AAV-Mediated Gene Targeting Is Significantly Enhanced by Transient Inhibition of Nonhomologous End Joining or the Proteasome *In Vivo*

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Abstract

Recombinant adeno-associated virus (rAAV) vectors have clear potential for use in gene targeting but low correction efficiencies remain the primary drawback. One approach to enhancing efficiency is a block of undesired repair pathways like nonhomologous end joining (NHEJ) to promote the use of homologous recombination. The natural product vanillin acts as a potent inhibitor of NHEJ by inhibiting DNA-dependent protein kinase (DNA-PK). Using a homology containing rAAV vector, we previously demonstrated *in vivo* gene repair frequencies of up to 0.1% in a model of liver disease hereditary tyrosinemia type I. To increase targeting frequencies, we administered vanillin in combination with rAAV. Gene targeting frequencies increased up to 10-fold over AAV alone, approaching 1%. *Fah*^{-/-}*Ku70*^{-/-} double knockout mice also had increased gene repair frequencies, genetically confirming the beneficial effects of blocking NHEJ. A second strategy, transient proteasomal inhibition, also increased gene-targeting frequencies but was not additive to NHEJ inhibition. This study establishes the benefit of transient NHEJ inhibition with vanillin, or proteasome blockage with bortezomib, for increasing hepatic gene targeting with rAAV. Functional metabolic correction of a clinically relevant disease model was demonstrated and provided evidence for the feasibility of gene targeting as a therapeutic strategy.

Introduction

ADENO-ASSOCIATED VIRUS (AAV) is one of the safest viral vectors for use in human gene therapy (Buning *et al.*, 2003). The field of AAV gene therapy research continues to be dominated by gene addition approaches using cDNA cassettes driven by heterologous promoter and enhancer sequences to augment expression of a mutated disease gene. Yet, drawbacks to this methodology abound including unregulated and transient episomal expression (Garrick *et al.*, 1998; Russell and Kay, 1999), random integration (Hendrie *et al.*, 2003), increased oncogenic risks (Donsante *et al.*, 2007), and the inability to treat genetically dominant disorders arising from misfolded or abnormally functioning proteins.

AAV-based gene-targeting paradigms differ in that patient genomes are manipulated *in vivo* using site-specific recombination to correct underlying mutations. In this way, gene function is restored within the context of endogenous regulatory elements, thus eliminating problems with inadequate or inappropriate expression. AAV gene-targeting vectors can correct different genomic loci, show both targeted and stable expression through integration, and cover a

greater number of applicable human diseases (Hendrie and Russell, 2005). Single-stranded AAV genomes utilize homologous recombination machinery to correct mutations through targeted site-specific integration (Parekh-Olmedo *et al.*, 2005).

Our lab has demonstrated the utility of recombinant adeno-associated virus (rAAV) for performing gene targeting in an ENU-generated point mutation mouse model of hereditary tyrosinemia type I (HTI) (Paulk *et al.*, 2010), a fatal disease arising from a breakdown in tyrosine catabolism at fumarylacetoacetate hydrolase (FAH). In both HTI patients and *Fah*^{-/-} mice, hepatotoxic metabolites accumulate, causing death in a cell-autonomous manner. Treatment with 2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione (NTBC), an inhibitor that blocks upstream of FAH, prevents metabolite accumulation and rescues the phenotype (Al-Dhalimy *et al.*, 2002). FAH⁺ cells have a strong selective advantage in the HTI mouse liver (Overturf *et al.*, 1996) that can be exploited for selection of repaired hepatocytes *in vivo*. One advantage of the *Fah* model for gene-targeting studies is that the readout is functional phenotype restoration, not merely correction of an artificial reporter system with no

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bearing on physiology. When rAAV-*Fah* vectors are administered to *Fah*^{-/-} mice, only corrected hepatocytes survive and repopulate the liver. Our repair vectors contain a 4.5-kb genomic *Fah* fragment centered on the nucleotide needed for correction in the *Fah*^{-/-} mouse. Hepatic correction frequencies of up to 0.1% were previously achieved with this model, a level that was subtherapeutic without selection (Paulk *et al.*, 2010).

