Modular adeno-associated virus (rAAV) vectors used for cellular virus-directed enzyme prodrug therapy

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The pre-clinical and clinical development of viral vehicles for gene transfer increased in recent years, and a recombinant adeno-associated virus (rAAV) drug took center stage upon approval in the European Union. However, lack of standardization, inefficient purification methods and complicated retargeting limit general usability. We address these obstacles by fusing rAAV-2 capsids with two modular targeting molecules (DARPin or Affibody) specific for a cancer cell-surface marker (EGFR) while simultaneously including an affinity tag (His-tag) in a surface-exposed loop. Equipping these particles with genes coding for prodrug converting enzymes (thymidine kinase or cytosine deaminase) we demonstrate tumor marker specific transduction and prodrug-dependent apoptosis of cancer cells. Coding terminal and loop modifications in one gene enabled specific and scalable purification. Our genetic parts for viral production adhere to a standardized cloning strategy facilitating rapid prototyping of virus directed enzyme prodrug therapy (VDEPT).

Vir.al gene therapy and the subcategory of virus-directed enzyme prodrug therapy (VDEPT) provide many opportunities and have been heralded as universal tools for therapy. Despite the potential, clinical success has been delayed due to difficulties in managing the many factors coming into play in the design, production and application of such complex systems. Gene therapy relies on an externally provided transgene also named ‘gene of interest’ (GOI) which is delivered to a target tissue or a specific cell type. Upon reaching the target the goals diverge. Gene sublementation therapy typically aims at maintaining the gene and its expression in the target cell as long as possible to replace a defective gene and/or alter the phenotype of the target cell. In contrast, VDEPT, as presented here, may also be used to kill tumor cells. This two-step method first delivers the gene for a non-endogenous enzyme to a target tissue via a viral vector. Following transduction, the enzyme is expressed, and the ability to specifically activate subsequently administered and otherwise inert prodrugs to potent drugs allows for a cell- or tissue- specific therapy with reduced systemic side effects. So far, a large variety of delivery methods have been developed including non-viral and viral, genome-integrating or non-integrating systems. Among the non-integrating viral systems usage of the adeno-associated virus (AAV) has been extensively studied. Serotype 2 (AAV-2) has gained significant interest since its great potential for gene therapy has been demonstrated in a successful clinical trial involving retinal infusion for patients with Leber’s congenital amaurosis. Most importantly, in 2012 a recombinant AAV serotype 1 became an approved drug in the European Union for treating patients with lipoprotein lipase (LPL) deficiency.

The AAV-2 is a small, non-enveloped virus belonging to the family of Parvoviridae, and more specifically to the genus Dependovirus. It relies on a helper virus for replication such as adenovirus or herpes simplex virus. The 4.7 kb single-stranded DNA genome harbors the replicative (rep) and capsid forming (cap) genes. The capsid is built from 60 subunits of the viral coat proteins VP1, VP2 and VP3, whereby VP1 and VP2 include the VP3 sequence. Remarkable progress has been made in tailoring this virus to the needs of a gene therapy vector. The need of a helper virus has been eliminated by providing the required genes on an external plasmid. Moreover, the initially favored broad natural tropism was later not only diminished but also successfully changed towards specific targets. Consequently, recombinant adeno-associated viruses (rAAV) are increasingly becoming the vector of choice for a wide range of gene therapy approaches. However, manufacturing AAV-2 particles for
cancer gene therapy still has several drawbacks. Most strategies guiding these viral vectors to specified cell surface receptors involve either insertion of small, directly binding peptides or the use of adaptors that mediate between ligand and receptor. To our knowledge, only one recent retargeting-approach used direct genetic coupling of a protein moiety (designed ankiny repeat protein, DARPin), which is directed towards the Her2 (ErbB2) receptor.

An additional hindrance for using rAAV particles as gene therapy carriers are unmodified, e.g. wild-type capsids, which emerge from the production method and tend to accumulate predominantly in the liver and bone marrow, rendering a systemic prodrug approach unfavorable and toxic for off-target cells. Furthermore, the preferred prodrug and enzyme choice for tumor therapy has yet to be defined in the context of the rAAV strategy, the tumor marker, and the cancer cell type, which requires more detailed cell-based studies. For example, the combination of thymidine kinase and ganciclovir is well established for selection, but cytotoxic deaminase and 5-fluoro-cytosine offers the potential advantage that the activated drug can pass the membrane and thus elicit a killing bystander effect, which might prove essential to eradicate a tumor.

