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The Impact of Pre-existing Immunity on the Non-clinical Pharmacodynamics of AAV5-Based Gene Therapy

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Adeno-associated virus (AAV)-based vectors are widely used for gene therapy, but the effect of pre-existing antibodies resulting from exposure to wild-type AAV is unclear. In addition, other poorly defined plasma factors could inhibit AAV vector transduction where antibodies are not detected. To better define the relationship between various forms of pre-existing AAV immunity and gene transfer, we studied valoctocogene roxaparvovec (BMN 270) in cynomolgus monkeys with varying pre-dose levels of neutralizing anti-AAV antibodies and non-antibody transduction inhibitors. BMN 270 is an AAV5-based vector for treating hemophilia A that encodes human B domain-deleted factor VIII (FVIII-SQ). After infusion of BMN 270 (6.0×10^{13} vg/kg) into animals with pre-existing anti-AAV5 antibodies, there was a mean decrease in maximal FVIII-SQ plasma concentration (C_{max}) and AUC of 74.8% and 66.9%, respectively, compared with non-immune control animals, and vector genomes in the liver were reduced. In contrast, animals with only non-antibody transduction inhibitors showed FVIII-SQ plasma concentrations and liver vector copies comparable with those of controls. These results demonstrate that animals without AAV5 antibodies are likely responders to AAV5 gene therapy, regardless of other inhibiting plasma factors. The biological threshold for tolerable AAV5 antibody levels varied between individual animals and should be evaluated further in clinical studies.

INTRODUCTION

Adeno-associated viruses (AAVs) are members of the parvovirus family, which are non-enveloped viruses that have a single-stranded DNA genome and can be readily modified into a vector delivery system for gene therapy.[1] At a minimum, there are 11 serotypes described for AAVs that can infect cells from multiple tissue types; however, human hepatocytes remain the preferred target for the production of secreted, systemically acting therapeutic proteins following gene transfer.[2,3] AAVs are thought to be relatively common in the environment, and seroprevalence studies show that up to 90% of human populations have been exposed to AAVs, resulting in capsid-directed humoral immunity.[4–7]

One potential consequence of prior exposure to AAVs is the development of neutralizing antibodies (NAbs), which may limit the transduction efficiency of AAV-based gene therapies.[8,9] Several species, including dogs, monkeys, and humans, have varying levels of circulating AAV antibodies.[10–12] Antibodies specific for AAVs may neutralize transduction of AAV gene therapy vectors or may simply be binding antibodies with no neutralizing activity but are readily detectable by ELISA-based methods regardless. For some common serotypes, the prevalence of antibodies in humans may reach 60% but is reported to be lower for AAV5, with a range of 3.2% to 40% and varying by geographic location.[4,5,13–15]

In addition to AAV antibodies, non-antibody neutralizing factors to AAVs can be detected in human plasma using *in vitro* cell-based assays that measure any form of interference with the transduction process.[4,16] The nature of these inhibitors is less defined and could range from small molecules (from concomitant medications, for example) to inflammatory peptides secreted by innate immune cells.[17–19] Accordingly, the range of potential mechanisms of action by NAbs or other plasma factors is broad and could include inhibition of AAV vector uptake, modulation of endosomal and nuclear trafficking, influence on capsid processing, and suppression of genome release.[20]

Hemophilia A is a congenital X-linked bleeding disorder resulting from a mutation of the gene encoding coagulation factor VIII (FVIII).[21] Hemophilia A patients are at high risk for prolonged and excessive bleeding that may be life-threatening; therefore, they are often treated with prophylactic administration of exogenous FVIII. Valoctocogene roxaparvovec (BMN 270) is an investigational AAV5-based gene therapy vector for the treatment of hemophilia A. The vector encodes B domain-deleted human FVIII (hFVIII-SQ) with a codon-optimized DNA sequence under the control of a liver-specific promoter for continuous hepatocyte expression.[22,23] An ongoing phase 1/2 dose escalation study is currently assessing the safety, efficacy, and

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Table 1. Experimental Study Design

Group	No. of Subjects	Purpose	AAV5 TAb/TI Status	TI Titer Range	BMN 270 Dose Level (vg/kg)
1	3	non-immune control	TAb−/TI−	negative	6×10^{13}
2	4	non-antibody transduction inhibitor	TAb−/TI+	2 to <5	6×10^{13}
3	3	non-antibody transduction inhibitor	TAb−/TI+	5 to 10	6×10^{13}
4	5	neutralizing AAV5 antibodies	TAb+/TI+	>5	6×10^{13}

TAb, total antibodies; TI, transduction inhibition; vg, vector genome; kg, kilogram.

immunogenicity of BMN 270 in patients with severe hemophilia A (J. Pasi et al., 2017, ISTH, abstract). Interim study results demonstrated that BMN 270 achieved the first successful gene transfer in hemophilia A patients, which was associated with a substantial decrease in the median annualized bleeding rate for subjects previously on prophylactic replacement therapy from 17 (range, 0–40) before gene transfer to 0 (range, 0–7), as evaluated starting 2 weeks post-infusion.