To improve upon earlier results and further increase correction to more therapeutic levels, we sought to transiently inhibit unwanted repair pathways such as nonhomologous end joining (NHEJ) to promote the sole use of homologous recombination (HR) machinery. NHEJ is the primary pathway of double-strand break (DSB) repair in postembryonic human cells (van Gent *et al.*, 2001). AAV vectors do not contain an endonuclease to create DNA DSBs and free chromosomal ends, thus naturally occurring breaks must be used when integrating. If NHEJ utilization is prevented, theoretically HR proteins should take over to repair the break with the homologous AAV repair template. Vanillin (3-methoxy-4-hydroxybenzaldehyde), a natural phytochemical, acts as a potent inhibitor of NHEJ by binding to and inhibiting DNA-dependent protein kinase (DNA-PK) (Durant and Karran, 2003). Vanillin is highly selective for DNA-PK and has been shown to have no measurable effect on other steps of NHEJ or on various other unrelated protein kinases (Durant and Karran, 2003).

Through work using proteasomal inhibitors, it was shown that the majority of AAV particles do not deliver their genomic payload to the nucleus (Johnson and Samulski, 2009). Indeed, several labs have shown that proteasomal inhibitors are capable of enhancing AAV transduction both *in vitro* and *in vivo* (Johnson and Samulski, 2009; Finn *et al.*, 2010; Monahan *et al.*, 2010), but the mechanism by which this occurs remains unknown. Several hypotheses have been put forward including that they increase AAV trafficking efficiency, block cytoplasmic or nuclear capsid degradation indirectly improve genome stability; decrease T-cell activation/proliferation; or decrease capsid antigen presentation. The only proteasomal inhibitor currently approved for clinical use is bortezomib (VelcadeTM), and we therefore sought to uncover whether this compound would work alongside vanillin in our AAV gene-targeting studies by improving AAV transduction.

Materials and Methods

Mouse strains and animal husbandry

The ENU-generated *Fah*^{5981SB} mouse (herein described as *Fah*^{-/-}) models human HTI with a single point mutation in exon 8 of *Fah* (Culiat *et al.*, 2005). Neonates die from acute liver failure if NTBC is not continually administered. NTBC treatment at 4 mg/L in drinking water prevents hepatorenal injury and rescues the phenotype. NTBC withdrawal allows modeling of HTI as mice rapidly develop hepatorenal disease over the course of several weeks and die of end-stage liver disease in 6–8 weeks (Grompe *et al.*, 1995). These mice have been backcrossed 10 generations onto a C57BL/6J background. The various *Fah*/*Ku70* mice were generated by crossing *Fah*^{-/-} mice with *Ku70*^{+/-} mice (Gu *et al.*, 1997) to establish *Fah*^{-/-}/*Ku70*^{+/-} breeders. All mice described are maintained on irradiated high-fat, low-protein mouse chow (Lab Diet, Brentwood, MO, cat. no. Picolab 5LJ5) *ad libitum* to

decrease flux through the tyrosine pathway. The Institutional Animal Care & Use Committee of Oregon Health & Science University approved all mouse procedures.

Plasmid vectors and recombinant AAV production

Plasmid construction and design strategy were described previously (Paulk *et al.*, 2010). Standard transfection and viral isolation protocols described previously (Grimm, 2002), were used with the following modifications. Unpurified virus was sedimented and resuspended in benzonase buffer for freezing at -80°C. After three freeze-thaw cycles in a dry ice/ethanol bath, the suspension was benzonase-treated (EMD Chemicals, San Diego, CA). Virus was pelleted and precipitated with 1 M CaCl₂, followed by 40% PEG-8000 treatment and resuspension in a sodium/HEPES buffer and rotated overnight at 4°C. After resuspension, purification by sequential cesium chloride density centrifugation was performed as described (Choi *et al.*, 2007) with the following modifications. Peak fractions from both gradients were determined by optical refractometry. Positive final fractions were extensively dialyzed against phosphate-buffered saline lacking Ca²⁺ or MgCl₂ (Gibco, Grand Island, NY) and supplemented with 5% (w/v) D-sorbitol. Vector titering was performed by dot blot.

