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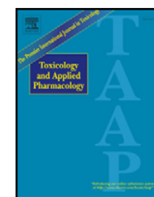

Safety profile, efficacy, and biodistribution of a bicistronic high-capacity adenovirus vector encoding a combined immunostimulation and cytotoxic gene therapy as a prelude to a phase I clinical trial for glioblastoma

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ABSTRACT

Adenoviral vectors (Ads) are promising gene delivery vehicles due to their high transduction efficiency; however, their clinical usefulness has been hampered by their immunogenicity and the presence of anti-Ad immunity in humans. We reported the efficacy of a gene therapy approach for glioma consisting of intratumoral injection of Ads encoding conditionally cytotoxic herpes simplex type 1 thymidine kinase (Ad-TK) and the immunostimulatory cytokine fms-like tyrosine kinase ligand 3 (Ad-Flt3L).

Herein, we report the biodistribution, efficacy, and neurological and systemic effects of a bicistronic high-capacity Ad, i.e., HC-Ad-TK/TetOn-Flt3L. HC-Ads elicit sustained transgene expression, even in the presence of anti-Ad immunity, and can encode large therapeutic cassettes, including regulatory elements to enable turning gene expression "on" or "off" according to clinical need. The inclusion of two therapeutic transgenes within a single vector enables a reduction of the total vector load without adversely impacting efficacy. Because clinically the vectors will be delivered into the surgical cavity, normal regions of the brain parenchyma are likely to be transduced. Thus, we assessed any potential toxicities elicited by escalating doses of HC-Ad-TK/TetOn-Flt3L (1×10^8, 1×10^9, or 1×10^{10} viral particles [vp]) delivered into the rat brain parenchyma. We assessed neuropathology, biodistribution, transgene expression, systemic toxicity, and behavioral impact at acute and chronic time points. The results indicate that doses up to 1×10^9 vp of HC-Ad-TK/TetOn-Flt3L can be safely delivered into the normal rat brain and underpin further developments for its implementation in a phase I clinical trial for glioma.

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Introduction

Glioblastoma multiforme (GBM) is the most common primary brain tumor in adults and is highly invasive, with a median survival of just 15–21 months (Buckner, 2009; Grossman et al., 2009; Wen and Kesari, 2008). The standard of care for GBM is comprised of surgical resection, radiotherapy, and chemotherapy (Stupp et al., 2005, 2006, 2008). The highly infiltrative nature of GBM makes total tumor resection almost impossible and the tumor inevitably recurs. In comparison to the primary tumor, recurrent GBMs are known to harbor numerous mutations/gene rearrangements and have the potential to express novel proteins that could serve as tumor neoantigens and add to the phenomenon of

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immune escape (DuPage et al., 2011; King et al., 2011). Recurrent GBM is refractory to both chemotherapy and radiotherapy and eventually leads to the patient's death. Immunotherapeutic approaches constitute a promising adjuvant treatment for GBM and are at present under preclinical and clinical study (Ali et al., 2005; Candolfi et al., 2009a, 2009b; Curtin et al., 2005, 2009; Ghulam Muhammad et al., 2009; Wei et al., 2009).

We previously developed a gene therapy regime based on two first-generation adenoviral vectors (Ads), each encoding a therapeutic transgene, for the treatment of GBM. The conditionally cytotoxic herpes simplex type 1 thymidine kinase (TK) (Ali et al., 2004, 2005; Candolfi et al., 2009b; Curtin et al., 2009) kills proliferating tumor cells in the presence of the prodrug ganciclovir (GCV), and human soluble fms-like tyrosine kinase ligand 3 (Flt3L) recruits bone marrow-derived dendritic cells into the brain tumor milieu, triggering an anti-GBM immune response (Candolfi et al., 2009b; Curtin et al., 2009; Ghulam Muhammad et al., 2009). We have shown that the administration of Ad-TK + Ad-Flt3L into the tumor mass leads to long-term survival in rats bearing intracranial syngeneic CNS-1, 9L, and F98 GBM (Ali et al., 2005; Ghulam Muhammad et al., 2009), as well as mice bearing intracranial syngeneic GL26 GBM, GL261 astrocytoma, and B16-F10 intracranial melanoma (Curtin et al., 2009). Additionally, the combination of Ad-TK and Ad-Flt3L induces GBM-specific immunological memory that improves survival in intracranial multifocal and recurrent models of GBM (Candolfi et al., 2009b; Curtin et al., 2009; King et al., 2008a, 2008c, 2011). Furthermore, we have recently demonstrated that the TK/Flt3L gene therapy approach can induce an adaptive immune response capable of recognizing a brain tumor neoantigen in a model of recurrent GBM (King et al., 2011).

Adenoviruses are the most commonly used vectors for cancer gene therapy. Despite the promising safety profile demonstrated in preclinical studies (Edelstein et al., 2007), their efficacy in the clinical scenario can be hampered by the high prevalence of anti-Ad immune response in the human population (Bangari and Mittal, 2006; Schmitz et al., 1983). Most adults (85%) exhibit systemic immunity against Ad after naturally occurring infections with wild type Ad (Bangari and Mittal, 2006; Harvey et al., 1999; Schmitz et al., 1983). Recent work has shown that the generation of high-capacity adenoviral vectors (HC-Ads) lacking all viral transcriptionally-active regions abolishes the anti-Ad-specific immune system activation and elicits long-term transgene expression (Barcia et al., 2007; Parks et al., 1996; Xiong et al., 2006). Previously, we have demonstrated sustained transgene expression of HC-Ads under tight tetracycline regulation following intratumoral delivery in a rat syngeneic GBM model, even in the presence of systemic pre-existing immune responses against Ads (King et al., 2008b; Muhammad et al., 2010; Puntel et al., 2010b).

We recently developed a bicistronic regulatable HC-Ad-TK/TetOnFlt3L and demonstrated high therapeutic efficacy with no signs of toxicity in a rat syngeneic model of glioma after intra-tumoral administration (Puntel et al., 2010b). Nevertheless, filing for an Investigational New Drug (IND) request to the United States Food and Drug Administration to administer the bicistronic HC-Ad therapy to human patients requires a preclinical toxicity assessment in healthy animals (FDA, 2006). Thus, we performed detailed studies of the biodistribution, immune response against the therapeutic transgenes and adenovirus, and systemic effects after delivery of the bicistronic HC-Ad into the brain parenchyma of naïve rats.

The present study consisted of a dose escalation of a bicistronic HC-Ad-TK/TetOn-Flt3L delivered into the brain parenchyma of naïve rats. We assessed the biodistribution of the HC-Ad vector genomes, therapeutic transgene expression, and potential systemic and neurological toxicity. This is the first study involving a single bicistronic HC-Ad vector encoding a conditional cytotoxic gene and a tightly regulatable immunostimulatory gene in a preclinical safety study. Our data demonstrate the high safety profile of the bicistronic HC-Ad vector platform with a maximum tolerated dose of 1×10^9 vp. This warrants further development of the bicistronic HC-Ad-TK/TetOn-Flt3L platform for its eventual implementation in a phase I clinical trial for GBM in human patients.

Materials and methods

High-capacity adenoviral vector. Details of the molecular characterization, rescue, and amplification of the HC-Ad vector were published previously (Palmer and Ng, 2008, 2011; Southgate et al., 2008; Xiong et al., 2006). Briefly, HC-Ad-TK/TetOn-Flt3L constitutively expresses the herpes simplex type 1 thymidine kinase (TK) under the control of the hCMV promoter, and human soluble fms-like tyrosine kinase 3 ligand (Flt3L) under the control of the tightly regulatable mCMV-TetOn inducible expression system developed by us (Xiong et al., 2006).

Animals. Adult male Lewis rats (220–250 g, Harlan, Indianapolis, IN) were used. Rats were kept in controlled conditions of light (12 h light–dark cycles) and temperature (20–25 °C). The rats received water and standard rodent chow ad libitum.

Intracranial HC-Ad injections and experiment endpoints. Starting 2 days before treatment and ending after 4 weeks, rats were fed doxycycline-mixed chow (DOX chow) ad libitum (Modified LabDiet® Laboratory Rodent Diet 5001 with 1000 ppm Doxycycline, PMI® Nutrition International/Purina Mills LLC, Richmond, IN). Groups of rats were injected unilaterally in the right striatum with 1×10^8, 1×10^9, or 1×10^{10} viral particles (vp) of HC-Ad-TK/TetON-Flt3L. The vector was injected in a final volume of 3 µl of saline using a 10 µl Hamilton syringe fitted with 26-gauge needle. The stereotactic coordinates were as follows: 1 mm anterior and 3.2 mm lateral to the bregma, and the injection volume of 3 µl was delivered in 3 locations (1 µl each) at the depths −5.5, −5.0, and −4.5 mm from the dura. Twenty-four hours after treatment, the rats received ganciclovir (GCV, 25 mg/kg, i.p.; Roche Laboratories, Nutley, NJ), twice daily for up to 10 consecutive days. The control group of rats received 3 µl of saline at the same stereotactic coordinates as the treatment groups.

Groups of rats were evaluated at 5 days, 1 month, 4 months, and 1 year for biodistribution of HC-Ad vector genomes, neurotoxicity, peripheral blood cell counts, serum biochemistry, and circulating levels of anti-adenovirus neutralizing antibodies and anti-TK antibodies. Additionally, behavior was assessed at 1 month, 4 months, and 1 year. All animal procedures were carried out in accordance with NIH guidelines for the care and use of laboratory animals and approved by the Cedars-Sinai Medical Center Institutional Animal Care and Use Committee.

