; sense 5'-GAGCAGGAACUGAAAG CCAUUGUCA-3', antisense 5'-UGACAAUGGCUUUC AGUUCUGCUC-3'). A universal, non-targeting siRNA (Life Technologies) served as a negative control. The functionality of all siRNAs was assessed previously [22]. At 24 h after transfection, the cells were transduced with the adenoviral vectors AdEE4-TK or AdEE4 (Figure 1) at an MOI of 100 TCID₅₀/cell. At 24 h after transduction, the cells were infected with Ad5 at an MOI of 0.01 TCID₅₀/cell and cultivated in the presence or absence of 1.2 μM GCV for an additional 2 days before extraction of DNA and determination of wt Ad5 genome copy numbers.

Inhibition of adenovirus replication by amiRNAs and/or HSV-TK expression/GCV treatment was assessed by seeding 3×10^4 A549 cells into the wells of 96-well plates, followed by transduction with the adenoviral vectors encoding HSV-TK and one or more amiRNA copies at an MOI of 100 TCID₅₀/cell. After 24 h, the cells were infected with wt Ad5 at an MOI of 0.01 TCID₅₀/cell and cultivated in the presence of GCV at concentrations

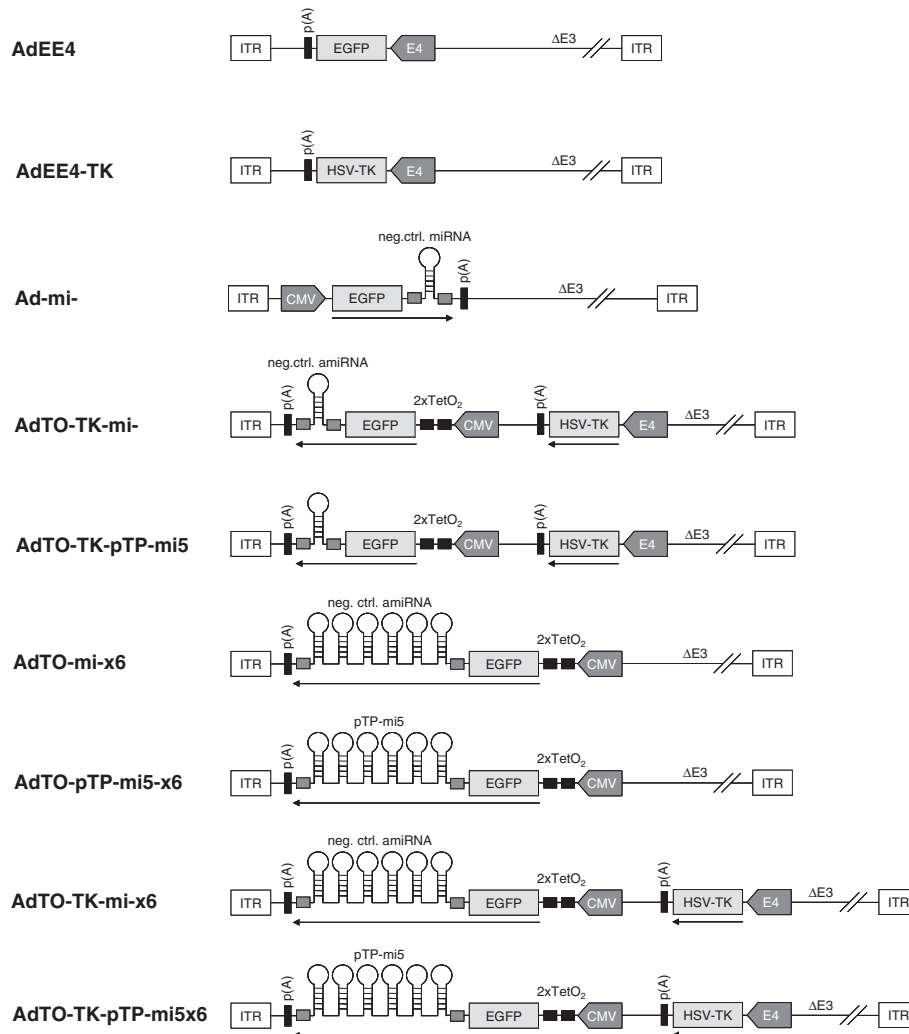


Figure 1 Schematic representation of adenoviral vectors used in this study. All vectors were based on the Ad5-derived vector, pAd/PL-DEST™ (Life Technologies), and they lack the E1 and E3 genes. Expression cassettes were inserted into the deleted E1 region in the antisense orientation with respect to the left inverted terminal repeat (ITR). Expression cassettes contained the indicated open reading frames (light grey) for the enhanced green fluorescent protein (EGFP) or the herpes simplex virus 1 thymidine kinase (HSV-TK). Gene expression was driven by the cytomegalovirus promoter (CMV) or the adenoviral E4 promoter (E4). Promoters are indicated as boxed grey arrows. The 2 copies of the tetracycline repressor-binding sequence are indicated as 2xTetO₂. Mmu-miR-155-derived sequences flanking the hairpins that give rise to mature amiRNAs are indicated as small, dark grey boxes. Polyadenylation sequences are indicated as p(A).

ranging between 0 and 1.2 μ M for up to 6 days. The cultures were subjected either to DNA isolation for determination of wt Ad5 genome copy numbers or to TCID₅₀ analysis.