Because pre-existing AAV immunity may limit the transduction efficiency of AAV-based gene therapies,[3,9] subjects in the BMN 270-201 trial were screened and excluded on the basis of either pre-existing AAV5 antibodies and/or non-antibody inhibitors.[24] Total antibodies (TAbs) to AAV5 were detected in plasma using a bridging electrochemiluminescent immunoassay, and AAV5 transduction inhibition (TI), irrespective of whether it was mediated by AAV5 antibodies or non-antibody inhibitors, was determined in a cell-based TI assay utilizing an AAV5-luciferase reporter vector and HEK293T/17 cells.[16] The caveat of detecting TI by using a cell-based assay is that the obtained results may be cell origin-[25] or reporter gene-specific; thus, the neutralizing potency of plasma *in vitro* may not always translate to neutralizing potency *in vivo*. In addition, non-antibody transduction inhibitors present in human plasma generally had lower TI titers than neutralizing AAV antibodies,[16] suggesting that the magnitude of effect between these two classes of neutralizing factors may differ *in vivo*.

To inform the most efficient screening strategy for future large, multicenter clinical studies, we studied the various types of pre-existing immunity and their effect on the efficiency of gene transfer in non-human primates. In particular, it is unclear which level of neutralizing AAV5 antibodies, if any, would completely block transduction *in vivo* rather than diminish FVIII-SQ to lower but meaningful plasma levels. In addition, the physiological relevance of non-antibody transduction inhibitors detected in a cell-based TI assay remains incompletely understood. Therefore, the objective of this non-clinical study was to determine the pharmacodynamics of gene delivery and hFVIII-SQ expression following a single infusion of BMN 270 into cynomolgus monkeys with varying pre-existing levels of neutralizing AAV5 antibodies and non-antibody transduction inhibitors.

RESULTS

Detection of Pre-existing AAV5 Immunity in Monkeys (Screening Phase)

To study the effect of pre-existing AAV5 immunity on BMN 270-mediated gene transfer, this study aimed to specifically enroll cynomolgus monkeys that met pre-defined criteria for seropositivity. To this end, 60 individual monkeys were screened using both AAV5 TAb and AAV5 TI assays prior to the study. For the AAV5 TAb assay, it was confirmed by surface plasmon resonance that both cynomolgus monkey immunoglobulin G (IgG) and IgM can bind strongly to at least one component of the combined detection reagent (protein A/G/L), suggesting that AAV5-specific antibodies of both isotypes are detectable in the TAb assay (Figure S1).

Fifteen monkeys (Figure S2A) were subsequently enrolled in one of four groups (Table 1). Group 1 contained non-immune control animals without AAV5 antibodies and without detectable TI (TAb−/TI−). Groups 2 and 3 contained animals without AAV5 antibodies but with detectable AAV5 TI (TAb−/TI+), likely mediated by plasma factors referred to here as "non-antibody transduction inhibitors." Lower TI titers (2–4) were enrolled in group 2, and moderate TI titers (5–10) were enrolled in group 3. Group 4 contained animals with neutralizing AAV5 antibodies (TAb+/TI+) with various TI titer levels (>5).