Neonatal vector administration

For comparative treatment studies, P1 *Fah*^{-/-} neonates were injected with 1 × 10¹¹ vector genomes (vg) per mouse (in 10-μl volume) of rAAV2/8-*Fah* by intravenous (IV) injection into the superficial temporal vein. All mice were maintained on NTBC throughout. Livers were harvested at weaning, 21 days post-treatment. For random integration studies, P1 *Fah*^{-/-} neonates received 4 × 10¹⁰ vg/mouse of both rAAV2/8-*Fah* (Paulk *et al.*, 2010) and rAAV2/8-LSP-eGFP (Wang *et al.*, 1999) in a 20-μL volume by IV injection into the superficial temporal vein. Neonates were treated with NTBC until weaning and then withdrawn from it to select for corrected hepatocytes. Serum and liver tissue were collected after serial transplantation.

Adult vector administration

Adult *Fah*^{-/-} mice (age ≥ 13 weeks) were administered 2 × 10¹¹ vg/mouse of rAAV2/8-*Fah* in 100-μl volume by IV lateral tail vein injection. NTBC treatment was continued for 1 week following vector administration during capsid uncoating, and then withdrawn for 3 weeks up to 3 months depending on the study. Liver tissue and serum were collected at the end of each experiment.

Vanillin administration

For each experiment, a fresh vanillin (Alfa Aesar, Ward Hill, MA) stock solution of 10 mg/ml solubilized in dH₂O was prepared, sterile filtered, and kept at 55°C to keep vanillin in solution. This solution was then administered at 100 mg/kg/d by intraperitoneal (IP) injection for 7 days starting on the day of vector administration.

Bortezomib administration

For each experiment, a fresh bortezomib (Melone Pharmaceutical, Edison, NJ) stock solution of 5 mg/ml solubilized

in dimethyl sulfoxide (DMSO) was prepared, sterile filtered and diluted in 0.9% sterile saline for co-administration with AAV at 0.5-mg/kg by IV lateral tail vein injection (100- μ l total volume).

4,5-Dimethoxy-2-nitrobenzaldehyde administration

For each experiment, a fresh 4,5-dimethoxy-2-nitrobenzaldehyde (DMNB; Sigma, Milwaukee, WI) stock solution of 10 mg/ml solubilized in DMSO was prepared, sterile filtered, and diluted in 0.9% sterile saline. This solution was then administered at 100 mg/kg on days 2 and 5 after vector administration by IP injection.

Transplantation

For serial transplantations, livers of corrected donor mice were perfused as described (Grompe *et al.*, 1992) and 4.5×10^5 random hepatocytes were transplanted into six recipients, each by intrasplenic injection as described (Ponder *et al.*, 1991).

Liver immunohistochemistry

In all experimental mice, a minimum of four liver sections from varying depths and randomly selected liver lobes were analyzed for the number of FAH+ nodules. Since nodules represent the clonal expansion of a single corrected hepatocyte, nodule frequency was corrected for nodule size. Briefly, the surface area of each liver section was measured by scanning the glass slide along with a size standard using an Epson Perfection V700 scanner at 400-dpi resolution. Adobe Photoshop 7.0 software was then used to select and count pixels of each liver section. Pixel counts were then converted to numbers of hepatocytes based on the diameter of a mouse hepatocyte ($\sim 25 \mu\text{m}$). Correction factors were then applied to frequencies of FAH+ nodules in a section containing x hepatocytes based on the following assumptions, as described previously (Wang *et al.*, 2002): 1) hepatocyte nodules are spherical; 2) all nodules in a given sample are approximately the same size; 3) the number of hepatocytes in the largest clone represents the middle of that nodule. Immunohistochemical fixation and staining protocols for FAH and hematoxylin and eosin (H&E) were completed as described (Wang *et al.*, 2002). Two separate, blinded investigators performed quantitation. Microscopy was performed on a DM IL LED microscope (Leica, Buffalo Grove, IL) using Leica LAS Image Analysis Software. Blood for serology was collected by terminal cardiac puncture and biochemical measurements were completed as described (Grompe *et al.*, 1995).