Starting as a team in the international Genetically Engineered Machine (iGEM, iGEM Foundation, MA, USA) competition, a yearly, worldwide and well-known contest in the field of synthetic biology, we addressed the aforementioned obstacles in rAAV gene therapy. The foundational base of iGEM is a system of so-called BioBricks - standardized and interchangeable genetic elements, coding and non-coding, which share a common pre- and suffix allowing an idempotent BioBrick assembly as a uniform and fast cloning strategy. We combined the latest achievements of rAAV-2 research using this modular BioBrick approach. For targeting we chose two protein A26. Both targeting molecules were selected to bind EGFR protein-protein interactions throughout the cell24. Consisting of (DARPins) originate from ankyrin repeat proteins which mediate specificity for the cancer cell-surface marker human epidermal carcinomas with nanomolar affinity and have already been visualized using the B1 antibody, which binds a linear epitope into the viral capsid34,35 or insert small peptides into surface-exposed loops29. In order to specifically redirect rAAV-2 particles to cancer cell surface markers, we alternatively fused two different targeting molecules to the N-terminus of the viral coat protein VP2 and incorporated at the same time a small purification tag into a surface-exposed loop. Based on previous publications, both methods likely neither interfere with capsid assembly, genome packaging, nor infection14. However, so far all alterations of the viral capsid or its encapsidated genome involved tedious cloning steps and the different genetic elements were difficult to exchange. We therefore adapted the helper virus free AAV-2 system to the BioBrick RFC 10 standard21. As a result, all exchangeable genes were cloned on separate plasmids flanked by specific prefix and suffix sequences which provide specific restriction enzyme recognition sequences (Figure 1a). These BioBricks can be assembled using a standardized idempotent cloning strategy (Figure 1b). Next to the wild-type similars, we also constructed our viral modifications and extensions in the same assembly standard creating a highly modular system in which the targeting motives, the loop-integrated purification tag, as well as all elements of the encapsidated genome can be easily exchanged (details will be published elsewhere).

For targeting, we used a mosaic virus approach, in which the VP2 capsid protein is knocked out from the RepCap plasmid with a point mutation in the start codon. A modified VP2 is then provided on an additional plasmid under the control of the CMV promoter. Next to simplifying cloning, this method ensures that the modifications in the VP2 construct, which comprises also the reading frame of VP3, only occur in the capsid at the frequency of the VP2 protein, and that the N-terminal modifications of VP2 are strictly coupled with the loop modifications. Using this approach, we created three different rAAV-2 particles carrying two previously described modifications in the exposed 587-loop. The first modification diminishes the natural tropism towards heparan sulfate proteoglycan (HSPG) due to mutation of two interaction mediating arginine residues (R585A and R588A)12. The second modification is the insertion of a His6 purification tag at amino acid position 587, an exposed position known to tolerate insertion of small peptides. Since HSPG and His6 are always combined, these modification are denoted as AAV-HisHSPG and the resulting viral particle served as the control for the targeting experiments. Including these two modifications, two further particles were created which additionally display EGFR receptor targeting molecules – either the Affibody-ZEGFR1907 or the DARPin_E0129 – fused to the N-terminus of VP2, and which are named AAV-2_Affibody and AAV-2_DARPin, respectively, because a full name such as AAV-2_Affibody_ZEGFR1907 is barely readable (Figure 1c). These rAAV variants were produced in HEK-293 cells, harvested from serum-free cell culture supernatant, purified and analyzed for their correct assembly and targeting properties.

Assembly analysis. First, we determined whether the fusion proteins of VP2-DARPin or VP2-Affibody, respectively, had been incorporated into the viral capsids. Western blots of viral particle samples were visualized using the B1 antibody, which binds a linear epitope common to all three VP proteins, and protein bands of expected size were detected either corresponding to VP2-Affibody-Flag (Figure 2a, lane A_2, approx. 75 kDa) or VP2-DARPin-Flag (Figure 2b, lane B_2, approx. 85 kDa). Comparison with AAV-2_Affibody and AAV-2_DARPin, respectively, because a full name such as AAV-2_Affibody_ZEGFR1907 is barely readable (Figure 1c). These rAAV variants were produced in HEK-293 cells, harvested from serum-free cell culture supernatant, purified and analyzed for their correct assembly and targeting properties.

Results Design, modularization, and production. Several different approaches have been reported to either introduce larger proteins into the viral AAV-2 capsid14,15 or insert small peptides into surface-exposed loops29. In order to specifically redirect rAAV-2 particles to cancer cell surface markers, we alternatively fused two different targeting molecules to the N-terminus of the viral coat protein VP2 and incorporated at the same time a small purification tag into a surface-exposed loop. Based on previous publications, both methods likely neither interfere with capsid assembly, genome packaging, nor infection14. However, so far all alterations of the viral capsid or its encapsidated genome involved tedious cloning steps and the different genetic elements were difficult to exchange. We therefore adapted the helper virus free AAV-2 system to the BioBrick RFC 10 standard21. As a result, all exchangeable genes were cloned on separate plasmids flanked by specific prefix and suffix sequences which provide specific restriction enzyme recognition sequences (Figure 1a). These BioBricks can be assembled using a standardized idempotent cloning strategy (Figure 1b). Next to the wild-type similars, we also constructed our viral modifications and extensions in the same assembly standard creating a highly modular system in which the targeting motives, the loop-integrated purification tag, as well as all elements of the encapsidated genome can be easily exchanged (details will be published elsewhere).