The inhibitory effect of the HSV-TK/amiRNA expression cassette on the replication of vector AdTO-TK-pTP-mi5x6 was determined by transducing 1.2e + 05 T-REx-293 cells with the vector at an MOI of 0.01 TCID₅₀/cell. At the time of transduction, amiRNA expression was induced by adding 1 μ g/ml doxycycline to the medium, and GCV was added at concentrations ranging between 0 and 1.2 μ M. At 48 h after transduction,

the cultures were subjected to DNA isolation and determination of vector copy numbers by qPCR.

Determination of adenovirus genome copy numbers

Determination of wt Ad5 DNA levels was performed by qPCR using a TaqMan primer/probe set specific for the E1A gene (E1A-fwd 5'-GACGGCCCCCGAAGATC-3', E1A-rev 5'-TCCTGCACCGCCAACATT-3', and E1A-p 5'-CGAGGAGGCGGT'TTCGCAGA-3'). For the determination of DNA levels of recombinant adenoviral vectors, a TaqMan primer/probe set specific for the hexon gene was used (hexon-fwd 5'- CACTCATATTTCTT

ACATGCCCACTATT-3', hexon-rev 5'-GGCCTGTTGGGCATAGATTG-3', hexon-probe 5'-AGGAAGTA ACTCACGAGA ACTAATGGGCCA-3'). Adenovirus genome copy numbers were determined using serial dilutions of an adenoviral reference DNA as a standard.

FACS analysis

EGFP expression rates were determined by FACS analysis. Cells transduced with EGFP-expressing adenoviruses were harvested by trypsinization, resuspended in normal cell culture medium, and pelleted by centrifugation at 1200 rpm for 5 min. Thereafter, cells were washed once with phosphate buffered saline (PBS) and fixed with 1% formaldehyde in PBS. Samples were analyzed with a FACS Calibur analyzer (Becton Dickinson, Heidelberg, Germany), and percentages of fluorescent cells and mean fluorescence intensities (MFIs) were calculated.

Statistical analysis

All the data are expressed as mean \pm standard deviation (SD). In cases in which the dataset consisted of only 2 groups of samples, Student's *t*-test was applied to test for statistical significance. For the analysis of larger datasets, one-way ANOVA, corrected with Bonferroni's post-hoc test, was applied. A *p*-value less than 0.05 was considered statistically significant.

Results

Adenovirus-directed siRNAs increase the HSV-TK/GCV-mediated anti-adenoviral effect

We have previously shown that siRNAs or adenoviral vector-encoded amiRNAs targeting viral mRNAs coding for essential viral DNA synthesis components (i.e., the viral DNA polymerase and pTP, respectively) can inhibit wt Ad5 replication *in vitro* [21,22]. We have also demonstrated that the targeted expression of HSV-TK in wt Ad5-infected cells renders adenovirus amenable to inhibition by GCV, through the suppression of viral DNA synthesis [29]. Thus, it is conceivable that a combination of the 2 approaches can lead to additive effects.

To obtain evidence for such additive effects, we transfected A549 cells with the panel of siRNAs directed against the hexon, viral protease, IVa2, pTP, and viral DNA polymerase mRNAs selected in the previous study. Subsequently, cells were transduced with the adenoviral HSV-TK expression vector, AdEE4-TK, or its respective negative control vector, pADEE4 carrying an EGFP gene instead of the HSV-TK gene, and were treated with 1.2 μ M GCV. This concentration is in the range of patient serum levels after treatment with typical doses of GCV [36-38], and has previously been shown by us to inhibit wt Ad5 replication in cells expressing HSV-TK from AdEE4-TK, while leaving cells not infected with wt

Ad5 unaffected [29]. Finally, cells were infected with wt Ad5, and 48 h after infection, wt Ad5 genome copy numbers were determined. Transfection of siRNA alone inhibited wt Ad5 replication to an extent comparable to that obtained in our previous study (Figure 2A, left panel). As already demonstrated, siRNAs targeting early transcripts (among them the pTP and DNA polymerase mRNA-targeting siRNAs) were much more efficient than those targeting late transcripts (i.e., those coding for the hexon protein and the viral protease). The highest inhibition rates were obtained with the DNA replication-targeting anti-pTP and anti-DNA polymerase siRNAs, with the latter leading to an inhibition rate of 2 orders of magnitude (Figure 2A, left panel). Alternatively, HSV-TK expression alone decreased wt Ad5 genome copy numbers by 2.3 orders of magnitude (Figure 2A, right panel). However, wt Ad5 genome copy numbers declined even further upon concomitant transfection of cells with the siRNAs (Figure 2A, right panel). Again, the viral DNA replication-affecting siRNAs led to the most prominent additive effects. These effects were not only visible as decreased wt Ad5 genome copy numbers, but also as a reduction in the output of infectious virus progeny (Figure 2B).

Combined HSV-TK and amiRNA expression increases the anti-adenoviral effect in the presence of GCV

These results prompted us to generate a combinatorial adenoviral vector harboring the HSV-TK expression unit, such as that present on AdEE4-TK, and an amiRNA expression cassette, as found in AdTO-pTP-mi5. In our previous study [21], an amiRNA (pTP-mi5) targeting the Ad5 pTP mRNA was identified as the most potent amiRNA for inhibition of wt Ad5 DNA replication *in vitro*. Thus, in the present study, we merged the adenoviral HSV-TK expression vector, AdEE4-TK, with the adenoviral pTP-mi5 expression vector, AdTO-pTP-mi5, to create the adenoviral vector AdTO-TK-pTP-mi5 (Figure 1). A corresponding negative control vector (AdTO-TK-mi-) carrying an expression cassette for a negative control amiRNA instead of pTP-mi5 was also constructed. We decided to use replication-deficient adenoviral vectors for the combined HSV-TK/amiRNA expression and delivery, because this type of vector may prove advantageous in an envisioned therapeutic application. Because of the shared organ tropism of the adenoviral vector and the wt virus, such a vector may ensure the delivery of the expression cassettes into those cells that are also the preferred targets of the wt virus.