Statistical analysis

Statistical analyses were conducted with GraphPad Prism software v.4.0 (GraphPad, San Diego, CA). Experimental differences were evaluated by student two-tailed *t*-test assuming equal variance. *P* values < 0.05 were considered statistically significant.

eGFP copy number quantitative PCR

Total DNA was isolated from randomly dissected liver tissue with a MasterPure DNA Purification kit (Epicentre Biotechnologies, Madison, WI). Genomic DNA (75 ng) was

subjected to a two-step PCR amplification under the following conditions: one cycle at 95°C for 3 min; 45 cycles at 95°C for 15 sec and 68°C for 40 sec; and primers, *eGFP* F: 5'-ACTTCAAGATCCGCCACAAC-3'; *eGFP* R: 5'-GAACTCAGCAGGACCATGT-3'; *Actb* F: 5'-CCACCCCAGCAAGGACACTG-3'; *Actb* R: 5'-GCTCCCTAGGCCCTCCTGT-3'. Dilutions of *eGFP* plasmid into mouse genomic DNA were used to generate copy number standards. Results were normalized to β -actin expression. PCR was performed on an iQ5 Multicolor Real-Time PCR (Bio-Rad, Hercules, CA), using iQ5 Standard Edition Software, v.2.0.

Results

Transient inhibition of either NHEJ or the proteasome increases gene targeting

To examine the effects of pharmacological inhibition of NHEJ or the proteasome on gene targeting, 13-week-old *Fah*^{-/-} adult mice were subjected to one of four treatments: AAV alone, AAV and vanillin, AAV and bortezomib, or AAV and both vanillin and bortezomib. rAAV2/8-*Fah* was given at a dose of 2×10^{11} vg/mouse by IV tail vein injection. Based on pharmacokinetic dosage data on vanillin in a model of neuropathic pain (Beaudry *et al.*, 2010), we administered vanillin at 100 mg/kg/d by IP injection for 7 days starting on the day of vector administration. Using published dosages of bortezomib from promising studies in hemophilic mice (Monahan *et al.*, 2010), we co-injected bortezomib with rAAV at 0.5 mg/kg by IV tail vein injection. Mice were maintained on NTBC for 1 week following vector administration, and then it was withdrawn for 3 weeks. Livers were harvested from mice and scored for FAH+ nodules (Fig. 1).

FIG. 1. Transient non-homologous end joining (NHEJ) or proteasome inhibition enhances gene targeting. Frequencies of corrected nodules were quantified by counting the number of FAH+ clones per x hepatocytes ($1/x$) from adult mice harvested 4 weeks posttreatment. Mean and SD are shown, with the number of independent animals analyzed above each bar. Black, adeno-associated virus (AAV); white, AAV + vanillin; gray, AAV + bortezomib; lines, AAV + vanillin + bortezomib.

FIG. 2. Liver immunohistochemistry. (a) Fumarylacetoacetate hydrolase positive (FAH+) staining from adult *Fah*^{+/+} control mouse; (b) FAH- staining from adult *Fah*^{-/-} control mouse; (c) FAH stain on adult *Fah*^{-/-} mouse treated with AAV, (d) AAV + vanillin, (e) AAV + bortezomib, or (f) AAV + vanillin + bortezomib; (g) hematoxylin and eosin (H&E) stain on adult *Fah*^{-/-} mouse treated with vanillin; (h) H&E stain on adult *Fah*^{-/-} mouse treated with bortezomib; (i) FAH stain on adult *Fah*^{-/-} serial transplant recipients; (j) FAH stain on *Fah*^{-/-}*Ku70*^{+/+} neonate treated with AAV; (k) FAH stain on *Fah*^{-/-}*Ku70*^{+/-} neonate treated with AAV; (l) FAH stain on *Fah*^{-/-}*Ku70*^{-/-} neonate treated with AAV. Scale bar = 10 μ m.