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pSB1C3_001-RepCapΔHSPG-VP2_KO, pSB1C3_001VP2ΔHSPG&His6-DARPin-Flag and pSB1C3_001VP2ΔHSPG&His6-Affibody-Flag. Blotted samples from the two targeting viral particles were additionally probed with the anti-Flag antibody (Figure 2a, b, lanes A_3, B_3) confirming correct sizes of both VP2 fusion proteins. Rough estimations of the band size and density using ImageJ (ImageJ 1.47, NIH) revealed that the modified VP2 fusion proteins represented approximately 11–14% of the total band intensity (corrected for protein mass; in detail: AAV-2_Affibody: VP1 29%, VP2_Affibody 14%, VP3 57%; AAV-2_DARPin: VP1 27%, VP2_DARPin 11%, VP3 62%).

Viral particle purification and surface display. Since the viral particles harvested from the supernatant of serum-free cultured HEK-293 producer cells are diluted and contain some cell debris as well as unassembled capsid proteins, which both interfere with cellular assays, concentration and purification measures are required. Using ultrafiltration devices with a molecular weight cut-off (MWCO) of 1 MDa we enriched rAAV-2 capsids about 27-fold (Figure 3a) and at the same time, the high MWCO facilitated depletion of unassembled viral coat proteins. A slight enrichment of single capsid proteins in the filtrate might have emerged either from broken capsids or cells during ultrafiltration (Figure 3b). Given the fact that the coat protein VP2 is non-essential for viral assembly, a small population of produced viral particles lacks the VP2 fusion proteins. These unmodified capsids can cause negative side effects such as unspecific transduction especially when used at high multiplicities of infection. To easily deplete vector preparations from these unwanted particles, we took advantage of the His_6-purification tag inserted into the VP2-Affibody and the VP2-DARPin. Concentrated preparations of the targeted viruses displaying (His)_6-tags were incubated with Ni-NTA resin. Unmodified particles were removed by washing and the targeting molecule displaying capsids were eluted with imidazole. To regain physiological conditions, the imidazole containing buffer was exchanged for phosphate buffered saline (PBS) using centrifugal concentrators. Using this method we were able to deplete concentrated vector preparations from unmodified rAAV-2 particles which represented approximately 20% of the total capsid number (Figure 3c).

Successful guidance of viral vectors to EGFR-overexpressing cancer cells requires accessible targeting moieties. To assess surface display of the Affibody and the DARPin, the respective modified rAAV-2 samples were subjected to an enzyme-linked immunosorbent assay (ELISA). ELISA plates were coated with anti-Flag-antibody and incubated with equal capsid titer of ultrafiltration-purified rAAV-2 samples. Both targeted viruses showed a comparable high display of the Affibody or DARPin (Figure 3d). These results demonstrate incorporation of the Affibody_ΔEGFR:1907 and the DARPin_E01 into AAV-2 capsids and their display based on fusions to VP2.

Target cell binding and infectivity. The key feature of tumor-tissue guided viral vectors is the coupling of infection to the binding of markers expressed on the cell surface. First we investigated binding of viral targeting vectors to EGFR-overexpressing A431 cells in an ELISA-type experiment. Cell-bound viral particles were detected using the AAV-2 capsid specific antibody A20, which is specific for assembled capsids. While the signal for AAV-2_Affibody remained at background level, both targeted viruses showed a comparable high display of the Affibody or DARPin (Figure 3d). These results demonstrate incorporation of the Affibody_ΔEGFR:1907 and the DARPin_E01 into AAV-2 capsids and their display based on fusions to VP2.
AAV-2_DARPin to transduce different cell lines. Three cell types with an EGFR surface density ranging from very low (approx. 10^4, MCF7) over intermediate (approx. 2 \times 10^5, HeLa) to very high (approx. 4 \times 10^6, A431) were incubated with equal genomic titers of targeting capsids. Since the infection pathway of the AAV-2 involves converting its single-stranded genome into a double-strand, the number of infectious particles is directly proportional to the number of double-stranded viral genomes inside the cells after 24 h. Thus, the infectious titer can be determined via quantitative real-time PCR. As depicted in figure 4b, AAV-2 Affibody and AAV-2_DARPin very specifically transduced A431 target cells at very high rates (up to 700-fold, compared to MCF7 cells) and HeLa cells to an intermediate level (up to 200-fold). In absolute genome copy numbers, MCF7 cells were barely transduced. At the same time AAV-2^HSPG could be detected in A431, HeLa and MCF7 only at negligible low background levels. These results demonstrate that Affibody and DARPin guided viral capsids are suitable for high-specificity targeting towards high-level EGFR displaying cancer cells and that the rate of infection is strongly dependent on the level of EGFR receptor expression.