The screening results from all 60 monkeys also confirmed that the TI titer range of non-antibody inhibitors was generally lower than that of AAV5 antibodies (Figure S2B), which was similar to observations made in human plasma.[16] All detected non-antibody transduction inhibitors in monkey plasma had a TI titer of less than 10, whereas TI titers of more than 10 were always associated with the presence of AAV5 antibodies. Thus, different types of pre-existing AAV5 immunity have different *in vitro* neutralizing potency, suggesting that the effect on BMN 270 transduction *in vivo* could be different as well.

hFVIII-SQ Protein Concentration in Monkey Plasma

To compare the effect of the different types of pre-existing AAV5 immunity *in vivo*, the same BMN 270 dose (6.0×10^{13} vector genomes (vg)/kg) was administered to animals in all four groups via intravenous injection on a single occasion on day 1. After dosing, blood samples were collected weekly for determination of hFVIII-SQ protein levels in plasma. The highest FVIII-SQ concentration in plasma (C_{max}) was observed between days 22 and 36 in each group (Figure 1; Table 2). In the non-immune control animals (group 1), C_{max} FVII-SQ protein concentration ranged from 24.3 to 38.2 ng/mL. In animals with non-antibody transduction inhibitors, plasma FVIII-SQ C_{max} concentrations were similar to or higher than those in the control animals, with a range of 16.3–113 ng/mL (group 2) and 22.4–43.2 ng/mL (group 3). In sharp contrast, 2 of 5

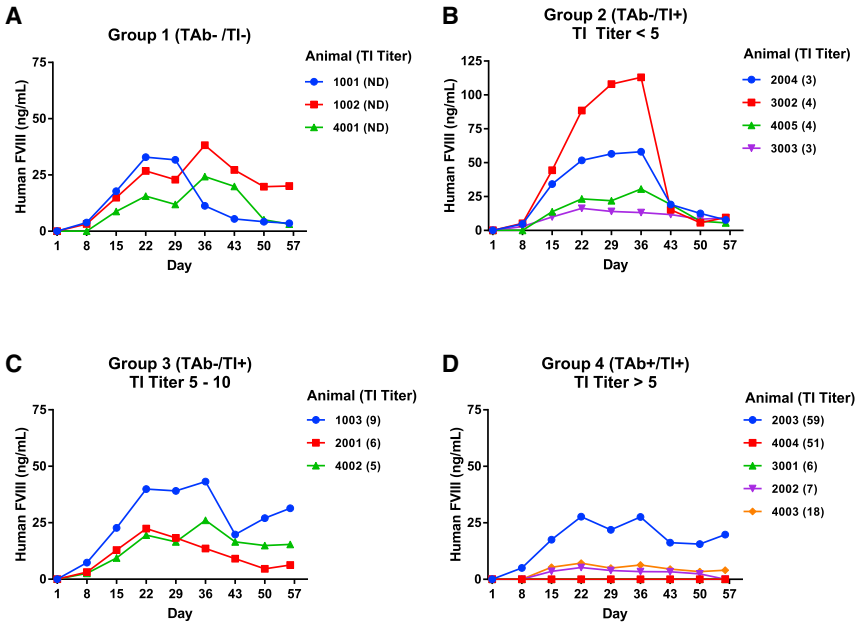

Figure 1. Measurement of FVIII-SQ Protein Concentration in Monkey Plasma

(A–D) Citrated plasma samples from individual animals were collected at the indicated time points and used to quantitate human FVIII-SQ protein plasma concentration (nanograms per milliliter) using a sandwich enhanced chemiluminescence (ECL) assay. Individual graphs are shown for (A) three group 1 non-immune control animals (TAb−/TI−), (B) four group 2 animals with non-antibody transduction inhibitors (TAb−/TI+) that showed low TI titers (<5; note the change in y axis scale), (C) three group 3 animals with non-antibody transduction inhibitors (TAb−/TI+) that showed moderate TI titers (5–10), and (D) five group 4 animals with neutralizing AAV5 antibodies (TAb+/TI+; TI titers > 5).

animals with neutralizing AAV5 antibodies (group 4) did not produce any FVIII-SQ above the lower limit of quantitation (LLOQ), and an additional 2 of 5 animals showed notably reduced FVIII-SQ plasma levels. Only 1 of 5 animals in group 4 (animal 2003) produced FVIII-SQ at levels comparable with those in group 1. Group 4 C_{max} plasma FVIII-SQ concentration ranged from 0 to 27.7 ng/mL. Baseline (day −7) results were below the LLOQ in all animals from all groups, confirming that the assay had no cross-reactivity for monkey FVIII.

To more quantitatively compare plasma FVIII-SQ concentrations between groups, we evaluated the mean C_{max} FVIII-SQ plasma concentration (Figure 2A) for each group. The mean (SD) C_{max} FVIII-SQ protein concentration for group 1 non-immune control animals was 31.8 (7.0). For group 2 and group 3 animals with only non-antibody transduction inhibitors, there was no decrease in mean (SD) C_{max} FVIII-SQ protein concentration, with measures of 54.5 (42.7) and 30.6 (11.1), respectively; however, the mean (SD) C_{max} FVIII-SQ concentration in group 4 animals with neutralizing AAV5 antibodies was 8.0 (11.5), demonstrating a 74.8% decrease in mean C_{max} FVIII-SQ plasma concentration versus group 1 control animals.