Because efficient amplification of adenoviral vectors containing an adenoviral DNA replication-targeting amiRNA cassette in packaging cells requires the shut-down of amiRNA expression in these cells, amiRNA expression in our system is under the control of a

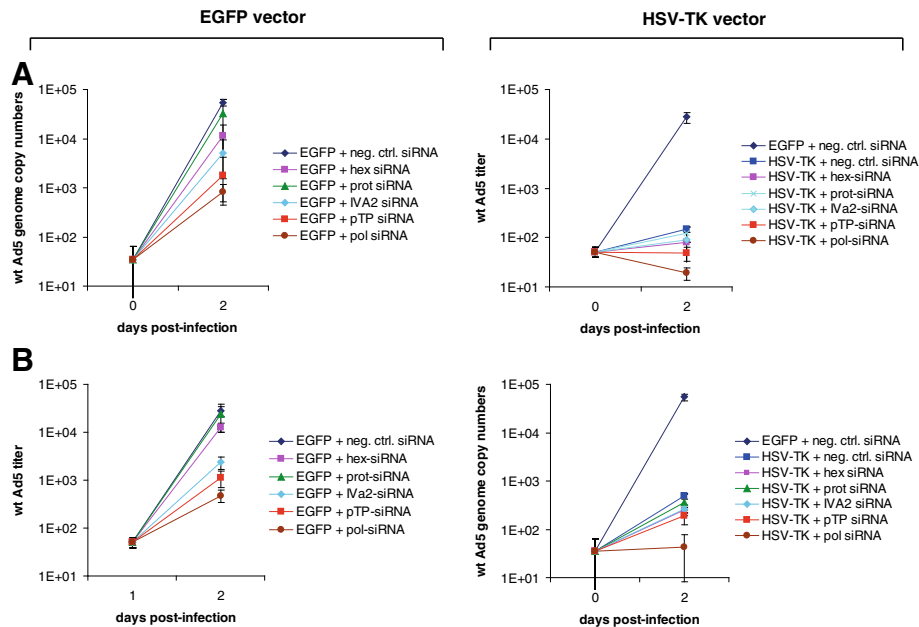


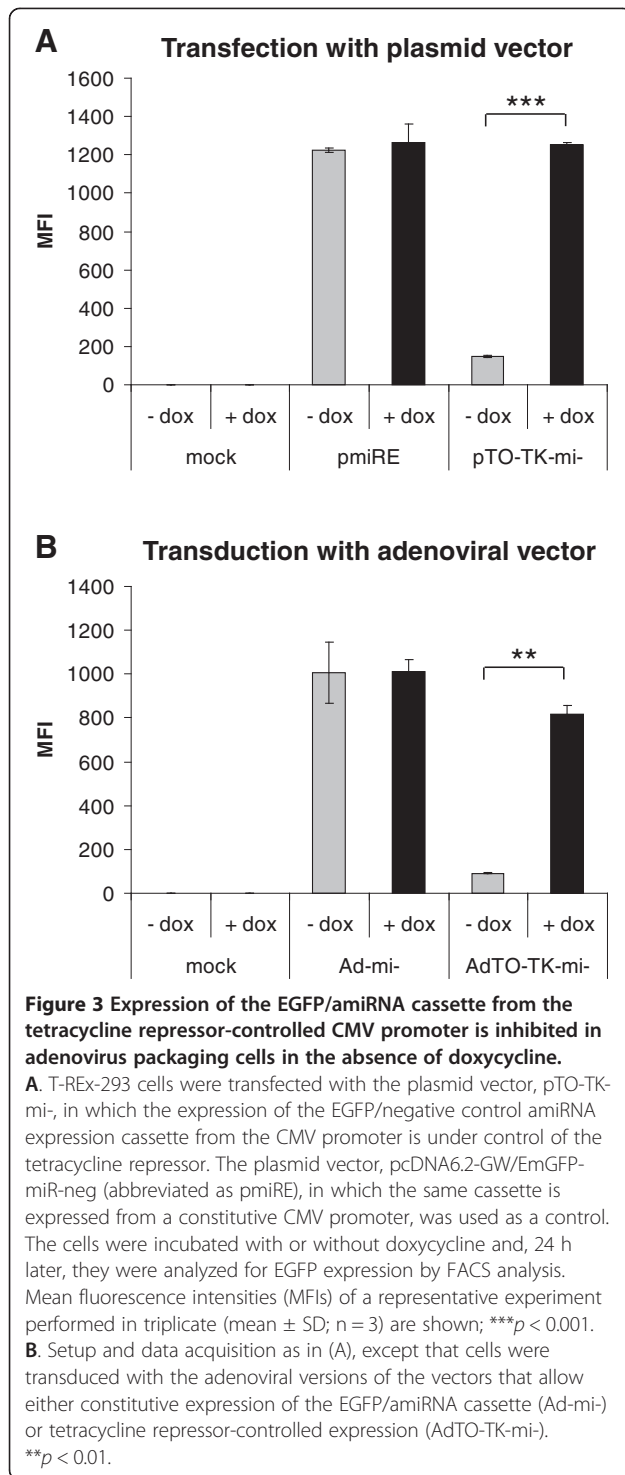
Figure 2 A combination of HSV-TK expression/GCV treatment and siRNA-mediated silencing of adenoviral gene expression leads to an additive inhibitory effect on adenoviral DNA replication. A549 cells were transfected with 30 nM siRNA directed against the hexon (hex), protease (prot), IVa2, pTP, and viral DNA polymerase (pol) transcripts. A nontargeting siRNA served as a negative control. At 24 h after transfection, cells were transduced with either the adenoviral HSV-TK expression vector, AdEE4-TK (right panels), or the analogous EGFP expression vector, AdEE4 (left panels), at an MOI of 100 TCID₅₀/cell. Another 24 h later, cells were infected with wt Ad5 at an MOI of 0.01 TCID₅₀/cell, and GCV was or was not added at a concentration of 1.2 μM. At 48 h after infection with wt Ad5, the cultures were analyzed for wt Ad5 multiplication. **A.** Genome copy numbers were determined by qPCR using a primer/probe set specific for the E1A gene present only on wt Ad5. **B.** The output of infectious virus progeny was determined by TCID₅₀ assays. Data represent the means ± SD of 3 independent transfections/infections.