**In vitro tumor cell killing.** In a second step, the ability of the targeting/prodrug activation system to kill target cells was exemplarily evaluated by testing the AAV-2_Affibody in an MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) cell viability assay of transduced cells. The assay (Figure 5) revealed that more than 90% of the target cells were efficiently killed after four days utilizing cytotoxic deaminase (CD)-armed AAV-2_Affibody particles in combination with 500 \(\mu\)M 5-fluorocytosine (5-FC). With 250 \(\mu\)M 5-FC the cell viability was reduced by more than 50%. In contrast, AAV-2^HSPG particles without binding moiety, or incubation with the prodrug alone affected the cell viability at most to 20%, but typically only to an insignificant level (Figure 5, AAV-2^HSPG and cells only).

Since cell internal DNA damage signals generated by activated genotoxins such as 5-fluorodeoxyuridine monophosphate or ganciclovir-triphosphate (activated forms of 5-FC and GCV) are sensed and ultimately transduced to apoptotic signals, the activity of the effector caspases 3 and 7 was measured in rAAV-2 treated cells (Figure 6). The 'Apo-ONE Homogeneous Caspase-3/7 Assay' revealed that AAV-2_DARPin and AAV-2_Affibody efficiently induce apoptosis in A431 target cells while caspase activity in off-target HeLa cells remained at background levels. The combination of the AAV-2_DARPin together with the cytotoxic deaminase showed the most potent apoptosis induction in A431 cells, with comparable efficacies for 500 \(\mu\)M and 250 \(\mu\)M 5-FC (Figure 6c, approx. 12 times more caspase activity relative to AAV2^HSPG), followed by AAV-2_DARPin particles armed with the mouse guanylate kinase – herpes simplex virus thymidine kinase (mGMK-TK30) (Figure 6d, approx. 8 times more caspase activity relative to AAV2^HSPG). Apoptosis induction through Affibody-guided vectors showed a comparable potency to DARPin guided vectors at 500 \(\mu\)M prodrug concentrations, but at 250 \(\mu\)M 5-FC or GCV AAV-2_DARPin superseded AAV-2_Affibody by a factor of 2.5 (Figure 6a, c) and 5 (Figure 6b, d) for 5-FC and GCV, respectively.

**Apoptosis induction and targeting specificity in mixed cell culture.** In a final step, we evaluated the ability of AAV-2_Affibody and AAV-2_DARPin for differential targeting. EGFR-overexpressing A431 cells were either combined with HeLa or MCF7 cells in a mixed cell culture. In cells undergoing early apoptosis, Annexin V is translocated from the inner side of the plasma membrane to the outer layer and thus becomes surface exposed. Hence staining with an Annexin V antibody reveals cells in an early apoptotic state. Since experiments showed that A431 cells undergoing apoptosis lose the majority of their EGFR receptors (Figure 7, lower right panel, A431 untreated 84% EGFR positive) camptothecin induced apoptotic A431 cells were included as control (Figure 7, lower right panel, A431 + camptothecin 27% EGFR positive). As a consequence, cells were simultaneously infected and treated with 250 \(\mu\)M 5-FC, and flow cytometry analysis was carried out 48 h later. Targeted A431 cells and off-target HeLa or MCF7 cells were mixed in a 1:4 ratio. Flow cytometry analysis with simultaneous staining for EGFR

**Figure 2** | Western blots of viral capsids equipped with targeting modules, concentrated by centrifugal ultrafiltration. Viral particles were separated on a 7.5%–15% SDS gel and blotted onto a PVDF membrane. Particles with deleted HSPG motive served as controls (lanes A_1, B_1) using B1 antibody. (a) Western blot analysis of AAV-2_Affibody. Capsid proteins were detected using B1 antibody (lane A_2). VP2-Affibody was specifically detected via anti-Flag antibody (lane A_3) (b) Western blot analysis of AAV-2_DARPin. Capsid proteins were detected using B1 antibody (lane B_2). VP2- DARPin was specifically detected via anti-Flag antibody (lane B_3).
expression and Annexin V revealed specific apoptosis induction in EGF receptor overexpressing A431 cells with comparable efficacies for AAV-2-Affibody and AAV-2-DARP in (Figure 7, left panels). Control AAV-2ΔHSPG particles did not mediate prodrug activated apoptosis (Figure 7, middle panels), showing almost the same staining pattern as cells which were not treated with any of the viral particles (Figure 7, middle panel, untreated cells). Taken into account that A431 cells undergoing apoptosis lose most of their EGF receptors and thus stain as apoptotic but EGFR negative, these results demonstrate that retargeted AAV-2_Affibody and AAV-2_DARP in viral particles discriminate between cells depending on EGFR expression and primarily induce EGFR dependent apoptosis. This