Rat brain tumor model. A total of 4500 rat GBM CNS-1 cells were stereotactically implanted in the right striatum of syngeneic Lewis rats as described previously (Ali et al., 2005; King et al., 2008c; Puntel et al., 2010b). Six days later, rats received an intratumoral injection of 1×10^8 vp HC-Ad or saline. The total volume delivered was 1.5 µl (delivered equally in three locations ventral from the dura: 5.2, 5.0, and 4.8 mm) into the tumor mass. Starting 2 days before HC-Ad administration, rats were orally gavaged with 30.8 mg DOX/kg/day for 4 weeks. One day after treatment, rats were orally gavaged with 560 mg/kg/day of valacyclovir (VAL, valacyclovir hydrochloride hyclate, Cat# V9889-1300G, Sigma-Aldrich, St. Louis, MO, USA) for 15 days. VAL is an orally prescribed prodrug of acyclovir with a high bioavailability (54.5%) compared with ganciclovir (5%–9%). Both acyclovir and ganciclovir are monophosphorylated by TK, enabling progression to a triphosphorylated active molecule that acts as a nucleoside analog and inhibits DNA polymerase activity. All experimental procedures were carried out in accordance with the animal care and handling practices at the University of Michigan (Ann Arbor, MI, USA).

Serum biochemistry and complete blood cell counts (CBC). At 5 days, 1 month, 4 months, or 1 year after HC-Ad-TK/TetOn-Flt3L injection, blood was collected from each animal during euthanasia. The samples

were sent to the laboratory of Antech Diagnostics (Irvine, CA) for analysis of routine hematological and biochemical parameters. Alternatively, complete blood count (CBC) analysis was performed on an IDEXX ProCyte Dx (IDEXX Laboratories, Westbrook, ME, USA), and plasma biochemistry, collected using sterile EDTA tubes (Beckton-Dickinson) and a 10 min, 2000 RCF centrifugation, was performed on a VetTest 8008 (IDEXX laboratories) (Supplementary Figs. S6b and S7b). Additionally, enzyme-linked immunosorbent assays (ELISAs) were performed on plasma samples in the DOX dosing experiments described in Supplementary Figs. S6a and S7a; kits for TNFα, IL1β, and IL6 were purchased from R&D Systems (Duoset Kits, catalog numbers DY510, DY501, DY506; R&D Systems, Minneapolis, MN, USA).

Circulating neutralizing anti-adenovirus antibodies. The level of adenovirus-specific neutralizing antibodies was assessed as described previously (Muhammad et al., 2010; Puntel et al., 2010a, 2010b). Briefly, serum samples were heat-inactivated at 56 °C for 30 min and serially diluted two-fold in minimal essential medium (Invitrogen, Carlsbad, CA) containing 2% fetal bovine serum (FBS). The range of dilutions was 1:2 to 1:4096. Each 50 μl serum dilution was incubated with 1×10^7 infectious units (i.u.) of first generation adenoviral vector expressing β-galactosidase (Ad-β-Gal) in a 10 μl volume for 90 min at 37 °C. The 50 μl of sample containing sera and virus was then added to the wells of a 96-well plate containing pre-seeded (1.5×10^4) HEK 293 cells per well and were incubated at 37 °C for 1 h. A further 50 μl of medium containing 10% FBS was added to each well, and the cells were incubated at 37 °C for 20 h before fixing with 4% paraformaldehyde in PBS (pH 7.4) and staining with 5-bromo-4-chloro-indolyl-β-D-galactoside (X-gal) (Sigma, St. Louis, MO). The neutralizing antibody titer for each animal is given as the reciprocal of the highest dilution of serum at which 50% of Ad-β-Gal-mediated transduction was inhibited. Positive and negative controls were used.

Anti-TK antibody assay. The titer of anti-TK antibodies in the sera of rats was assessed as described previously (King et al., 2011). Briefly, CNS-1 cells were infected with a first-generation adenoviral vector expressing TK (Ad-TK) at a multiplicity of infection of 200 i.u./cell or mock infected as a control. After 72 h, the cells were harvested and freeze/thawed. Cell lysates were added to a 96-well plate (Cat # 442404, NUNC, Rochester, NY) and incubated overnight at 4 °C. The serum samples were then diluted 1:4 and added to wells coated with cell lysates and incubated for 2 h at room temperature. The wells were washed and incubated for 1 h with rabbit anti-rat IgG/biotinylated secondary antibody (1:1000; Dako, Carpinteria, CA). Wells containing positive control rabbit sera were incubated with goat anti-rabbit IgG/biotinylated secondary antibody (1:1000; Dako). Finally, the wells were incubated with streptavidin-HRP (R&D Systems) and visualized with substrate solution (R&D Systems) at a wavelength of 500 nm. The percent change of optical density was calculated for each sample incubated with TK cell lysates compared to mock lysates.

Biodistribution of vector genomes. Biodistribution was assessed at 5 days, 1 month, 4 months, and 1 year after virus injection as described by us previously (Puntel et al., 2006, 2010a). Briefly, rats were perfused without fixative and 25 mg of tissue sample was harvested from the following locations: the brain injection site, contralateral brain hemisphere, cerebellum, brain stem, spleen, liver, testes, small gut, lung, heart, cervical lymph nodes, kidney, and lumbar spinal cord. Total DNA was purified and used for the quantitation of vector genomes by real-time quantitative PCR using a primer and probe specific for the cosmid sequences contained in the HC-Ad vector backbone as described by us previously (Puntel et al., 2006). The vector genomes are shown as a ratio of vector genomes/25 mg of tissue (n = 5 per group).

Neuropathological analysis. Neuropathological evaluation was performed at 5 days, 1 month, 4 months, and 1 year after viral injection. Following perfusion with oxygenated Tyrode's solution and 4% paraformaldehyde (PFA), brains were post-fixed in 4% PFA for 3 days. Fifty-micrometer serial coronal sections were cut at the immediate vicinity of the injection site and free-floating immunocytochemistry was performed as previously described (Candolfi et al., 2007; King et al., 2008b, 2008c) with markers for oligodendrocytes and myelin sheath (mouse monoclonal anti-MBP, 1:1000, Chemicon, Temecula, CA, USA, cat# MAB1580), dopaminergic nerve terminals (rabbit polyclonal anti-TH, 1:5000, Calbiochem, La Jolla, CA, cat# 657012), $CD8^+$ T cells (mouse anti-CD8, 1:1000, Serotec, Raleigh, NC, cat# MCA48G), macrophages and microglia (CD68/ED1, mouse anti-ED1, 1:1000, Serotec, Raleigh, NC, cat# MCA341R; IBA1, polyclonal rabbit anti-EBA1, 1:1000, cat# 019-19741, Wako Pure Chemical Industries), activated macrophages, microglia, and immune cells (mouse anti-MHC II, 1:1000, Serotec, Raleigh, NC, cat# MCA46G), TK (rabbit anti-TK, 1:10,000, custom made), and Flt3L (rabbit anti-Flt3L, 1:500, custom made). Nissl staining was performed to assess gross histopathological features of the brains. The stained sections were photographed with Carl Zeiss Optical Axioplan microscope using Axiovision Rel 4.6 and MOSAIX software (Carl Zeiss, Chester, VA). Quantification analysis of MHCII-, ED1/CD68-, and CD8-positive staining areas was also performed using the Axiovision Rel 4.8 image quantitation software.

Behavioral analysis. The neurobehavioral impact as a consequence of HC-Ad treatment was assessed 1 month, 4 months, and 1 year following the injection of the virus. Testing of amphetamine-induced rotational behavior, abnormalities in limb use asymmetry, and spontaneous motor and rearing behavior was done as described by us in detail (King et al., 2008a). Briefly, amphetamine-induced rotational behavior was measured using a RotoMax apparatus and software (AccuScan Instruments, Columbus, OH) for 90 min after subcutaneous injection of 1.5 mg/kg D-amphetamine sulfate (Sigma, St. Louis, MO). To measure forelimb use asymmetry, contacts made by each forepaw with the wall of a 20.3 cm wide clear cylinder were scored from videotape over a 10 min period in slow motion by two independent, experimentally blinded observers. Forelimb contact with the walls of the cylinder was scored measuring only initial contact with the cylinder walls. Baseline spontaneous locomotor and rearing activity was recorded for 30 min in a 40.6 × 40.6 × 38.1 cm closed box using photobeam breaks and optical sensors. Spontaneous locomotor and rearing activity was then monitored for 120 min after subcutaneous injection of 1.5 mg/kg D-amphetamine sulfate (Sigma). The data were scored as the total number of beam breaks summed over the observation period.

Measurement of doxycycline concentration in plasma using liquid chromatography with tandem mass spectrometry (LC–MS/MS).
For the plasma samples, 20 μl of each sample was mixed with 20 μl of methanol prior to adding 60 μl of tetracycline-containing acetonitrile. The samples were prepared in duplicate. To collect the supernatant for LC–MS/MS analysis, the suspensions were centrifuged at 15,000 rpm for 10 min. To ensure retention of doxycycline and its internal standard during LC–MS/MS, the final supernatant was mixed with water at 1:6 (v/v) ratio. LC–MS/MS analysis was performed using a 5 cm × 2.1 mm, 5 μm Zorbax C18 column (Agilent), a mobile phase A of 0.1% formic acid in purified water, a mobile phase B of 0.1% formic acid in acetonitrile, and a flow rate of 0.4 ml/min. This procedure was performed by the Pharmacokinetics Core at the University of Michigan.