tetracycline repressor/operator system. When produced in packaging cells, the tetracycline repressor shuts down amiRNA expression. As demonstrated in Figure 3, the regulatable expression system is functional in both the plasmid and adenoviral vectors constructed in this study: the production of EGFP, which can serve as a measure of amiRNA production because it is encoded by the same primary transcript that gives rise to the amiRNA (see Figure 1), was strongly suppressed in packaging cells upon expression of the tetracycline repressor. To investigate whether the combinatorial vector leads to increased inhibition of wt Ad5 replication, we transduced A549 cells with AdTO-TK-pTP-mi5 or the negative control vector, AdTO-TK-mi-, and infected them with wt Ad5. Concomitantly, cells were or were not treated with 1.2 μM GCV, and wt Ad5 genome copy numbers were determined 48 h post-infection with wt Ad5 (Figure 4A). In the absence of GCV, pTP-mi5 expression inhibited wt Ad5 replication by slightly more than 1 order of magnitude. HSV-TK expression in the presence of 1.2 μM GCV but in the absence of the pTP-targeting amiRNA led to a decrease of wt Ad5 genome copy numbers by 2.7 orders of magnitude. However, a combination of pTP-mi5 and HSV-TK expression and concomitant

treatment with GCV led to a further significant drop in wt Ad5 genome copy numbers. Neither the negative control amiRNA nor the expression of HSV-TK in the absence of GCV significantly inhibited wt Ad5 replication (data not shown). The additive effect of concomitant pTP-mi5 and HSV-TK expression became more prominent when we cultivated the wt Ad5-infected cells for longer periods, and measured wt Ad5 genome copy numbers at 6 days post-infection with wt Ad5 (Figure 4B). Here, the additional gain in the inhibition rate was greater than one order of magnitude.

Multipcopy amiRNA/HSV-TK expression inhibits wt adenovirus replication even at very low GCV concentrations

To create the final version of the vector, we improved the amiRNA/HSV-TK expression cassette by incorporating additional copies of pTP-mi5 hairpins into the EGFP/amiRNA transcription unit. This approach had previously resulted in increased knockdown of pTP gene expression [21] and hence, it was anticipated to be beneficial for the function of the vectors generated in this study. The final vectors (i.e., AdTO-TK-pTP-mi5×6 and its analog, AdTO-TK-mi5-×6, carrying the non-targeting



amiRNA expression cassette) were used to transduce A549 cells. Concomitantly, the cells were treated with GCV and infected with wt Ad5, as performed previously. Because HSV-TK expression and concomitant treatment with 1.2 μM GCV alone had already been proven to efficiently inhibit wt Ad5 replication, we assumed that

additional expression of the 6xpTP amiRNA cassette might allow for efficient inhibition of wt Ad5 replication at even lower GCV concentrations. Thus, we incubated the cells with increasing amounts of GCV (ranging from 0.15 to 1.2 μM) and determined wt Ad5 genome copy numbers at 48 h post-infection (Figure 5). As expected, at the highest GCV concentration of 1.2 μM, the additive effect of 6xpTP-mi5 expression on wt Ad5 inhibition was minor and, in the particular set of experiments shown here, not statistically significant (Figure 5D; compare green and red lines). However, the gain in wt Ad5 inhibition became clearly visible upon gradually lowering the GCV concentration (Figure 5A to C). In fact, even at the lowest concentration of 0.15 μM (Figure 5A), the total inhibition rate (~2.7 orders of magnitude) was comparable to that after treatment with the almost 10-fold higher GCV concentration of 1.2 μM in Figure 5D (compare red lines in Figure 5A to D). In contrast, the inhibition rate gradually decreased upon lowering the GCV concentration in the presence of the non-targeting negative control amiRNA (compare green lines in Figure 5A to D). In conclusion, simultaneous expression of the 6xpTP-mi5 expression cassette allowed for a 10-fold decrease in the GCV concentration, without resulting in a significant loss in the inhibition rate. At any GCV concentration, the combinatorial inhibitory effect was also clearly higher than the effect that was mediated by the 6xpTP-mi5 expression cassette alone (blue line in Figure 5A to D).

The additive effect mediated by the 6xpTP-mi5 expression unit also manifested as a further drop in the output of infectious virus progeny as determined by TCID₅₀ analysis (Figure 6). Again, this effect became most pronounced when GCV became limiting (i.e., at GCV concentrations < 1.2 μM) (compare red lines in Figure 6A to C with that in Figure 6D).