Figure 3 | Viral particle concentration, depletion of unassembled capsid proteins, affinity purification and surface display of targeting modules. (a) Four days post transfection the supernatant from HEK-293 producer cells was harvested (supernatant) and concentrated via centrifugal ultrafiltration (concentrate, filtrate). The number of packed viral particles was determined via qPCR (n = 3, mean ± SD). (b) Samples from viral particle producing HEK-293 cells (supernatant), ultrafiltration filtrate and concentrate and fresh cell medium (neg. control) were incubated on an ELISA plate. Assembled particles or individual capsid proteins were detected using the A20 or the B1 antibody, followed by incubation with HRP-coupled anti-mouse antibody (n = 3, mean ± SD) (c) Ultrafiltration concentrated viral particles were incubated with Ni-NTA resin washed and capsids were eluted with PBS containing 500 mM imidazole. Subsequently, 4 × 10⁹ purified and non-purified particles were incubated in an ELISA plate, coated with anti-His antibody. Viral particles were detected using the capsid specific A20 antibody, followed by HRP-coupled anti-mouse antibody (n = 4, mean ± SD) (d) 1.8 × 10⁹ His-affinity purified AAV-2_Affibody and AAV-2_DARP in particles were incubated on an ELISA plate coated with anti-Flag antibody. Assembled capsids were detected using the A20 antibody, followed by biotin-coupled isotype specific IgG3 antibody and streptavidin-coupled horseradish peroxidase (n = 3, mean ± SD).
justifies to further develop the concept in animal models and predict future applications in patients.

**Discussion**

In recent years, adverse reactions in patients treated with prodrug strategies had been reduced significantly and currently approximately 10% of worldwide marketed drugs can be classified as prodrugs, albeit almost all of them are activated by endogenous enzymes. By implementing a two-step process – systemic application of a non-toxic, non-bioactive prodrug and its conversion to a pharmacologically active agent delivered on-site of a therapeutically relevant target – systemic side effects can be minimized. In cancer chemotherapy, most classical drugs target rapidly proliferating cells, thus not only affecting neoplastic cells, but also harming natural regenerative processes.

At present, there are two major drawbacks of using viruses as gene shuttles for prodrug converting enzymes: First, the limited knowledge on how to specifically retarget the viral vectors towards cancer cells, and second, the limited knowledge to make the right choice for the enzyme prodrug combination. In addition, rapid prototyping is hindered by laborious cloning steps, which is key to the advance of disease-specific and personalized medicine.

Based on the AAV-2 scaffold, we present two modularly assembled recombinant viral vectors which present diminished natural tropism and rationally designed retargeting via general binding scaffolds towards cancer cell surface markers. These viral particles deliver genes coding for prodrug converting enzymes, which again can easily be swapped to test other enzyme prodrug pairs. In contrast to systems reported by others, our vector design and fast two-step purification process assures specific purification only of targeted viral particles (Figure 1c, 3b, 3c).

As cancer cell surface marker, we chose the epidermal growth factor receptor 1 also named ErbB1. This marker is a clinically validated prototype receptor belonging to the family of receptor tyrosine kinases vastly overexpressed in several different types of cancer. The EGF receptor is already exploited for cancer therapy with small
molecule inhibitors such as gefitinib (trade name Iressa) or erlotinib (trade name Tarceva) and antibodies such as cetuximab (IMC225, trade name Erbitux) in the case of colon as well as squamous cell carcinoma of the head and neck. For targeting, we tested the small and robustly folding DARPin_E01 and the Affibody_ZEGFR:1907, which both have reported EGFR binding affinities in the nanomolar range and originate either from the ankyrin proteins or from the Z-domain scaffold of the staphylococcal protein A. Although monoclonal antibodies have already been used as targeting devices for either the adeno-associated virus in an adapter mediated way or for the adenovirus in a covalently bound way, both the DARPin and the Affibody outperform them in terms of size, aggregation tendency, lack of disulfide bonds and glycosylation. Expressed as fusions to the viral coat protein VP2, both were readily displayed on the viral surface (Figure 3b) and were available for receptor target binding (Figure 4). Additionally we armed the viral particles with genes coding for two different prodrug converting enzymes: the cytosine deaminase (CD) and the mouse guanylate kinase – herpes simplex virus thymidine kinase (mGMK-TK30, ganciclovir), respectively. Note that the scale in panel c differs from the other panels.