Statistical analysis. Sample sizes were calculated in order to detect differences between groups with a power of 80% at a 0.05 significance level, using PASS 2008 (Power and Sample Size software; NCSS, Kaysville, UT). Data were analyzed using one-way analysis of variance followed by Tukey–Kramer Multiple-Comparison Test (NCSS or Graphpad Prism v5.0). Where data were not normally distributed, they were log-transformed. P values < 0.05 were used to determine the null hypothesis to be invalid. The statistical tests used are indicated in the figure legends.

Results

Neuropathology analysis

To examine the potential effects of HC-Ad-TK/TetOn-Flt3L delivery (Fig. 1a) on inflammation and normal brain architecture we performed an extensive neuropathological analysis of brain sections at 5 days (Fig. 1b), 1 month, 4 months (Supplementary Figs. S1a and S1b, respectively), and 1 year (Fig. 1c) after HC-Ad delivery. Naïve adult Lewis rats received 1×10^8, 1×10^9, or 1×10^{10} vp of the bicistronic HC-Ad vector or saline (Fig. 1a). GCV was administered twice daily for up to 10 days. The rats were fed DOX-containing chow for up to 4 weeks.

Five days after vector injection, neuropathological assessment revealed evidence of neurotoxicity at the highest dose tested (1×10^{10} vp). No evidence of brain tissue damage was detected in the brains of animals injected with the two lower doses (1×10^8 vp and 1×10^9 vp). Nissl staining showed local alterations in the morphology of the tissue at the site of injection at 5 days after delivery of HC-Ad-TK/TetOn-Flt3L, suggesting immune cell infiltration and microglial activation. Myelin basic protein (MBP) and tyrosine hydroxylase (TH) immunoreactivity indicated normal brain structures for the lower doses used while a significant disruption of myelinated nerve tangles and loss of striatal TH immunoreactivity was found for animals injected with the highest dose (i.e., 1×10^{10} vp). $CD8^+$ T cells were present in significant numbers in the high dose, and $ED1^+$ cells (activated microglia and macrophages), as well as major histocompatibility complex (MHC) II^+ cells (antigen presenting cells) were found within the injected hemisphere of the brain, especially in the vicinity of the injection site (Fig. 1b and Supplementary Figs. S2) at the acute time point. One month after HC-Ad-TK/TetOn-Flt3L

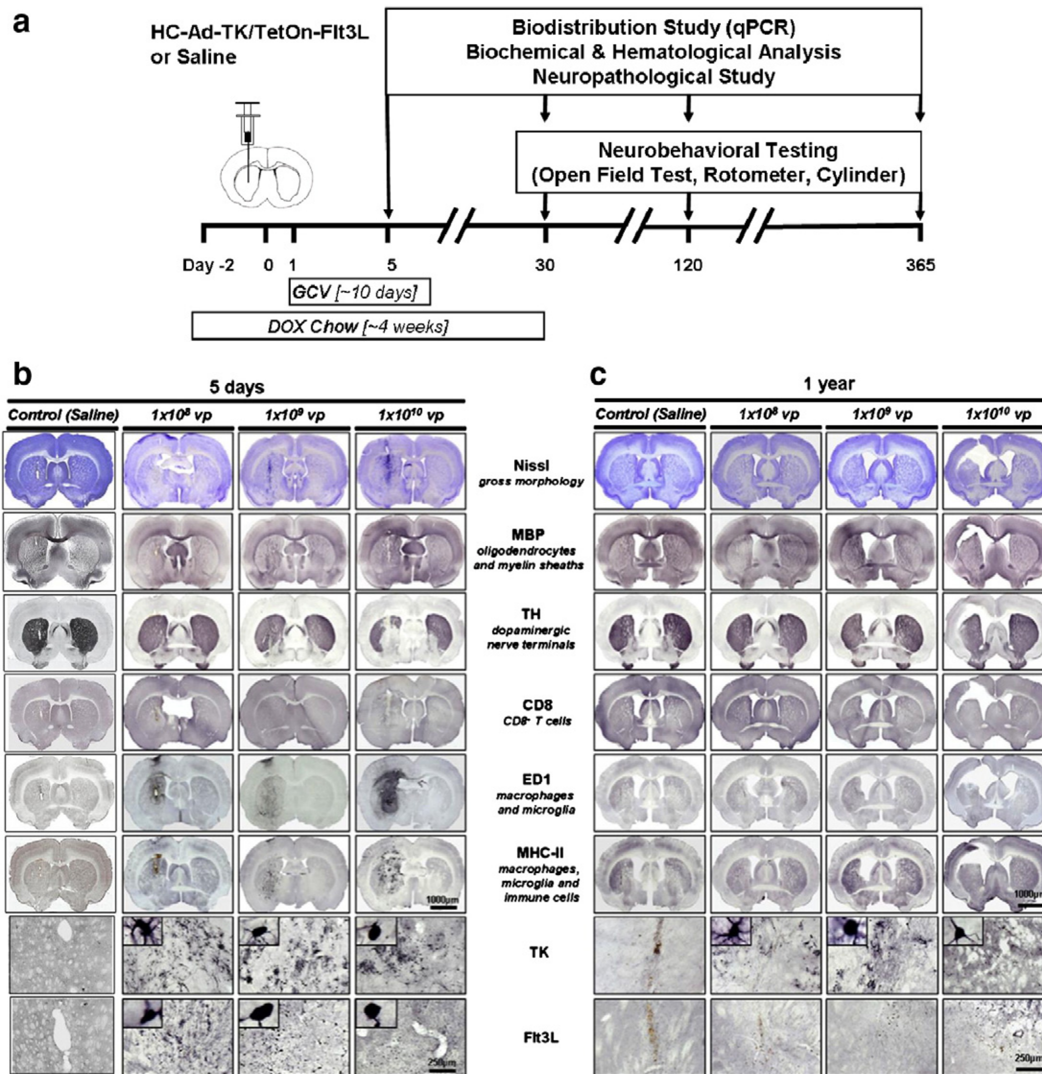

Fig. 1. Experimental design and neuropathological analysis following injection with HC-Ad-TK/TetON-Flt3L. Experiment design (a). Adult naïve Lewis rats were injected stereotactically in the right striatum with either saline or escalating doses of a bicistronic high-capacity adenoviral vector, HC-Ad-TK/TetON-Flt3L (1×10^8, 1×10^9, or 1×10^{10} vp); after 24 h, they received ganciclovir (GCV; 25 mg/kg, i.p.), twice daily for up to 10 days. All rats were fed doxycycline-mixed rodent chow (DOX chow) ad libitum for up to 4 weeks starting 2 days before treatment. Groups of rats were evaluated at 5 days (short-term), 1 month (medium-term), 4 months, and 1 year (long-term) for biodistribution of vector genomes, neuropathology, peripheral blood cell counts, serum biochemistry, and circulating levels of anti-adenovirus neutralizing antibodies and anti-TK antibodies. Neuropathology results for 5 days and 1 year are shown (b and c). The brains were processed for Nissl staining to show gross morphology, and immunocytochemistry using primary antibodies against myelin basic protein (MBP) to label oligodendrocytes and myelin sheaths, tyrosine hydroxylase (TH) to label striatal dopaminergic fibers, CD8 to detect CD8 + T cells, ED1 for labeling macrophages and activated microglia, MHC-II to label MHC II + macrophages, microglia and immune cells, thymidine kinase (TK) or fms-like tyrosine kinase 3 ligand (Flt3L) to detect expression of therapeutic transgenes in the striatum. Scale bar: 1000 μm for all but TK and Flt3L: 250 μm.

injection, Nissl staining revealed normal gross morphology for the lower doses of 1×10^8 vp and 1×10^9 vp, but tissue loss was detected in the brains injected with 1×10^{10} vp. MBP and TH denoted normal structure of the brain architecture for the two lower doses. In contrast, there were significant loss of myelinated nerves tangles and TH expressing neurons in the striatum of animals injected with the highest dose (1×10^{10} vp). Infiltration of $CD8^+$ T cells was seen with the highest vector dose which returned to normal levels after 1 month (Fig. S2). There were increased levels of immunoreactive ED1/CD68 and MHC II cells at the highest HC-Ad dose compared with saline (Supplementary Figs. S1a and S2). At 4 months and 1 year after delivery of 1×10^8 vp and 1×10^9 vp dose of HC-Ad-TK/TetOn-Flt3L we found an absence of scarring and complete restoration of the normal brain architecture. Although infiltration of $ED1^+$ or $MHCII^+$ immune cells was nearly absent in the brains injected with the highest dose (1×10^{10} vp), there was evidence of tissue damage and loss in the injected hemisphere (Supplementary Figs. S1b and Fig. 1c). Levels of MHCII, ED1/CD68, and CD8 immunoreactivity were quantified by computer-assisted image analysis software (Fig. S2). MHCII staining at 1 month was greater in the 1×10^9 vp and 1×10^{10} vp treated groups versus saline (*$P<0.05$; Supplementary Fig. S2b). CD8 staining at 5 days was greater in the 1×10^{10} vp treated group versus saline (*$P<0.05$; Supplementary Fig. S2i), and CD8 staining at 1 month was greater in the 1×10^9 vp and 1×10^{10} vp treated groups versus saline (*$P<0.05$; Supplementary Fig. S2j). Immunocytochemical characterization of transgene expression revealed abundant cells expressing TK within the brain striatum at day 5 (Fig. 1b) and 1 month after the injection of all three doses (Supplementary Fig. S1a). At 4 months (Supplementary Fig. S1b) and 1 year after the treatment (Fig. 1c), a few TK immuno-reactive positive cells remained very close to the injection site for all doses studied. Flt3L was detected at 5 days (Fig. 1b) and at lower levels at 1 month (Supplementary Fig. S1a) after injection; no Flt3L immunoreactivity was detected at either 4 months (Supplementary Figs. S1b) or 1 year after injection (Fig. 1c), this is due to the fact that Flt3L expression was turned "off" four weeks after HC-Ad delivery.