Nodules represent the clonal expansion of a single corrected hepatocyte, thus nodule frequency was corrected for nodule size (Wang *et al.*, 2002).

Figure 2 shows representative FAH immunohistochemistry from control (Fig. 2a, b) and rAAV treated mice (Fig. 2c–f). All cotreatment regimens had significantly higher gene-targeting frequencies than AAV alone. H&E staining on control mice treated only with vanillin (Fig. 2g) or only with bortezomib (Fig. 2h) showed a lack of overall toxicity.

Given that bortezomib was no better at enhancing gene targeting than vanillin, we decided to focus exclusively on vanillin cotreatment for the duration of the studies. Of note, attempts to replicate the adult cotreatment studies in neonates were not possible because bortezomib treatment was lethal during this developmental window (data not shown).

Gene targeting frequencies are sexually dimorphic

To examine effects of sex and age on gene targeting, P1 *Fah*^{-/-} neonates and 13-week-old *Fah*^{-/-} adult mice of different sexes were treated with either rAAV or rAAV and vanillin. rAAV2/8-*Fah* was given at 2×10^{11} vg/mouse by IV tail vein injection to adults, and 1×10^{11} vg/mouse by IV facial vein to neonates. Vanillin was administered at 100 mg/

kg/d by IP injection for 7 days starting on the day of vector administration. Neonates were maintained on NTBC until harvest at weaning to prevent metabolic selection of FAH+ cells. Adults were maintained on NTBC for 1 week following vector administration and then it was withdrawn for 3 weeks. Livers were harvested from mice and scored for FAH+ nodules (Fig. 3). In both ages of mice, males consistently showed significantly higher gene targeting than female littermates when treated with AAV alone. In neonates, a significant difference was seen in both genders with vanillin cotreatment, compared to AAV alone. Even with cotreatment, male neonates had significantly more gene targeting than female littermates. This trend continued in adult mice (Fig. 3), wherein males always showed significantly greater gene targeting than females, no matter the treatment cohort. Interestingly, adult females showed no significant difference between AAV treatment and AAV and vanillin combination therapy.

Toxicity profile of vanillin *in vivo*

To investigate whether the significant increases in gene targeting with vanillin treatment was the result of NHEJ inhibition or simply a toxic response to vanillin causing

FIG. 3. Sexually dimorphic responses to AAV and vanillin. Frequencies of corrected nodules were quantified by counting the number of FAH+ clones per x hepatocytes ($1/x$) from neonates harvested at 3 weeks and adults harvested 4 weeks posttreatment. Mean and SD are shown, with the number of independent animals analyzed above each bar. Black, AAV; white, AAV + vanillin; M, male; F, female.

hepatocellular turnover, we performed *in vivo* toxicity studies on mice treated only with vanillin. *Fah*^{-/-} adult mice were treated with vanillin alone at 100 mg/kg/d by IP injection for 7 days. Mice were maintained on NTBC during the experiment to mimic the experimental conditions used during vector combination therapy studies. Livers were immediately harvested after the final day of vanillin treatment and examined for signs of injury by immunohistochemistry and serology. Results by H&E showed only a slight loss of hepatic glycogen retention but no signs of injury (Fig. 2g). Serology to examine potential hepatic toxicity showed normal bilirubin and transaminase levels (Table 1, column d), further demonstrating the safety and nontoxic attributes of vanillin.