Figure 6 | Apoptosis induction in A431 relative to HeLa cells. 2 × 10³ A431 or HeLa cells, respectively, were transduced with 3 × 10⁸ genomic viral particles on day one. On day three, the culture medium was exchanged and prodrug solutions were added. On day six, caspase-3 and caspase-7 activity was measured using the Apo-ONE Homogeneous Caspase-3/7 Assay (Promega). Samples were transferred to a 96 well microplate, and fluorescence was measured using a microplate reader. The caspase activity ratio of A431 to HeLa cells for each prodrug concentration is given relative to the respective AAV-2 ΔHSPG ratio (n = 3, mean ± SD). As labeled, panels (a) to (d) show experiments with combinations of Affibody or DARPin and CD (5-FC) or mGMK-TK30 (ganciclovir), respectively. Note that the scale in panel c differs from the other panels.
or MCF7 control cells, underwent apoptosis upon addition of the respective prodrug (Figure 6 and 7). Taken together AAV-2_Affibody, as well as AAV-2_DARPin both with diminished natural tropism and armed with prodrug converting enzymes were able to specifically transduce target but not control cells in mono-, as well as in mixed cell culture. In combination with a recent study from Münch et al.\textsuperscript{14} who used a DARPin specific for Her2/neu for rAAV mediated delivery of TK in an artificial cell system with HER2/neu stable expressing CHO cells and a tumor-cell xenograft model, we corroborate the versatility of the rAAV approach. In line with demands to avoid animal experiments, we demonstrate that essential data for targeting and prodrug activation can be obtained in cell culture. However, we also acknowledge that clinicians demand animal testing before clinical testing can be initiated. Münch and coworkers observed residual off-targeting assuming unmodified viral particles in the vector stock as a reason. Using our all-in-one approach, which couples a loop exposed His-tag with the presentation of the targeting moiety in one protein, this challenge can be overcome. Following this strategy, modified rAAV-2 particles can specifically and rapidly be purified from serum-free cell culture supernatant with a single step centrifugal ultrafiltration followed by an easy to perform affinity chromatography. By doing so, recombinant vector stocks can not only be depleted from unmodified capsids but at the same time the need for expensive and time consuming batchwise purification and standardization of the targeting moiety can be overcome. Additionally, we included two genes coding for prodrug converting enzymes which – when packaged into the targeting vectors – will be specifically expressed inside targeted cancer cells and thus facilitate their cell death by conversion of non-toxic prodrugs into toxic compounds.

Taken together, we present here the first example of a standardized, recombinant and easy to purify AAV-2 based viral targeting vector which addresses every demand of a virus-directed enzyme prodrug therapy by combining N-terminal capsid fusion with loop modification employing two modern targeting moieties and two enzyme—prodrug combinations. Using this comprehensive approach might not only help fighting various devastating types of cancer characterized by EGFR overexpression, but also build up a basis for targeting a plethora of other cell surface markers through standardized exchange of the guiding molecules.

**Figure 7** Effect of rAAV-2 infection on apoptosis induction in mixed cell cultures. 20 × 10^3 A431 cells were mixed with 80 × 10^3 HeLa cells or 80 × 10^3 MCF7 cells, and the mixture was transduced with either His-tag affinity purified AAV-2_Affibody, AAV-2_DARPin or AAV-2_DHPG capsids delivering the cytosine deaminase. Cells only served as a negative control and A431 cells incubated with 20 µM camptothecin for 4 h served as a positive control for apoptotic cells. After addition of the viral particles, cells were incubated in DMEM containing 250 µM 5-FC. 48 h later, cells were detached, washed and incubated with an anti-EGFR-Alexa488 antibody on ice for 1 h. An anti-Annexin-V-PE antibody was added and cells were analyzed by flow cytometry. For every sample 30,000 events were recorded. Dead cells were excluded using initial gating based on forward scatter height versus sideward scatter height. The anti-Annexin-V fluorescence is plotted against the EGFR fluorescence and the percentages of cells in each quadrant are given below each plot. Experiments show that EGFR expression of A431 cells decreases upon apoptosis (right panel). rAAV without targeting does not mediate prodrug activated apoptosis (middle panels). Targeted AAV primarily induces EGFR dependent apoptosis, but a bystander effect might harm nearby cells.
Methods

Generation of plasmids. All plasmids used, except pHelper (Stratagene) share the same pUC-derived backbone (pSB1C3 backbone) and were assembled using pGEM standard BioBrick assembly. The Rep cap insert in pSB1C3_001-RepCap was derived from pRSB1C3 and were assembled using Gem standard BioBrick assembly. The rep cap insert in pSB1C3_001-RepCap was derived from pRSB1C3 (Stratagene). The exchange region around aa 587 harbors two arginine residues (Arg585, Arg588) which are largely responsible for the natural HSPG-tropism of AAV-2 capsids. The viral brick - a short synthetic gene construct - containing a His tag, was assembled at this position also containing arginine to alanine mutations which largely abolish the binding of rAAV-2 capsids to the primary AAV-2 receptor on the cell surface. Targeting plasmids pSB1C3_001VP2-MADGUG-DARPIN-Flag and pSB1C3_001VP2-MADGUG-Flag were used. Affibody-Flag vectors were derived from the pRepCap plasmid, harboring the VP2 open reading frame of a CMV promoter. DARPin and Affibody were ordered as synthetic gene constructs flanked by pGEM pre- and suffixes (GeneArt, Life technologies). Vector plasmids pSB1C3-CD and pSB1C3-MGMK-TK30 harboring the AAV-2 left and right inverted terminal repeats (ITRs, from pAAV-MCS, Stratagene), a CMV promoter, a human beta-globin intron, the cytomegalovirus immediate early promoter (CD) or the fused genes of mouse granulocyte kinase (mGKM) and a herpex simplex virus thymidine kinase mutant (TK30)48, as well as the human growth hormone (hGH) polya antigen. The mGKM-TK30 was kindly provided by M. E. Black (Washington State University, USA). The CD gene was amplified from E. coli XL1 blue using primers CD-prefx 5'-CGTCTAGATGGCCGGCGTGTCGAATAACGCTTTACAAACAATTATTAAC-3' and CD-suffix 5'-CCAGAGAAGCCATCGATTACAAACGTACCGGTCTG-3'. Details on the modularization and cloning strategy will be published elsewhere (manuscript in preparation).