Analysis of clinical laboratory parameters

In order to detect any systemic side effects after HC-Ad-TK/TetOn-Flt3L delivery, we performed a thorough hematological and biochemical analysis of blood samples collected from the rats 5 days, 1 month, 4 months, and 1 year after treatment. The results, assessed using the markers aspartate aminotransferase (AST), alanine aminotransferase (ALT), bilirubin, urea (BUN), and creatinine, indicated normal liver and renal function at each time point across all vector doses compared to saline-injected animals (Table 1 and Supplementary Tables S1, S2, S3). Red and white blood cell counts in the HC-Ad-TK/TetOn-Flt3L injected animals were also within normal range, indicating that Flt3L transduction of the brain tissue does not substantially affect the levels of circulating blood cells.

Table 1

Biochemical and hematological parameters at 5 days after injection with escalating doses of HC-Ad-TK/TetOn-Flt3L into the brain parenchyma of naïve rats. At 5 days post treatment, blood was collected during euthanasia and a comprehensive panel of serum chemistry and hematologic parameters was performed by Antech Diagnostics (Irvine, CA). The median, minimum, and maximum values for each parameter at 5 days after treatment with escalating doses of HC-Ad-TK/TetOn-Flt3L are shown.

Parameters	Naive		Saline		HC-Ad-TK/TetOn-Flt3L					
					1×108		1×109		1×1010	
	Median	Range	Median	Range	Median	Range	Median	Range	Median	Range
Total protein (g/dl)	5.7	5.6–6.1	5.8	5.7–7.1	5.8	5.4–6.5	5.8	5.4–6.5	5.6	5.4–6.2
Albumin (g/dl)	2.9	2.8–3	2.9	2.5–3	2.8	2.6–3.1	2.8	2.5–3.1	2.8	2.6–3.1
Globulin (g/dl)	2.8	2.7–3.1	3	2.8–4.2	3	2.8–3.5	2.9	2.7–3.6	2.8	2.5–3.2
AST (IU/l)	87	63–422	110	67–593	157	69–348	134	65–308	127	63–265
ALT (IU/l)	51.5	46–89	41	30–161	43	31–74	38	4–96	38	16–55
Alkaline phosphatase (IU/l)	309	242–361	199	102–241	241	154–396	255	143–455	209	71–272
Total bilirubin (mg/dl)	0.1	0.1–0.1	0.2	0.1–0.6	0.2	0.1–0.7	0.2	0.1–0.3	0.2	0.1–0.4
BUN (mg/dl)	19.5	18–21	24	16–85	24	14–34	22	14–56	21	15–66
Creatinine (mg/dl)	0.3	0.2–0.3	0.5	0.4–1.5	0.5	0.4–0.6	0.5	0.2–0.7	0.4	0.3–0.5
Phosphorus (mg/dl)	8.1	7.2–11.2	13.9	9.6–22.1	14.2	10.8–20.8	12.3	9.3–23.4	11.8	9–19.4
Glucose (mg/dl)	86.5	47–105	162	57–310	142	87–346	180.5	103–302	201	107–292
Calcium (mg/dl)	10.4	9.9–10.9	10.6	8.9–11.5	10.8	9.4–12.1	10.6	9.6–12.4	10	9.2–11.5
Sodium (mmol/dl)	142.5	140–144	138	134–141	141	137–145	140	136–144	140	129–144
Potassium (mmol/dl)	6.3	5.4–7.2	10.6	8.1–13.4	9.8	7.1–12.8	8.1	4.9–11.8	12.7	7.4–19.1
Chloride (mmol/dl)	98	97–99	98	92–101	97	91–102	98	95–102	101	95–108
Cholesterol (mg/dl)	80	75–96	79	73–118	82	70–124	80	68–93	73	63–91
CPK (IU/l)	675.5	170–7517	493	193–23,079	689	208–15,555	657	164–13,064	981	172–8543
WBC count ($\times 10^3/\mu l$)	8.8	7.3–10	5.8	4.1–7.6	5.7	3.8–8.7	5.6	3.9–8.6	6.2	4–7.7
RBC count ($\times 10^6/\mu l$)	8.2	7.9–8.6	9	8.2–9.5	8.6	8–9.5	8.7	7.4–11	9.55	8–10.4
Hemoglobin (g/dl)	13.7	13.3–14.6	15	14–15.8	14.8	13.7–15.2	14.6	12.4–17.4	15.6	13.4–17
MCV (fl)	51	49–52	55	52–62	60	54–62	60	53–62	56	54–59
MCH (pg)	16.8	16.5–17.5	16.6	15.9–17.1	17	15.9–17.3	17	15.9–17.6	16.35	16.1–16.9
Neutrophils (%)	13	10–15	18	9–32	14	6–27	12	8–21	18	10–59
Absolute neutrophils (/μl)	1074	870–1500	928	369–2432	833	300–1806	600	312–1512	1023	470–4130
Lymphocytes (%)	85	83–87	79	64–89	82	68–93	86	75–90	80	40–87
Absolute lymphocytes (/μ/)	7611.5	6059–8300	4814	3649–5808	4675	3382–7052	4704	3471–7396	5031	2800–6391
Monocytes (%)	1	1–2	1	1–3	2	1–5	2	1–4	1	1–3
Absolute monocytes (/μl)	95.0	87–174	58	41–228	165	50–345	130	39–264	70	55–231
Eosinophils (%)	1	0–1	1	0–2	0	0–1	0	0–1	1	0–1
Absolute eosinophils (/μl)	88.0	0–100	52	0–94	0	0–86	0	0–47	60.5	0–77
Basophils (%)	0	0–0	0	0–1	0	0–1	0	0–1	0	0–1
Absolute basophils (/μl)	0	0–0	0	0–66	0	0–86	0	0–86	0	0–77
Platelet ($\times 10^3/\mu l$)	488.5	337–666	793	746–968	886	623–1018	890	625–1156	775.5	322–1561

Note: Red Blood Cell Morphology appeared normal in all groups. Abbreviations used: AST, aspartate aminotransferase; ALT, alanine aminotransferase; BUN, blood urea nitrogen; CPK, creatine phosphokinase; WBC, white blood cell; RBC, red blood cell; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin.

Behavioral impact after delivery of HC-Ad-TK/TetOn-Flt3L into the naïve rat brain parenchyma

In order to rule out the occurrence of chronic neurological deficits, naive animals injected with escalating doses of HC-Ad-TK/TetOn-Flt3L or saline were studied by performing a panel of neurobehavioral tests at 1 month (Supplementary Figs. S3), 4 months (Supplementary Figs. S4), and 1 year (Fig. 2). No abnormalities were detected with the doses of 1×10^8 vp and 1×10^9 vp at any of the time points tested with regard to total locomotor activity (Fig. 2a, and Supplementary Figs. S3a, S4a), rearing activity (Fig. 2b, and Supplementary Figs. S3b, S4b), right limb-use asymmetry (Fig. 2c, and Supplementary Figs. S3c, S4c), and amphetamine-induced rotational behavior (Fig. 2d and Supplementary Figs. S3d, S4d). However, there was evidence of neurobehavioral abnormalities in the groups of animals injected with the highest dose (i.e., 1×10^{10} vp), with regard to the mean asymmetry score (amphetamine-induced rotational behavior) at 4 months post injection (Supplementary Fig. S4d; *$P<0.05$) and the right limb use at 1 year post injection (Fig. 2c; *$P<0.05$).

Biodistribution of HC-Ad-TK/TetOn-Flt3L vector genomes following delivery into the naïve rat brain parenchyma

In order to characterize the biodistribution of the HC-Ad-TK/TetOn-Flt3L vector genomes, tissue samples from 13 sites or organs of naïve rats injected with escalating doses (1×10^8 vp, 1×10^9 vp, or 1×10^{10} vp) were analyzed. Rats were euthanized at 5 days (Figs. 3a–c), 1 month (Supplementary Figs. S5a–c), 4 months (Supplementary Figs. S5d–f), or 1 year (Figs. 3d–f). Bicistronic HC-Ad genomes were found restricted to the injected brain hemisphere at all time points tested (*$P<0.05$; Fig. 3 and Supplementary Fig. S5). The levels of Ad-TK/TetOn-Flt3L vector genomes remained stable 5 and 30 days after vector delivery. A decrease in HC-Ad vector genomes was detected 4 months after HC-Ad delivery. Importantly, HC-Ad-TK/TetOn-Flt3L vector genomes were below detectable limits in all peripheral organs, including other regions of the CNS and the liver, even at the highest dose tested at all time points.

Circulating neutralizing anti-adenovirus antibodies

Blood samples were collected from each animal during euthanasia to measure circulating anti-adenovirus neutralizing antibodies. Serum levels of anti-Ad neutralizing antibodies were indistinguishable from control levels at all-time points with the total HC-Ad vector dose of 1×10^8 vp (Fig. 4). Animals injected with the 1×10^9 vp and 1×10^{10} vp doses exhibited low titers of circulating anti-Ad neutralizing antibodies at the 1 month time point (Fig. 4b; *$P<0.05$), and at 4 months in the 1×10^{10} vp group (Fig. 4c; *$P<0.05$). However, all vector doses (1×10^8, 1×10^9 and 1×10^{10} vp) were indistinguishable from control samples at 1 year. In accordance with previous studies, neutralizing antibody titers at 64 or below were considered low and titers at or above 128 considered as high (Nwanegbo et al., 2004).