The combinatorial HSV-TK/amiRNA expression cassette inhibits adenoviral vector replication

In the presence of GCV and in the absence of the tetracycline repressor, the combinatorial expression cassette comprising the EGFP/amiRNA and HSV-TK transcription units should not only have a negative effect on the replication of a wt adenovirus present in the same cells, but also on the adenoviral vector itself by which it is carried. To investigate this inhibitory effect, we transduced T-REx 293 cells, which carry the adenoviral E1 genes and thus promote the replication of otherwise replication-deficient adenoviral vectors, with the combinatorial vector, AdTO-TK-pTP-mi5x6. We cultivated the cells with or without doxycycline for an additional 48 h to determine the amiRNA-mediated inhibitory effect, and treated them with increasing amounts of GCV (ranging between 0 and 1.2 μM) to investigate the HSV-TK-mediated effect. GCV treatment alone, in the absence of

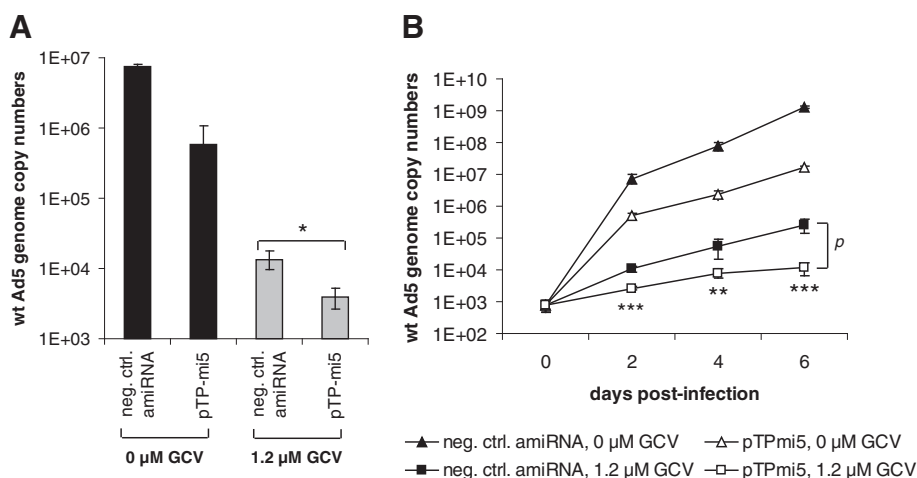


Figure 4 A combination of HSV-TK expression and pTP-mi5 expression leads to an additive inhibitory effect on adenoviral DNA replication upon treatment with GCV. A549 cells were transduced with the adenoviral HSV-TK/pTP-mi5 expression vector, AdTO-TK-pTP-mi5, or the respective control vector, AdTO-TK-mi-, expressing a negative control amiRNA at an MOI of 100 TCID₅₀/cell. At 24 h post-transduction, cells were infected with wt Ad5 at an MOI of 0.01 TCID₅₀/cell, and GCV was or was not added at a concentration of 1.2 μM. **A.** Wt Ad5 genome copy numbers were determined at 48 h post-infection with wt Ad5 by qPCR using a primer/probe set specific for the E1A gene present only on wt Ad5. **B.** The infection kinetics for wt Ad5 was monitored over a time span of 6 days by determining genome copy numbers in the same way as described for (A). Data represent the means ± SD of 3 independent transductions/infections. **p* < 0.05, ***p* < 0.01, ****p* < 0.001.

pTP-mi5 expression (i.e., cells not treated with doxycycline), was only effective at the highest GCV concentration of 1.2 μM (Figure 7). Here, the amount of vector DNA was decreased by ~1.5 orders of magnitude. No significant inhibition was seen at lower concentrations of GCV, ranging from 0.15 to 0.6 μM. The expression of the pTP-mi5 amiRNA alone (doxycycline-treated cells, 0 μM GCV) decreased vector copy numbers by about one order of magnitude. The expression of pTP-mi5 in addition to GCV treatment led to a clear increase in the overall inhibitory effect when low concentrations of GCV were used (compare -dox samples with + dox samples). Thus, in agreement with the previous results (Figure 5), the combinatorial amiRNA/HSV-TK expression cassette was of the highest benefit when GCV was limiting. Leaky expression from adenoviral promoters is an issue in the application of adenoviral vectors as gene delivery vehicles, and recombination with wt adenovirus may transform replication-deficient, E1- and E3-deleted adenoviral vectors into replication-competent versions. Thus, taken together, the combinatorial HSV-TK/amiRNA expression cassette presented here may constitute a tool for the decrease of adenoviral background gene expression and aid in the control of unintended vector replication, or, when present in cells that become infected with wt adenovirus, may inhibit wt adenovirus replication and spreading.

Discussion

We previously demonstrated the inhibition of adenovirus replication both by RNAi-based methods and

through the targeted expression of HSV-TK in adenovirus-infected cells with concomitant GCV treatment. Both approaches targeted viral DNA replication, albeit in different ways. While the siRNAs/amiRNAs decreased the number of functional viral proteins that are needed for the initiation (pTP) or progression of viral DNA replication (DNA polymerase), GCV-ppp acted downstream of these steps in a functionally different way (i.e., as a nucleoside analog by preventing the DNA polymerization process). CDV is mechanistically related to GCV-ppp since it blocks the same step during virus replication [39], and we have demonstrated that the expression of a pTP RNA-targeting amiRNA in adenovirus-infected cells and concomitant treatment with CDV leads to additive inhibitory effects [21]. Thus, it was conceivable that a combination of pTP gene silencing via an amiRNA and HSV-TK expression/GCV treatment can result in a similar effect. This assumption was supported by results demonstrating that siRNAs targeting viral transcripts required for DNA replication increased the HSV-TK/GCV-mediated effect (Figure 2). In these experiments, we did not only include the siRNAs with the highest previously proven inhibitory effect on adenoviral DNA replication, but also the ones that had resulted in poor antiviral effects in our previous study (i.e., the hexon and viral protease RNA-targeting siRNAs). We hypothesized that a strong inhibition of viral DNA replication by HSV-TK expression/GCV treatment (which is more effective than that mediated by viral DNA polymerase or pTP mRNA-targeting siRNAs) should decrease viral DNA genome copy numbers and, consequently, hexon and protease