Viral particle production and purification. Production of recombinant adenovirus serotype 2 particles (rAAV-2) was generally based on the adenovirus helper-free AAV packaging strategy. Viral like particles targeting vectors were generated/generated by transfection of 4× 10^6 HEK-293 cells cultured in 7 cm cell culture dishes with four plasmids at a total DNA amount of 15 μg per dish using linear polyethylenimine (25 kDa, Polysciences Inc) as described previously. Targeting plasmids pSB1C3_001-MADGUG-VP2, pSP1C3-DARPIN, pSB1C3_001SADGUG, pVP2-MADGUG-Affibody, RepCap (pSB1C3-RepCap_001-MADGUG-VP2), vector plasmids (pSB1C3-CD and pSB1C3-MGMK-TK30) and the helper plasmid (pHelper, Stratagene) were transfected in a molar 4:1:3:5:5 ratio. For the generation of viral vectors control, a triple-repli-competition strategy, the helper and the respective vector plasmid was miniated. The supernatant was removed after 24 h, allowing the matrix to exchange for FreeStyle 293 Expression Medium (Gibco) to allow serum-free production of rAAV-2 particles. Four days post transfection, the supernatants were harvested and twice cleared from cell debris by centrifugation (700 xg, 10 min) at 4 °C. Ruthenium-red stained pellets were washed and concentrated and purified using Vivaspin 20 centrifugal concentrators (1 MDa MWCO, Sartorius) and PBS, making use of the high molecular weight cut-off membrane, which allows most contaminating molecules and proteins to pass through, but holds back rAAV-2 particles.

His-tag affinity purification. Modified viral particles were concentrated and clarified as described above, and purified by immobilized metal-ion affinity chromatography (IMAC). For batch binding to the matrix, 5 ml samples were incubated with 500 μl Ni-NTA agarose resin (Qiagen) were coated with an anti-Flag antibody (F1804, Sigma; 1:50 000 dilution) for 1 h on an orbital shaker. The slurry was transferred to a gravity-flow RotiBlock/PBS, 0.05% Tween 20 (v/v). After every incubation step, the plate was washed three times with PBS. Last, 0.5 μl of peroxidase substrate o-Phenylenediamine (1 mM, Sigma) in substrate buffer (50 mM citric acid, 100 mM disodium hydrogen phosphate, 0.01% H2O2, pH 5.5) was added per well. The reaction was stopped after 15 min with 1 M sulfuric acid and 50 mM sodium sulfate and quantified via absorption at 492 nm using a microplate reader (Tecan Sunrise).

Determination of capsid, genomic and infectious titers. Capsid titers were determined using the AAV Titration ELISA (Progen) according to the manufacturer’s instructions. Genomic titers of rAAV-2 particles were determined using specific real-time PCR (qPCR) as described previously. Briefly, an aliquot of purified viral vectors was supplemented with 5 μl DNase I and DNase I buffer (Fermentas), incubated for 3 h at 37 °C, heat inactivated for 10 min at 65 °C, and finally diluted 100-fold. 5 μl of these DNase I resistant particles containing the viral DNA supplemented with 7.5 μl Quantifast SybrGreen MasterMix (Qiagen) and CMV_forward/reverse primers (10 μm each) were used as template in a qPCR reaction on a RotorGene 3000 (Qiagen). Viral genome titers were calculated from a standard curve of 13–13×10^4 copies of a CMV promoter-containing plasmid Infectious viral Titers were determined as described by Rohr et al.18, but using the Quantifast SybrGreen PCR Kit (Qiagen) on a RotorGene qPCR cycler. Briefly, 10 cells per well in a 24 well cell culture plate were seeded and incubated for 24 h with equal amounts of viral vectors. Subsequently, the cells were harvested and digested with proteinase K (Sigma) and residual single-stranded DNA was removed using S1 nuclease (Fermentas). Double-stranded DNA which originated from infectious particles harboring viral sDNA was then quantified using qPCR.