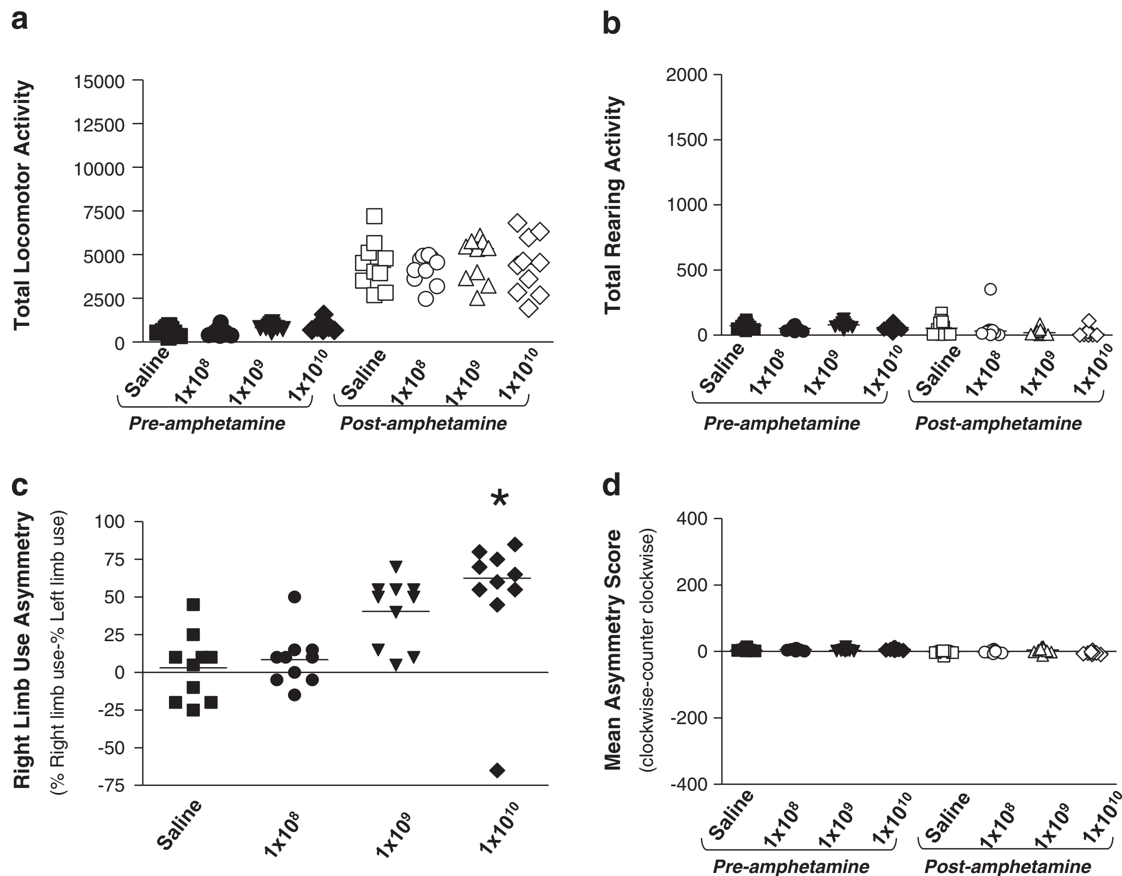

Fig. 2. Behavioral assessment of rats after 1 year of treatment with bicistronic high-capacity adenoviral vector, HC-Ad-TK/TetON-Flt3L. Behavioral assessment was performed before and after amphetamine treatment in the animals 1 year after intracranial administration of escalating doses of HC-Ad-TK/TetON-Flt3L. Saline-treated, age-matched rats were used as controls. (a) Total locomotor activity; (b) total rearing activity; (c) right limb use asymmetry; (d) asymmetry in rotational behavior. HC-Ad-TK/TetON-Flt3L, bicistronic high-capacity adenovirus; TK, thymidine kinase; Flt3L, fms-like tyrosine kinase ligand 3. Data were analyzed using one-way analysis of variance followed by Tukey's post-test (NCSS). *$P<0.05$ vs. saline.

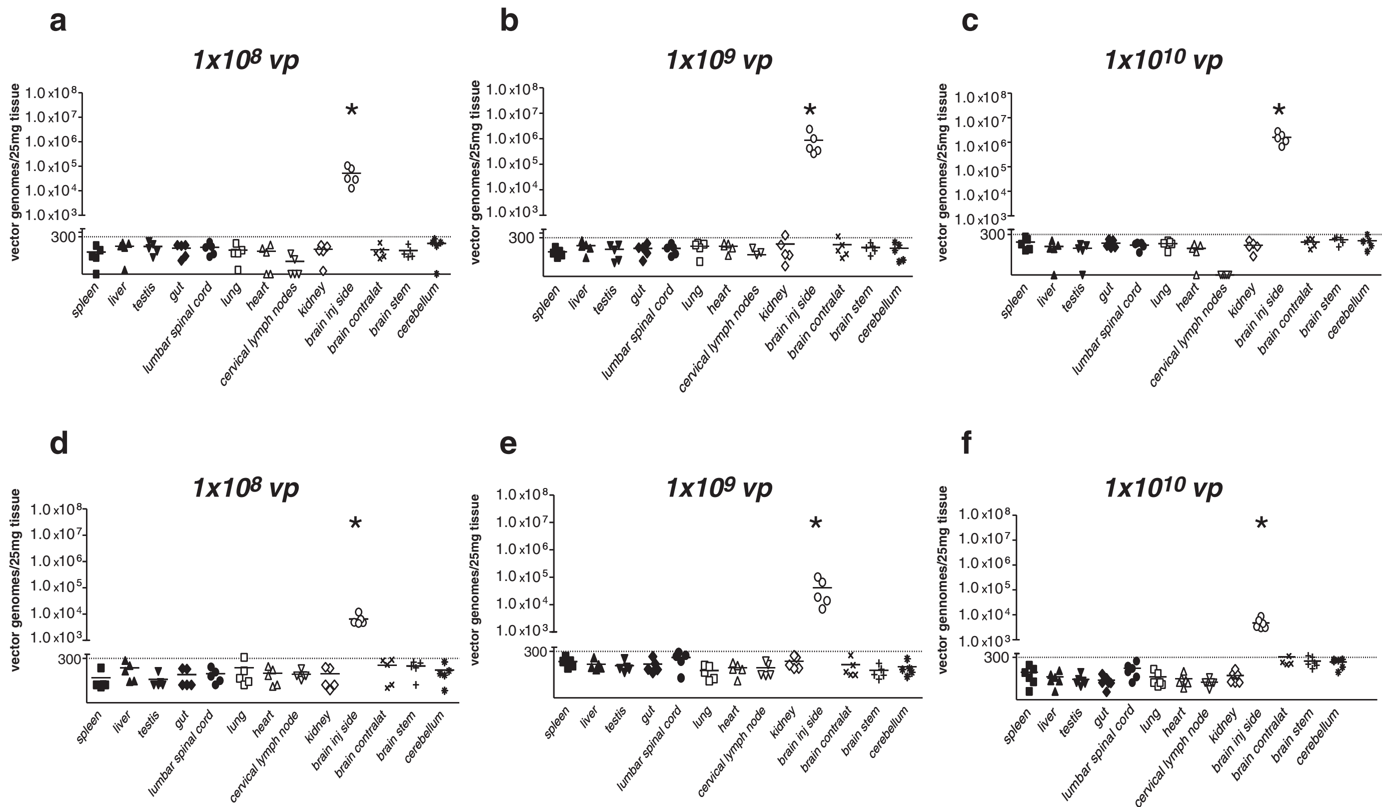

Fig. 3. Biodistribution of HC-Ad-TK/TetOn-Flt3L vector genomes at 5 days and 1 year after delivery into the brain parenchyma of naïve rats. Three escalating doses of HC-Ad-TK/TetOn-Flt3L, (a, d) 1×10^8 vp, (b, e) 1×10^9 vp, and (c, f) 1×10^{10} vp, were stereotactically injected into the striatum of naïve Lewis rats. Ganciclovir (25 mg/kg, i.p.) was injected twice daily for up to 10 days and DOX chow was administered ad libitum for up to 4 weeks. The animals were euthanized at experimental end points and tissue samples (25 mg) were harvested from a total of 13 organs or sites: brain injection site, brain contralateral side, brain stem, cerebellum, lumbar spinal cord, cervical lymph nodes, heart, lung, spleen, liver, kidney, small gut, and testes. 5 μl of the isolated DNA from each tissue sample was used for qPCR to assess the biodistribution of the vector genomes. Vector genomes' quantification results are the average of triplicates for each DNA sample, and are shown as a ratio of vector genomes/25 mg of tissue. Dotted line indicates detection limit. Results are based on n = 5 per group. The biodistribution study at 5 days (a,b,c) and 1 year (d,e,f) post HC-Ad-TK/TetOn-Flt3L delivery showed that the vector genomes were restricted to the brain side of injection. Data were analyzed using one-way analysis of variance followed by Tukey–Kramer Multiple-Comparison Test (NCSS). Where data were not normally distributed, they were log-transformed. *P<0.05.