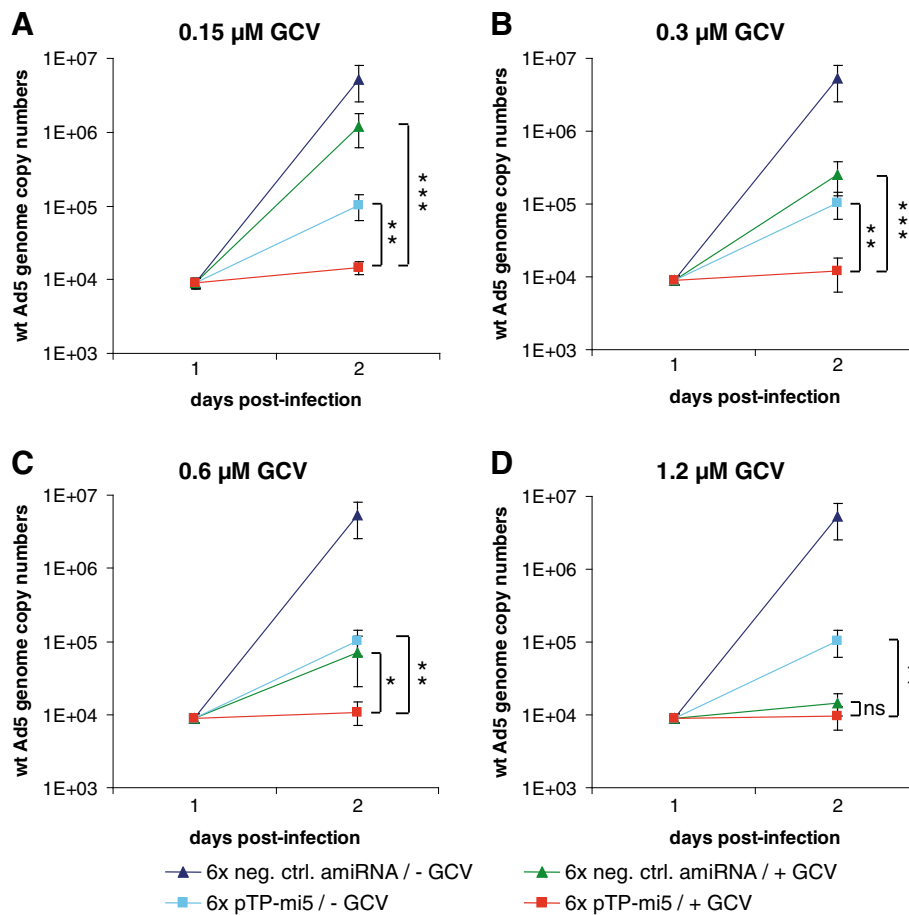


Figure 5 Improvement of the amiRNA expression cassette by concatemerization of amiRNA hairpins and inhibition of adenoviral DNA replication at decreased GCV concentrations. A549 cells were transduced with the adenoviral HSV-TK/pTP-mi5 expression vector, AdTO-TK-pTP-mi5x6 (carrying 6 copies of pTP-mi5), or the respective control vector, AdTO-TK-mi-x6 (carrying 6 copies of a negative control amiRNA) at an MOI of 100 TCID₅₀/cell. At 24 h post-transduction, cells were infected with wt Ad5 at an MOI of 0.01 TCID₅₀/cell, and GCV was or was not added at concentrations of 0.15 μM (A), 0.3 μM (B), 0.6 μM (C), or 1.2 μM (D). Wt Ad5 genome copy numbers were determined at 48 h post-infection with wt Ad5 by qPCR using a primer/probe set specific for the E1A gene present only on wt Ad5. Data represent the means \pm SD of 3 independent transduction/infection experiments, each performed in triplicate. In each panel, data points for cells that were not treated with GCV are given as a reference. ** $p < 0.01$, *** $p < 0.001$.

gene copy numbers. This, in turn, should result in a decrease in the otherwise vast amounts of hexon and protease transcripts present in adenovirus-infected cells, which may allow the siRNAs to silence their respective target genes more effectively. However, similar to our previous study, the hexon and protease RNA-targeting siRNAs were relatively poor inhibitors of virus multiplication under these conditions. These results reflect those obtained in experiments in which we inhibited viral DNA synthesis by siRNAs, but were unable to further increase the overall antiviral effect by also targeting the hexon and protease transcripts [22]. Thus, the data presented here support our previous observations that argue against a concomitant targeting of different viral processes (e.g., viral DNA synthesis and capsid generation) as the method of choice to inhibit adenovirus multiplication but, rather,

for the targeting of only a single process such as viral DNA synthesis at different steps.