Genetic mutations in *Ku70* mimic chemical affects of vanillin *in vivo*

Having established that vanillin treatment is not toxic, we sought to confirm the effects of blocking NHEJ with a genetic

approach. *Ku70*^{-/-} mice, which genetically lack the ability to perform NHEJ through loss of functional *Ku70*, were bred with *Fah*^{-/-} mice to create double mutants. P1 *Fah*^{-/-} *Ku70*^{-/-} neonates were treated with 1×10^{11} vg/mouse of rAAV2/8-*Fah* by IV facial vein and compared to identically treated *Fah*^{-/-} *Ku70*^{+/+} and *Fah*^{-/-} *Ku70*^{+/-} littermates (Fig. 4). If NHEJ loss was truly responsible for the increase in gene targeting seen with vanillin cotreatment, then AAV treatment alone in mice genetically incapable of NHEJ should show similar gene correction frequencies. As hypothesized, *Fah*^{-/-} *Ku70*^{+/+} and *Fah*^{-/-} *Ku70*^{+/-} neonates treated with AAV had normal levels ($\sim 0.1\%$) of gene correction (Fig. 2j,k). Only the *Fah*^{-/-} *Ku70*^{-/-} littermates (Fig. 2l) had significantly ($p < 0.03$) higher gene correction frequencies at the levels seen in *Fah*^{-/-} mice treated with AAV and vanillin ($\sim 1\%$).

Random integration frequencies remain unaffected by transient NHEJ inhibition

Although phenotypic reversion indicated successful site-specific gene targeting, random integration of AAV genomes could also occur. Current data in the field are conflicting as to whether inhibition of NHEJ would worsen (Song *et al.*, 2004) or improve (Fattah *et al.*, 2008) random integration frequencies. Since the majority of postembryonic DNA damage is repaired by NHEJ, it is plausible that a transient block to NHEJ would decrease the frequency of random integration. To assess whether random integration frequencies change with vanillin treatment, P1 *Fah*^{-/-} neonates were co-injected with 4×10^{10} vg/mouse of both rAAV2/8-*Fah* and rAAV2/8-LSP-eGFP (nonselectable serotype-matched control vector). Concomitantly, neonates were treated by IP injection with vanillin at 100 mg/kg for 7 days following viral administration. Postweaning, mice were subjected to NTBC withdrawal to select for corrected hepatocytes. To ensure no episomes remained and to select for integrants, 5×10^5 random hepatocytes were then serially transplanted into six secondary *Fah*^{-/-} recipients as described (Overturf *et al.*, 1997). After >12 weeks off NTBC, serum and liver tissue of the secondary transplant recipients were collected.

Liver function tests for bilirubin and transaminases demonstrated complete correction in all treatment cohorts when compared to untreated controls off NTBC (Table 1). Liver FAH immunohistochemistry illustrated the extensive

TABLE 1. SEROLOGICAL MEASURES OF FUNCTIONAL CORRECTION

Mice treatment	^a <i>Fah</i> ^{-/-} off NTBC	^b <i>Fah</i> ^{-/-} on NTBC	^c Wild-type	^d <i>Fah</i> ^{-/-} Van	^e <i>Fah</i> ^{-/-} AAV	^f <i>Fah</i> ^{-/-} AAV + Van	^g sTx recipients AAV + Van
Bili (mg/dl)	3.4 ± 1.3	0.2 ± 0	0.0 ± < 0.1	< 0.1 ± 0	< 0.1 ± 0	< 0.1 ± 0	0.2 ± 0.1
AST (U/L)	353 ± 86	121 ± 47	91 ± 27	98 ± 26	86 ± 8	91 ± 32	116 ± 13
ALT (U/L)	291 ± 51	59 ± 56	36 ± 5	38 ± 4	45 ± 1	53 ± 12	92 ± 10
n	6	5	5	3	3	7	6