Circulating anti-HSV1-TK antibodies in the serum of HC-Ad-TK/TetOn-Flt3L treated naïve rats

HSV1-TK is a viral protein and could potentially be immunogenic. In order to assess whether transduction of brain tissue with HC-Ad-TK/TetOn-Flt3L can induce a systemic immune response against HSV-1 TK, the levels of anti-HSV1-TK serum antibodies were determined by ELISA. For this purpose, blood samples were taken before euthanasia 5 days, 1 month, 4 months, and 1 year after the administration of HC-Ad-TK/TetOn-Flt3L in naïve rats (Fig. 5). Analysis of anti-HSV1-TK serum antibodies revealed the presence of TK-specific antibodies only in the serum of animals injected with 1×10^{10} vp at 4 months (Fig. 5c; *P<0.05) and 1 year (Fig. 5d; *P<0.05).

Efficacy study in rats bearing intracranial CNS-1 tumors treated with HC-Ad-TK/TetOn-Flt3L

Because doxycycline is a widely used antibiotic to treat infections in humans, we needed to determine if the doses which are currently approved by the FDA would be effective at turning on Flt3L expression for the HC-Ad-TK/TetOn-Flt3L after delivery into the brain. The level of DOX was calculated to be allometrically equivalent (Voisin et al., 1990) to doses in a 45.5 kg human of 200 or 300 mg DOX/day. The 200 mg DOX/day human dose was calculated to be equivalent to 30.8 mg/kg/day in rats, and the 300 mg DOX/day human dose we calculated to be equivalent to 46.2 mg/kg/day. Supplementary Fig. S6c shows Flt3L expression and neuropathology analysis, and Supplementary Fig. S6b shows complete blood cell counts (CBC) and plasma chemistry in rats orally gavaged with 30.8 mg DOX/kg/day. Supplementary Fig. S7 shows the same analysis but for the 46.2 mg DOX/kg/day group. We found that the lower dose of 30.8 mg DOX/kg/day was sufficient to turn on expression of Flt3L within the brain parenchyma. We tested the acute inflammatory response in the HC-Ad-TK/TetOn-Flt3L treated animals in the presence of high- and low-doses of DOX using ELISAs for TNFα, IL1β, and IL6. Our data indicated that the levels of these cytokines were below the detection limits of the assays and indistinguishable from saline-treated animals (data not shown). Liquid chromatography with tandem mass spectrometry (LC–MS/MS) analysis of the plasma DOX concentration using the oral gavage regime showed DOX levels of 1272 +/− 254 ng/ml (mean +/− SEM) in the 30.8 mg DOX/kg/day group, and 3055 +/− 295 ng/ml in the 46.2 mg DOX/kg/day group. These values are in line with the levels attained in human plasma after treatment with 200 mg/day of DOX (Beringer et al., 2012). We then proceeded to test the efficacy of the HC-Ad-TK/TetOn-Flt3L vector in a tumor model. Using the allometrically equivalent (Voisin et al., 1990) DOX dose of 30.8 mg/kg/day and a VAL dose of 560 mg/kg/day (calculated to be equivalent to the therapeutically relevant human dose of 6 g VAL/day (Chiocca et al., 2011)), rats (n = 5 per group) bearing intracranial tumors were treated with 1×10^8 vp of HC-Ad-TK/TetON-Flt3L or saline. DOX (30.8 mg/kg/day) was administered by oral gavage for 28 days following bicistronic vector treatment, and VAL (560 mg/kg/day) was administered also by oral gavage for 15 days following treatment (Fig. 6a). Our data indicate that the treatment led to long-term survival in approximately 40% of the rats (Fig. 6b; *P<0.05, log-rank test).

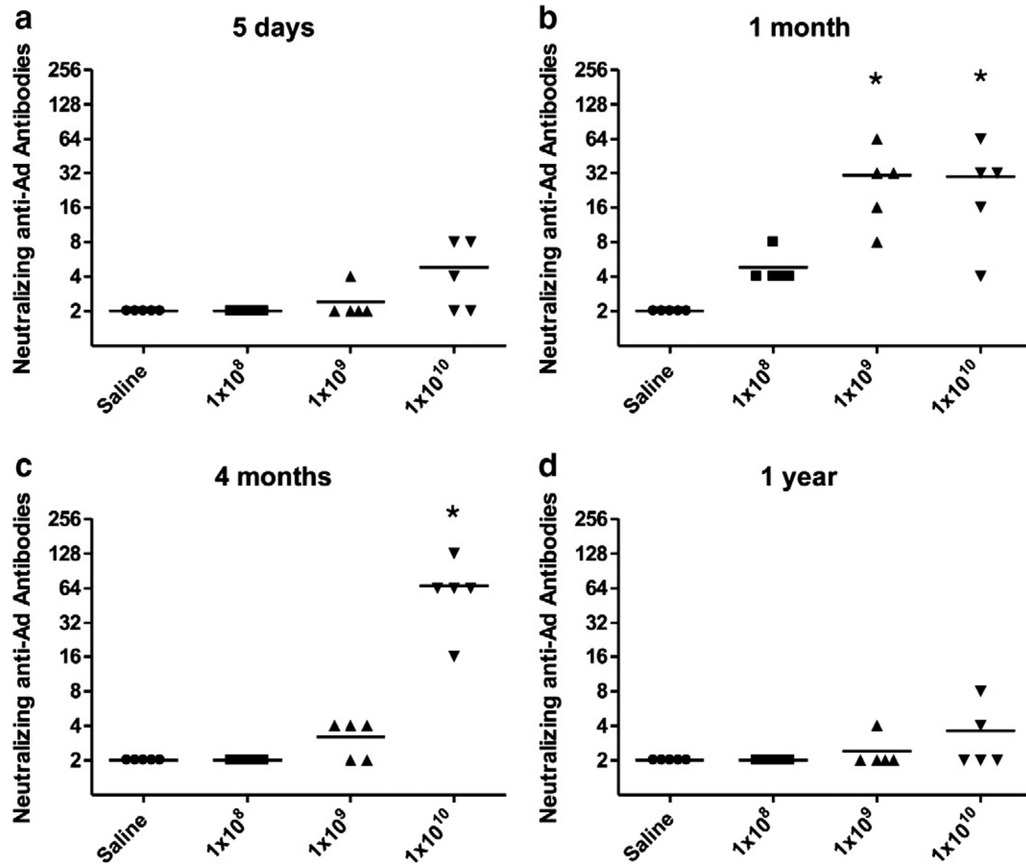

Fig. 4. Anti-adenovirus neutralizing antibodies in the serum of HC-Ad treated rats. The prevalence of anti-adenovirus neutralizing antibody in the serum of rats treated with intracranial injections of escalating doses of bicistronic HC-Ad-TK/TetOn-Flt3L or saline is shown. Ganciclovir (25 mg/kg, i.p.) was administered twice daily for up to 10 days and DOX chow was administered ad libitum for up to 4 weeks. The rats were euthanized at 5 days (a), 1 month (b), 4 months (c), and 1 year (d) after treatment and serum was collected. Low levels of neutralizing antibody were detected at the highest dose 1×10^{10} vp 4 months after HC-Ads delivery. *$P<0.05$ versus saline (one-way ANOVA followed by Tukey's multiple comparison test).

Neuropathological analysis of both saline-treated (Fig. 6c) and HC-Ad-TK/TetON-Flt3L-treated (Fig. 6d) rats confirmed the regression of the tumor mass in the vector-treated animals. Immune cell infiltration with macrophages, microglia, and CD8-positive cells was clearly evident (Fig. 6d), especially at the interface between the tumor cell mass and normal brain tissue.

Discussion

Due to the highly infiltrative nature of GBM, conventional therapies fail to significantly improve overall survival (Louis et al., 2007; Wen and Kesari, 2008), hence, there is a need to develop alternative therapeutic strategies. A body of literature describes the use of adenoviral vector-based gene therapies, including cytotoxic approaches and oncolytic vectors, to treat various types of cancer (Aguilar et al., 2011; Fukazawa et al., 2010; Harrington et al., 2010; Kroeger et al., 2010; Senzer et al., 2009; Teh et al., 2004). Results published from several clinical trials in the U.S. and Europe show that no treatment has been able to induce more than a modest increase in median survival (Chiocca et al., 2011; Maatta et al., 2009). As in other areas of cancer research, early phase I and II trials showed benefits. However, in a phase III trial employing adenovirus expressing HSV1-TK for the treatment of high-grade glioma performed throughout many European countries, the outcome was disappointing. Significant logistic shortcomings in this trial have led other investigators in the USA to continue trials using this approach for the treatment of both CNS and other cancers (Aguilar et al., 2011; Chiocca et al., 2011). Therefore, the evaluation of the use of adenoviral vectors for the treatment of cancer is still ongoing. The significant difference of the proposed approach is the combined use of tumor cell killing (mediated by HSV1-TK + ganciclovir) and immune-stimulation mediated by Flt3L elicited directly from the affected organ. This approach, pioneered by our research group, and described in preclinical experimental papers (Ali et al., 2005; Assi et al., 2012; Candolfi et al., 2009b, 2011, 2012; Castro et al., 2011; Curtin et al., 2005, 2008a, 2009; Ghulam Muhammad et al., 2009; King et al., 2008a, 2008c, 2011; Mineharu et al., 2012; Muhammad et al., 2010; Puntel et al., 2010b), will be tested for the first time in human patients using first generation adenoviral vectors (IND 14574) in a forthcoming phase I clinical trial. All evidence so far indicates that the combination of simultaneous tumor cell killing and immune stimulation (Pluhar et al., 2010) may provide the clinical benefit which still eludes the field.