Thus, it was conclusive to incorporate the individual modules for HSV-TK and pTP-mi5 expression into one common vector to concomitantly target adenoviral DNA synthesis at 2 different points. The combinatorial approach revealed an increase in the overall anti-adenoviral effect; however, this effect was modest when we inserted only one copy of the pTP-mi5 sequence into the vector (Figure 4). Because concatemerization of identical amiRNA sequences has been shown to increase knockdown rates [40,41], and concatemerization of pTP-mi5 has also previously resulted in enhanced silencing of Ad5 pTP [21], we added more copies to the vector. We did not observe any detrimental effects on cell viability that could potentially occur due to the higher number of amiRNAs potentially competing with

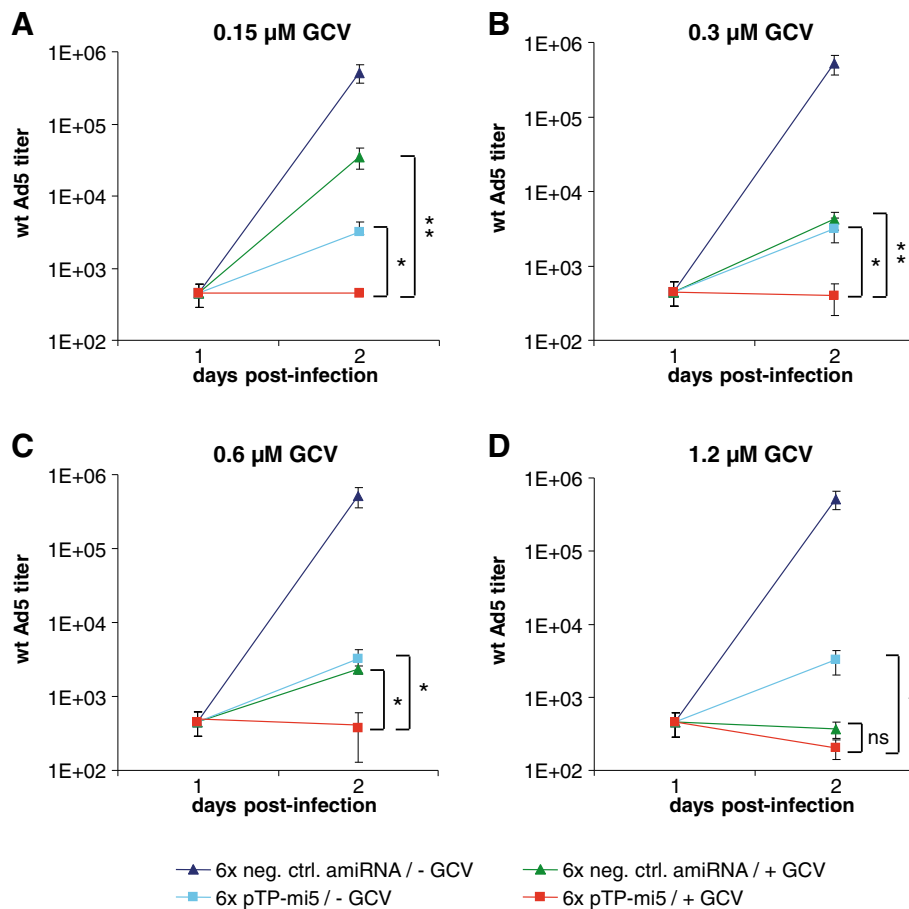
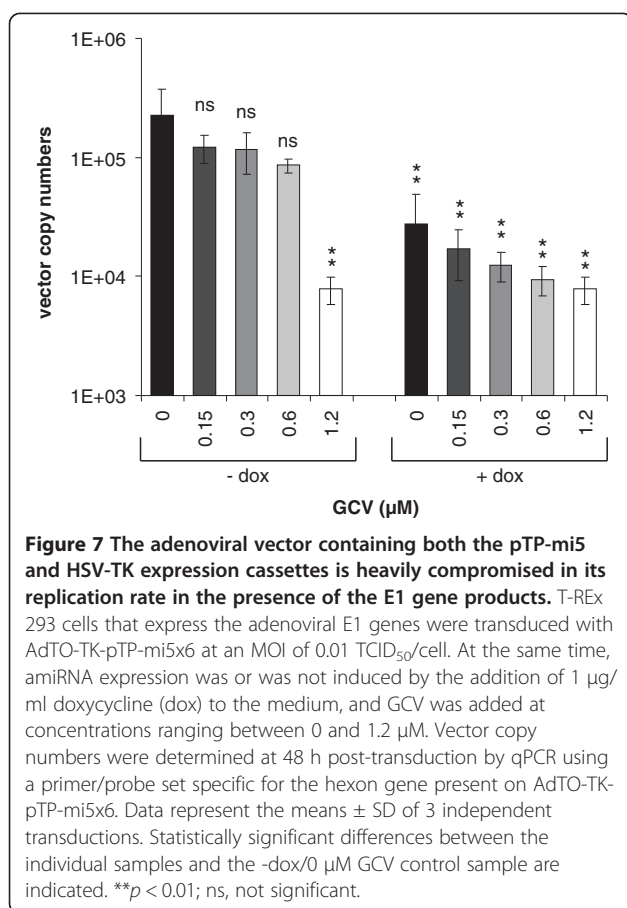


Figure 6 The concomitant expression of pTP-mi5 and HSV-TK results in an increased inhibitory effect on the generation of infectious virus progeny in the presence of GCV. A549 cells were transduced with the adenoviral HSV-TK/pTP-mi5 expression vector, AdTO-TK-pTP-mi5x6 (carrying 6 copies of pTP-mi5), or the respective control vector, AdTO-TK-mi-x6 (carrying 6 copies of a negative control amiRNA) at an MOI of 100 TCID₅₀/cell. At 24 h post-transduction, cells were infected with wt Ad5 at an MOI of 0.01 TCID₅₀/cell, and GCV was or was not added at concentrations of 0.15 μM (A), 0.3 μM (B), 0.6 μM (C), or 1.2 μM (D). The output of infectious virus progeny was determined by TCID₅₀ assays. Data represent the means ± SD of 3 independent transduction/infection experiments each performed in triplicate. **p* < 0.05, ***p* < 0.01.