AAV, adeno-associated virus; AST, aspartate aminotransferase; ALT, alanine aminotransferase; Bili, bilirubin; *Fah*, fumarylacetoacetate hydrolase; n, number of animals; NTBC, 2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione; sTx, serial transplant; U/L, units per liter; Van, vanillin. From left to right, values represent mean ± SD from untreated *Fah*^{-/-} mice off NTBC, untreated *Fah*^{-/-} mice on NTBC, untreated wild-type mice, *Fah*^{-/-} mice treated with vanillin alone for 7 days on NTBC, *Fah*^{-/-} mice treated with AAV alone and selected for 3 months off NTBC, *Fah*^{-/-} mice treated with AAV and vanillin and selected for 3 months off NTBC, and serially transplanted *Fah*^{-/-} mice selected for 3 months off NTBC, respectively. All values in columns (b–g) are significantly different from corresponding values in column (a) ($p < 0.05$).

FIG. 4. NHEJ inhibition is responsible for increases in gene targeting seen with vanillin. Frequencies of corrected nodules were quantified by counting the number of FAH+ clones per $\times$ hepatocytes ($1/\times$) from neonates harvested at 3 weeks posttreatment. Mean and SD are shown, with the number of independent animals analyzed above each bar. Black, *Fah*^{-/-} *Ku70*^{+/+}; white, *Fah*^{-/-} *Ku70*^{+/-}; gray, *Fah*^{-/-} *Ku70*^{-/-}.

repopulation in serial transplant recipients (Fig. 2i). Quantitative PCR was used to determine *eGFP* copy numbers in each of these highly repopulated mice (Table 2). The average copy number of irrelevant vector corrected for repopulation efficiency indicated that ~0.8% of gene-targeted hepatocytes also had a random rAAV2/8-LSP-*eGFP* integration event. This number was well within the range of reported random AAV integration estimates from the literature between 0.1% and 10% (Porteus *et al.*, 2003; Miller *et al.*, 2005; Nakai *et al.*, 2005; Vasileva *et al.*, 2006). As a result, it can be concluded that vanillin treatment did not significantly increase or decrease AAV random integration frequencies.

TABLE 2. *eGFP* COPY NUMBERS IN RECIPIENT MICE

Donor	sTx recipient	# copies/dGE	Average
1	1a	0.01	0.01
	1b	0.01	
	1c	0.01	
2	2a	0.00	0.006
	2b	0.01	
	2c	0.01	
Total			0.008
Random integration			0.008 = 0.8%

dGE, diploid genome equivalent; sTx, serial transplant. After neonatal co-injection of 1×10^{10} vg of rAAV2/8-*Fah* and rAAV2/8-LSP-*eGFP* and 7 days of vanillin treatment, FAH+ hepatocytes were selected for and then serially transplanted to remove episomes. Data represent the number of copies of *eGFP* per diploid genome equivalent in serial transplant recipients. Roughly 50% of the hepatocytes in repopulated recipients were donor derived at the time of harvest, so the frequency was corrected by a factor of 2 to give an estimated random integration frequency of 0.8%.

Discussion

In spite of advances made in gene therapy research, the disadvantages of traditional AAV gene addition methodologies remain: episomal expression will always be inherently unregulated, expression will be transient in cells that turn over, the risk of eventual transgene silencing will always be present, integration events will be random because they are not based on homology, random integration will produce increased mutagenic and oncogenic risks, and disorders caused by aberrantly functioning or misfolded proteins are untreatable by this approach. Our interest in AAV-mediated gene targeting stems from its ability to elegantly bypass these disadvantages through simple site-directed gene repair.

The precise nature of the interactions between NHEJ machinery and AAV are not clear and thus it was unknown whether inhibition of NHEJ would exacerbate (Song *et al.*, 2004) or improve (Fattah *et al.*, 2008) both random and targeted integration frequencies. We pursued both pharmacological and genetic approaches in various ages and genders of mice with a clinically relevant disease model to resolve the differences in the current literature. Our results show that transient NHEJ inhibition with vanillin both increased targeted AAV integration and surprisingly, had no significant effect on random AAV integration.