Using first-generation adenoviral vectors as vehicles for gene delivery, we have described the detailed mechanism and efficacy of a conditional cytotoxic/immune-stimulatory approach combining delivery of TK and Flt3L therapeutic transgenes directly into the tumor mass. We have demonstrated the therapeutic efficacy of this approach in rat and mouse syngeneic intracranial models of glioblastoma (Ali et al., 2005; Curtin et al., 2009; Ghulam Muhammad et al., 2009; King et al., 2008a, 2008c; Muhammad et al., 2010; Puntel et al., 2010b; Yang et al., 2010). The molecular mechanism of action of this treatment involves Flt3L-mediated recruitment of dendritic cells (DCs) into the GBM microenvironment; DCs phagocytose endogenous brain tumor antigens released in response to the killing of tumor cells induced by Ad-TK in the presence of GCV (Ali et al., 2005).

Gene therapy-induced cell death mediated by Ad-TK releases HMGB1, a TLR2 agonist which is necessary for the activation of DCs recruited to the tumor environment by Ad-Flt3L and the subsequent

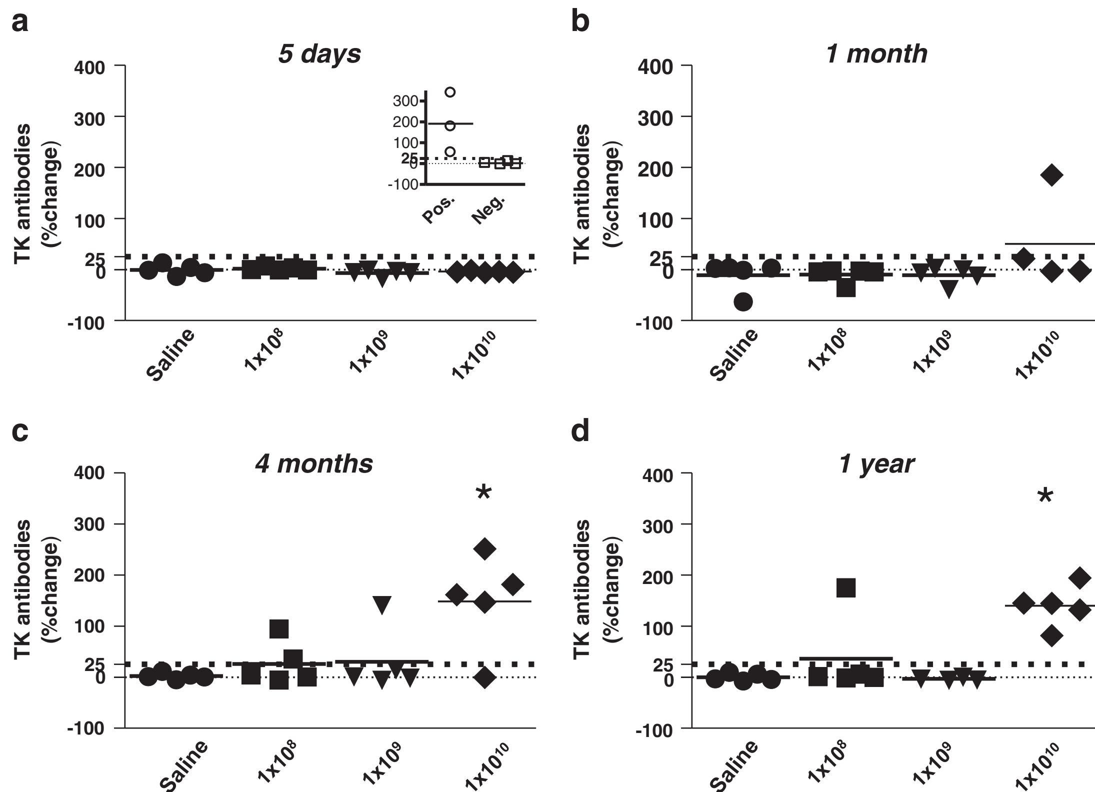

Fig. 5. Circulating anti-thymidine kinase antibodies. Escalating doses of HC-Ad-TK/TetOn-Flt3L, or saline were stereotactically injected into the striatum of Lewis rats. Ganciclovir (GCV) (25 mg/kg, i.p.) was administered twice daily for up to 10 days and doxycycline (DOX) containing chow was administered ad libitum for up to 4 weeks. The rats were euthanized at 5 days (a), 1 month (b), 4 months (c), and 1 year (d) after treatment and sera were collected. Serum from each animal was evaluated for the presence of circulating antibodies specific for thymidine kinase (TK). Circulating anti-TK antibodies were detected at the highest doses 1×10^{10} vp after 4 months and 1 year of the HC-Ad-TK/TetOn-Flt3L delivery into the naïve rat brain. The ELISA titer of anti-TK antibodies is represented as the % change in signal intensity from sera incubated with cell lysates from Ad-TK infected cells when compared to mock infected cells. Samples were considered positive when the % change was ≥25%; this threshold is indicated by the dashed line. Positive control and negative control were applied. Data were analyzed using one-way analysis of variance followed by Tukey's post-test (NCSS). *P<0.05 vs. saline.

activation of a systemic anti-GBM immune response (Curtin et al., 2009). DCs loaded in situ with brain tumor antigens then migrate to the draining lymph nodes, where they present tumor antigen to naïve T cells, thus inducing a brain tumor-specific immune response (Curtin et al., 2006, 2009) and generating immunological memory that protects against multifocal (King et al., 2008c) and recurrent brain tumors (King et al., 2011), as well as against tumors displaying new antigens (King et al., 2011). A multicenter phase I clinical trial for GBM has been approved by the FDA for testing this combined gene therapy strategy (BB-IND 14574; NIH/OBA Protocol # 0907-990; OSU Protocol # 10089).

The incorporation of both Flt3L and TK into a single HC-Ad vector would enhance the safety of the GMP manufacture by reducing the total viral load by 50% compared to the two-vector approach. As the maximum amount of vector that can be injected is limited by vector dose-mediated toxicity, our strategy maximizes therapeutic transgene expression per total viral load, thus reducing any putative brain inflammation. We observed inflammation in the brain of naïve animals injected in the normal brain parenchyma using 1×10^{10} vp of HC-Ad-TK/TetOn-Flt3L, suggesting that this is the maximum tolerated dose for this vector. Due to the complete deletion of all viral coding genes, HC-Ads do not display de novo expression of any viral antigens; therefore, cells infected with HC-Ads are not targeted by the anti-adenoviral immune response (Muhammad et al., 2010; Puntel et al., 2010b; Thomas et al., 2001). This facilitates long-term transgene expression in the brain, even in the presence of a systemic anti-adenoviral immune response (Muhammad et al., 2010; Puntel et al., 2010b; Thomas et al., 2000, 2001). Another advantage of the HC-Ad vector platform is its ability to encode large, complex expression cassettes, such as the inducible TetOn expression system (Xiong et al., 2006). The vector system described, i.e., gutless, helper dependent adenovirus vectors (HC-Ads) has never been used to treat human cancers. Further, since we are aiming to implement this approach in a phase I clinical trial in patients with glioblastoma multiforme (GBM) and the vectors will be injected at the time of surgery into the resection cavity, it is likely that normal brain cells will also be transduced. As gutless adenovirus vectors elicit stable transgene expression, regulation of Flt3L expression will enable turning expression off when no longer required; this adds to the safety of our approach (Curtin et al., 2008a, 2008c; Goverdhana et al., 2005). To this end, we have shown that the HC-Ad-TetOn vector system which we have developed induces sustained transgene expression in the presence of the inducer Dox, but transgene expression is negligible in the absence of Dox (Curtin et al., 2008b, 2008c; Goverdhana et al., 2005; Xiong et al., 2006). The HC-Ad vector system and therapeutic transgenes have never been tested in human clinical trials to date. Further, the regulatable promoter system will add safety and versatility to this therapeutic strategy. Doxycycline, needed to induce expression of Flt3L has been in clinical use for many years, and is usually well tolerated. In our trial, doxycycline will only be given to human patients for a limited time. The potential side effects (i.e., allergy to doxycycline, fever, nausea, vomiting) will be carefully monitored. Increased side effects of doxycycline in patients with brain tumors have not been reported. Consultation with our team of neurosurgeons that will be in charge of the clinical aspects of this clinical trial did not raise any further concerns that require special