endogenous miRNAs. In case of *in vivo* testing of this and related vectors, this point would have to be carefully addressed, though. The increased additive effects of combined HSV-TK/amiRNA expression obtained with this final vector were most pronounced when GCV was applied at very low concentrations (0.15 μM) (Figure 5). At these low concentrations, inhibition of adenoviral replication mediated by HSV-TK expression alone became marginal (Figure 5A); however, it was restored to normal levels upon concomitant knockdown of pTP gene expression by pTP-mi5. This observed effect is conclusive because, at very low concentrations, GCV-ppp needed for the blockage of DNA polymerization is expected to become limiting when, at the same time, high numbers of viral DNA replication complexes work in parallel to synthesize high numbers of viral DNA molecules. In contrast, the incorporation rate of GCV-ppp into nascent viral DNA strands should increase when only a few viral DNA molecules are generated.

In summary, our data suggest that wt adenovirus DNA replication can, in principle, be blocked most effectively by targeting 2 independent steps needed for viral DNA replication, namely (i) the formation of the initiation complex, and (ii) the actual DNA polymerization step. Targeting of these mechanistically different steps may not be restricted to the way we accomplished it, but may also prove a useful strategy when aiming to inhibit adenoviral DNA replication by novel “conventional” drugs or small-molecule inhibitors. In general, our strategy does not allow to cure wild-type virus-infected cells from the infection, and it cannot prevent them from cell death. However, it can effectively decrease the output of infectious virus progeny from these cells, and consequently prevent virus spreading.

In an envisioned therapeutic scenario, the delivery of anti-adenoviral amiRNAs, via a replication-deficient adenoviral vector, may have several unique advantages.



For example, it may allow for the amplification of amiRNA and HSV-TK expression cassette copy numbers upon exposure of the recombinant virus to the wt virus due to the initiation of vector replication. This transcomplementation effect has already been demonstrated earlier for the pTP-mi5 amiRNA expression cassette *in vitro* [21]. In addition, this effect may ensure a constant supply of recombinant vector as long as wt adenovirus is present. Moreover, due to the shared organ tropism of the adenoviral vector and its wt counterpart, delivery via an adenovirus-based vector may also permit the directing of the vector predominantly to those cells and organs that are also the preferred targets of the wt virus. Among those is the liver which functions as a major virion replicator during adenovirus infection and is readily transduced by adenoviral vectors [42]. Thus, it is conceivable, that strategies as the one presented here are able to reduce the output of infectious wt virus at least from this particular organ, and, consequently, inhibit spreading of the virus throughout the body. Because the immune response against the wild-type virus is heavily impaired or inexistent in immunocompromised patients, problems that can occur due to the elimination of adenoviral vectors by the

immune system should be less pronounced or absent in such an envisioned scenario. Alternatively, topical treatment of localized adenovirus infection, e.g., of the eye, where high local vector concentrations can be achieved, is conceivable for immunocompetent patients as well. Adenovirus-mediated epidemic keratoconjunctivitis (EKC) is associated with significant morbidity and possible long term consequences on visual acuity, and thus causes considerable economic losses.

Adenoviral vectors are among the most commonly used vectors for the delivery of genetic information *in vivo* [42]. In most cases, replication-deficient vectors whose E1 and E3 genes are deleted are employed. In theory, no viral genes should be expressed from these vectors. Leaky expression of viral genes from replication-deficient viral vectors, however, is known to occur due to the presence of binding sites for certain cellular transcription factors in adenoviral promoters [43]. This background expression from both early and late promoters can result in toxicity and immunity against adenoviral proteins [44-48] and, consequently, to short-lived transgene expression. The amiRNA generated from the combinatorial HSV-TK/amiRNA expression cassette, when included in such vectors, may potentially prevent leaky pTP expression from the adenoviral E2B promoter. It may, thus, inhibit any potential low-level replication and, consequently, late gene expression (which is dependent on viral DNA replication), thereby preventing the generation of highly immunogenic late gene products such as the hexon and fiber proteins. In fact, deletions within the E2B region that comprises the viral DNA polymerase and pTP genes lead to decreased downstream gene expression in E1-deleted adenoviral vectors [49,50], resulting in extended transgene expression and reduced liver toxicity [50,51].

The HSV-TK expression unit of the cassette, in conjunction with the amiRNA expression unit, may also help to bring any unforeseen high-level replication of adenoviral vectors under control, should they turn into replication-competent versions (i.e., upon unintended recombination with a wt virus). The elimination upon treatment with GCV would only affect the rearranged vector and not the parental vector because the latter would not promote the synthesis of significant amounts of HSV-TK. Thus, the combinatorial gene expression cassette presented here (or similar versions following the same principle) may also be considered for evaluation as safeguards for certain adenoviral vectors intended to be used in gene therapy studies.

Conclusions

In summary, the results presented in this study demonstrate that combined expression of HSV-TK and an amiRNA targeting the adenoviral pTP mRNA can

effectively inhibit wt adenovirus replication in the presence of the antiherpetic prodrug GCV. The enhanced inhibition rate accomplished with the combinatorial gene expression cassette can most likely be attributed to the targeting of 2 different steps in adenoviral DNA synthesis. This effect mediated by the expression cassette may not only be harnessed to inhibit wt adenovirus replication but also to prevent the replication of certain adenoviral vectors commonly used in gene therapy studies.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

MI performed the experiments, analyzed the data, and participated in the preparation of the manuscript; TL participated in the preparation of the manuscript; RK designed research, analyzed the data, and wrote the manuscript. All authors read and approved the final manuscript.

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