Our results with vanillin combination therapy are all the more promising when other attributes of vanillin are taken into consideration: vanillin is a known anticarcinogen (Akagi *et al.*, 1995), antioxidant (Burri *et al.*, 1989), anticlastogen (Sasaki *et al.*, 1990), antimutagen (Keshava *et al.*, 1998), antimicrobial (Davidson and Naidu, 2000), and analgesic (Beaudry *et al.*, 2010), and it has antineoplastic activity (McCann *et al.*, 2007). Moreover, vanillin is already a U.S. Food and Drug Administration (FDA)-approved food additive, thus an off-label use for vanillin in gene therapy seems feasible. Attempts to further improve gene targeting with vanillin analogs were unfruitful. DMNB, a known vanillin analog and even more potent disruptor of DNA-PK (Durant and Karran, 2003), was unsuccessful at improving gene targeting at doses tested in a 2-day time course (Supplementary Fig. S1; Supplementary Data are available online at www.libertpub.com/hum) and was not well tolerated in mice. Both 5- and 7-day courses of DMNB proved lethal.

In addition to transient NHEJ inhibition, transient proteasomal inhibition is a promising avenue in gene therapy research given the results seen in mouse and dog models of hemophilia treated with AAV and bortezomib (Monahan *et al.*, 2010). Our data with bortezomib highlights its potential as a second parallel approach to vanillin treatment, wherein bortezomib combination therapy improved gene-targeting frequencies in a safe and efficacious manner. Having already gained FDA approval for human multiple myeloma and mantle cell lymphoma studies, bortezomib combination therapies with AAV have potential for rapid approval and the benefit of pre-existing toxicity studies in humans (Richardson *et al.*, 2005).

As in other fields, gene therapists are beginning to appreciate sex differences in response to AAV therapies (Davidoff *et al.*, 2003; Ogura *et al.*, 2006; Ho *et al.*, 2008; Rebuffat *et al.*, 2010). For example, in hepatocytes there are more than 1000 genes that are differentially expressed between males

and females (Rinn and Snyder, 2005; Clodfelter *et al.*, 2006). However, no data exist to date for AAV gene-targeting vectors in animal models of any disease in which sex was controlled. Here we present the first data demonstrating that at all life stages, male mice displayed significantly more AAV-mediated gene targeting than their female counterparts. The detailed mechanisms responsible for sex differences in AAV liver transduction are still unclear but have at least been shown to involve androgen-dependent pathways (Davidoff *et al.*, 2003). The implications of our findings could affect the design and dosing strategies of future clinical trials.

Obstacles to AAV-mediated gene targeting remain. Notably, therapeutic levels of gene correction have proven elusive in disease models. However, our current work highlights the capacity of AAV-mediated gene targeting in a suitable selection-based disease. While disorders characterized by selection for gene-corrected cells overall are rare, there are other examples of this phenomenon: Fanconi's anemia (Battaile *et al.*, 1999), the copper storage disorder Wilson's disease (Allen *et al.*, 2004), bile-acid transporter defects (De Vree *et al.*, 2000), and junctional epidermolysis bullosa (Ortiz-Urda *et al.*, 2003) to name a few. If correction frequencies achieved here were increased just fivefold, they would become clinically relevant even for diseases that do not display a selective advantage. For example, it has been predicted that gene-targeting frequencies of 5% would have therapeutic benefit for patients with hemophilia A (Kay and High, 1999), and 10% for phenylketonuria patients (Fang *et al.*, 1994). Patients with diseases arising from point mutations are the most appropriate recipients for gene-targeting therapies. Historically, point mutations have been the most frequent genetic abnormality and source of acquired genetic disease (Bergeron, 2004).

This study establishes the utility of transient NHEJ inhibition with vanillin, or proteasome inhibition with bortezomib, for increasing hepatic gene targeting with AAV in all ages and sexes of mice. The drug doses used could be applied in humans; hence, these approaches may have human clinical relevance.

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Author Disclosure Statement

No competing financial interests exist for any of the authors of this study.

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