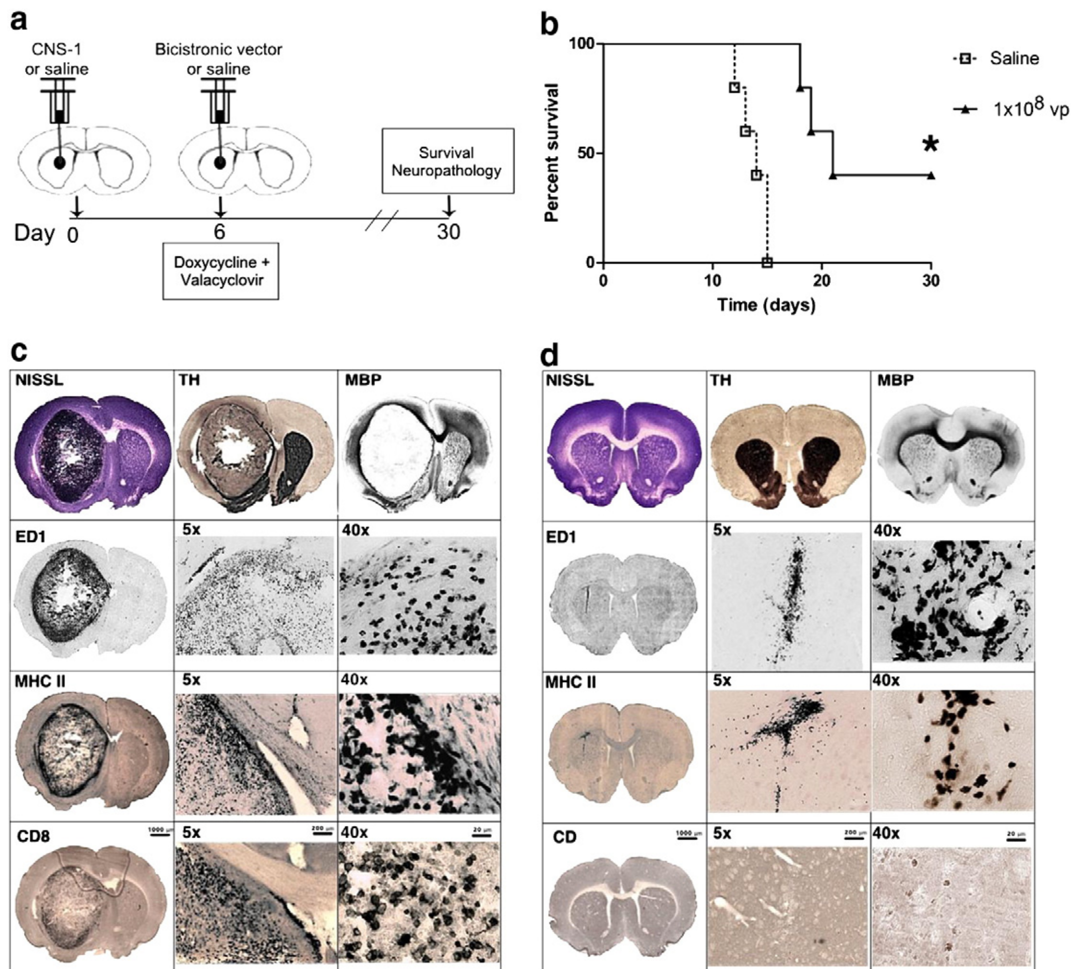

Fig. 6. Efficacy and neuropathology after intratumoral administration of 1×10^8 vp of the bicistronic HC-Ad. (a) HC-Ad-TK/TetOn-Flt3L (n = 5) or saline (n = 5) was delivered into CNS-1 tumors implanted into the striatum. (b) Kaplan–Meier survival curves of naïve tumor-bearing rats treated with bicistronic therapeutic HC-Ad. *P<0.05 versus saline (log-rank test). Representative brain sections of (c) saline-treated tumor-bearing animals and (d) long-term survivors were evaluated for structural integrity using Nissl staining, TH (tyrosine hydroxylase), and MBP (myelin binding protein) as well as infiltrating immune cells using ED1 (activated macrophages and microglia), MHCII (immune cells), and CD8 (cytotoxic T cells). Scale bars for whole sections: 1000 μm; scale bars for 5× images: 200 μm; and scale bars for 40× images: 20 μm.

consideration. Preliminary discussions with FDA have also failed to raise specific concerns relating to doxycycline administration to human brain tumor patients.

We recently demonstrated the therapeutic efficacy and high safety profile of the novel bicistronic vector HC-Ad-TK/TetOn-Flt3L in an intracranial syngeneic tumor-bearing model (Puntel et al., 2010b); however, an important aspect of characterizing new gene therapy agents is their tissue biodistribution (FDA, 2006), especially with an intracranial therapy because of the potential for irreversible neurological damage beyond the target site (Gonin and Gaillard, 2004). To directly address the efficacy of the bicistronic therapy at a lower and potentially safer dose of 1×10^8 vp, we implanted Lewis rats with 4500 CNS-1 cells into the striatum on Day 0, at 6 days after tumor implantation, rats were treated with 1×10^8 vp of HC-Ad-TK/TetOn-Flt3L vector delivered into the tumor mass. The prodrug valacyclovir (560 mg/kg) was administered via oral gavage from Day 7 to Day 21. To induce expression of Flt3L, doxycycline (30.8 mg/kg) was administered from Day 5 to Day 34 also via oral gavage. Oral gavage was utilized to mimic the route of administration used in human patients undergoing clinical trials (Chiocca et al., 2011). Of note, we based the dose and delivery route for valacyclovir on the dosing schedule reported in a previously approved clinical trial for GBM which utilized a first generation adenovirus vector encoding TK (Chiocca et al., 2011). The results from this experiment indicate that the therapy will still be efficacious using a lower HC-Ad dose in conjunction with equivalent doses and administration routes for VAL and DOX as have been previously used in humans (Chiocca et al., 2011; Micromedex, Retrieved December 2012).

In the present study, we investigated in naïve rats the biodistribution and general toxicity of a bicistronic high-capacity adenoviral vector HC-Ad-TK/TetOn-Flt3L administered into the brain striatum by stereotactic injection. Seeking to meet specific goals and criteria as recently delineated by the US Food and Drug Administration (FDA), we applied a real-time qPCR method to measure DNA content in various organs, evaluated serum biochemistry and blood cell counts, and tested the presence of circulating anti-TK-specific antibodies and neutralizing anti-Ad antibodies. We have previously demonstrated the safety and efficiency of the regulatory TetOn switch in the sustained transgene expression in the brain even in the presence of an immune response against its components (Xiong et al., 2008).

Therapeutic genes carried by HC-Ad vectors have been administered in pre-clinical models for different diseases, such as hemophilia A and B (Ehrhardt et al., 2003; Reddy et al., 2002), obesity (Morsy et al., 1998), familial hypercholesterolemia (Belalcazar et al., 2003; Kim et al., 2001; Oka et al., 2001; Pastore et al., 2004), ornithine transcarbamylase deficiency (Mian et al., 2004), diabetes (Kojima et al., 2003), and chronic viral hepatitis (Aurisicchio et al., 2000; Fiedler et al., 2004). Additionally, the efficacy of HC-Ad vectors has been assessed in animal models of sensory neuronopathies (Terashima et al., 2009), diabetic retinopathy (Lamartina et al., 2007), glycogen storage (Schillinger et al., 2005),

and hypertension (Schillinger et al., 2005). In anticipation of a phase I clinical trial for GBM using HC-Ad-TK/TetOn-Flt3L, we performed a dose-escalation study and executed a comprehensive analysis of toxicity in naïve rats. To our knowledge, these data represent the first toxicity study in naïve animals utilizing an intracranially injected single bicistronic HC-Ad vector.

Remarkably, vector genomes were restricted to the injection site of the brain in all rats tested at all-time points. This observation has profound implications for glioma gene therapy because the proposed treatment for GBM patients after surgical resection consists of injection of HC-Ad-TK/TetOn-Flt3L into the adjacent brain cavity following surgical resection of the tumor mass. Although considerably reduced from the levels at day 5, sustained levels of HC-Ad-TK/TetOn-Flt3L vector genomes persisted in the brain for up to 1 year after treatment, addressing the safety of the approach and suggesting the possible "reactivation" of the cytotoxic effects of TK and/or the immune stimulatory effects of Flt3L by the re-administration of GCV or DOX, respectively, if required (Xiong et al., 2006).

Using escalating doses of HC-Ad-TK/TetOn-Flt3L, we found 1×10^9 vp to be the maximum tolerated dose when injected into the naïve brain. The restoration of the normal brain architecture, the absence of severe long-term behavioral deficits at 1 year after injection, and the absence of significant chronic inflammation in the brain provide strong evidence regarding the safety of this single vector approach. We found 1×10^{10} vp to have toxic side effects, as assessed by neuropathology, behavioral testing, and the anti-Ad- and anti-TK-specific immune response.

CD8+, ED1/CD68+, and MHCII+ staining decreased at 4 months and 1 year after treatment and this may be attributed, at least partly, to the regulatable features of the engineered HC-Ad-TK/TetOn-Flt3L, which expresses Flt3L only in the presence of DOX. The absence of demyelination at all-time points attests to the overall safety of up to 1×10^9 vp of the bicistronic HC-Ad. Importantly, the delivery of up to 1×10^9 vp of bicistronic HC-Ad did not cause any alterations in biochemical or hematological parameters, demonstrating the lack of systemic toxicity of the HC-Ad-TK/TetOn-Flt3L mediated approach. Expression of Flt3L within the brain decreased over time, reflecting the inducible nature of the TetOn element. The presence of a humoral immune response against HSV1-TK only with the highest vector dose reinforces the conclusion that 1×10^9 vp is the maximum tolerated dose for this vector platform. Delivery of higher HC-Ad doses could lead to leakage of the vector into the ventricles or provoke local rupture of the blood brain barrier, with concomitant induction of anti-adenovirus neutralizing antibodies and anti-HSV1-TK circulating antibodies in the serum of the treated animals. In addition, the animals injected with 1×10^{10} vp, exhibited significantly different amphetamine-induced rotational behavior at 1 month and right limb asymmetry at 1 year, suggesting that neurotoxicity induced by the high HC-Ad dose could also lead to behavioral abnormalities in the treated animals.

In summary, the results reported in this study show that 1×10^9 vp of HC-Ad-TK/TetOn-Flt3L is the maximum tolerated dose that can be safely administered in the naïve brain parenchyma without adverse side effects. In the clinical scenario, we propose to deliver the therapeutic HC-Ad vector into the margins of the tumor bed; the data presented provide a compelling foundation for testing this novel therapeutic approach in a phase I clinical trial for patients with glioblastoma multiforme.

Author disclosure statement

We declare that we have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.taap.2013.02.001.

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