Cell culture. HEK-293, HeLa, and A431 (ATCC) cells were cultured in DMEM supplemented with stable glutamine, sodium pyruvate, 10% fetal calf serum, 1% penicillin/streptomycin (v/v), 4.5 g/l glucose (1.5 g/l for MCF7 cells) to a maximum confluence of 80% and then split 1:10 (HEK-293, MCF7, A431, or 1:50 (HeLa), respectively. All cell culture media and chemicals where purchased from PAA. Prodrug solutions containing Ganciclovir (GCV, Sigma) or 5-fluorocytosine (5-FC, Sigma) were prepared as ten-fold concentrates (2 mM and 5 mM) in PBS followed by sterile filtration.

Cell-binding assay. Binding of viral targeting vectors to the surface of A431 epidermoid carcinoma cells overexpressing EGFR19, was analyzed on whole ELISA. Cells were harvested, washed twice with PBS and transferred into a 96 well MaxiSorp plate (Nunc) at a concentration of 2,000 cells per well and spun down at 250 × g. The supernatant was removed and the plate dried for 4 h at room temperature. HeLa cells were used as control. Unspecific binding was blocked with 0.8% BSA/PBS for 1 h. Subsequently, cells were incubated with 1.8 × 10^4 viral particles for 2 h. Unbound capsids were removed via washing twice with PBS/0.05% Tween 20 (v/v). Unbound rAVs were detected as described under ELISA using a HRP-coupled anti-mouse antibody (sc-2005, SantaCruz, dilution 1:3,000) to detect bound A20 antibodies.

Caspase 3/7 assay. In order to assess apoptosis induction, 2,000 A31 or HeLa cells, respectively, were seeded overnight in 96 well microplates with an 1:5 × 10^4 genomic viral particles on day one. On day three, the culture medium was exchanged and prodrug solutions were added to cells. On day six, caspase-3 and -7 activity was measured using the Apo-ONE Homogeneous Caspase-3/7 Assay (Promega) according to the manufacturer’s instructions. Briefly, Apo-ONE buffer, supplemented with the Z-DEVD-R110 caspase substrate was added to cell culture medium in a 1:1 ratio. Plates were incubated at room temperature. Samples were transferred to a black 96 well microplate (Nunc) and fluorescence was measured using a microplate reader (Thermo Scientific Varioscan Flash, excitation wavelength 499 nm, emission wavelength 521 nm).

Flow cytometry. Specificity of rAAV-2 infection and apoptosis induction was examined in mixed cell cultures. A431 and HeLa, or A431 and MCF7 cells, respectively, were mixed in a 1:4 ratio and transduced with either AAV-2_DARPin or AAV-2_Affibody. AAV-2_DARPin and AAV-2_Affibody were used as controls. Additionally, A431 cells incubated with 20 μM camptothecin (Sigma) for 4 h served as a positive control for apoptotic cells. All viral capsids were purified as described under His-tag affinity purification. After addition of the viral particles, cells were immediately incubated in...
DMEM containing 250 μM 5-FC. 48 h later cells were detached using cell scrapers and washed twice in Annexin V binding buffer (BD Biosciences, additional 1% BSA). Subsequently, cells were incubated with an anti-EGFR-Alexa488 antibody (Cell Signaling, 1:20 dilution) on ice for 1 h. After 45 min an Annexin V-PE antibody was added (Promega, 1:100 dilution). Finally cells were washed once in Annexin V binding buffer and cells were analyzed by flow cytometry (FACS Calibur, Becton Dickinson). For every sample 30,000 events were recorded. Dead cells were excluded using initial gating with forward scatter height versus sideward scatter height.

**MTT assay.** In order to access cell viability, 10,000 A431 or HeLa cells, respectively, were seeded per well in a 96-well cell-culture plate and transduced with 3 × 10^6 genomic viral particles 24 h later. After 48 h, fresh medium supplemented with 5-FC was added to the cells and incubated for three additional days. On day six, cells were incubated in 100 μl fresh DMEM including 25 μl MTT solution (3.65 mg/ml MTT in PBS, Sigma) for 3 h. Subsequently, the culture medium was removed and the reduced formazan was solubilized for 30 min on an ELISA shaker using 200 μl DMSO (Sigma) and 25 μl Sorenson buffer (0.1 M Glycine, 0.1 M NaCl, pH 10.5). Absorbance at 570 nm was measured on a Tecan Sunrise microplate reader.

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Author contributions
S.H., T.B., H.J.W., A.F., B.K., V.M., S.B., P.S. and K.M.M. conceived the idea. T.B., H.W., A.F., B.K., V.M., S.B., P.S. cloned constructs and conducted initial experiments. S.H. designed and conducted final experiments and wrote the manuscript with the help of K.M.M., K.M.A. provided scientific input, K.M.M. supervised the project.

Additional information
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