To better compare overall FVIII-SQ plasma expression over time between study groups, the mean area under the concentration-time curve (AUC) from baseline through day 29 was also determined for each group (Figure 2B). Days 36 to 56 were excluded from AUC analysis because some animals showed signs of interference by anti-FVIII antibodies, which were confirmed to be absent in all animals through day 29 (described below). The mean (SD) FVIII AUC (ng·day/mL) from baseline to day 29 was similar or higher in group 2 (690 [503.3] ng·day/mL) and group 3 (412.3 [187.1] ng·day/mL) compared with group 1 (366.3 [142.5] ng·day/mL) but substantially lower in group 4 (121.3 [177.5] ng·day/mL), representing a 66.9% decrease versus group 1. Together, these results suggest an association between pre-existing neutralizing AAV5 antibodies and reduced levels of FVIII-SQ protein detected in plasma. No evidence for such an association was found for pre-existing non-antibody inhibitors of AAV5 transduction.

Relationship between FVIII-SQ Plasma Concentration and Titers of Neutralizing AAV5 Antibodies

FVIII-SQ protein levels in plasma were reduced but not completely abrogated in all animals with pre-existing AAV5 antibodies. Therefore, the relationship between achieved FVIII-SQ expression and neutralizing TI titers of pre-existing AAV5 antibodies was investigated in more detail for group 4 animals. TI titers determined during the pre-treatment screening phase were plotted against the individual FVIII-SQ AUC for each group 4 animal and normalized as a percentage of the group 1 control mean AUC (Figure 3). For practical reasons, immune screening was performed ~6 weeks ahead of dosing; however, the TI titers were also re-measured in animals selected for the study, using baseline samples collected 7 days prior to dosing. Thus, baseline sample results may more accurately reflect the magnitude of pre-existing immunity encountered during BMN 270 infusion (Figure 3B). No detectable FVIII-SQ in plasma was observed for animals 3001 and 4004 with pre-dose TI titers of 6;12 (screening;baseline) and 51;146, respectively, confirming that AAV5 antibodies within a wide TI titer range can potentially fully neutralize BMN 270 *in vivo*. In contrast, low concentrations of FVIII-SQ (5.19 and 7.71 ng/mL) were observed for animals 2002 and 4003 with pre-dose TI titers of 7;12 and 18;37, respectively, and a "normal" FVIII-SQ concentration (27.7 ng/mL) was observed for animal 2003 with pre-dose TI titers of 59;17. Hence, different plasma concentrations of FVIII-SQ were achieved in the presence of similar baseline TI titers (e.g., 3001 versus 2002 versus 2003), indicating that individual TI titer thresholds in animals with pre-existing neutralizing AAV5 antibodies may be variable.

Table 2. Summary of Pre-existing AAV5 Immunity, FVIII-SQ Plasma Levels, and Liver Vector Genomes

	Pre-existing AAV5 Immunity			FVIII-SQ Plasma Levels		Liver Vector Genomes	
Animal	Group	AAV5 Antibodies ECLU S/N (Result +/−)[a]	AAV5 TI Titer	FVIII-SQ C_{max} (ng/mL [Day])	FVIII-SQ AUC Days 1–29 (ng·Day/mL)	vg/μg	vg/Cell
1001	1 (TAb−/TI−)	0.72 (−)	-	32.9 (22)	493	2.35E+06	12.90
1002	1 (TAb−/TI−)	0.70 (−)	-	38.2 (36)	394	1.70E+06	9.33
4001	1 (TAb−/TI−)	0.51 (−)	-	24.3 (36)	212	1.76E+05	0.97
2004	2 (TAb−/TI+)	0.63 (−)	3	58.1 (36)	832	2.86E+06	15.74
3002	2 (TAb−/TI+)	0.38 (−)	4	113 (36)	1340	2.72E+06	14.96
3003	2 (TAb−/TI+)	0.52 (−)	3	16.3 (22)	252	3.68E+05	2.02
4005	2 (TAb−/TI+)	0.73 (−)	4	30.6 (36)	336	3.42E+05	1.88
1003	3 (TAb−/TI+)	0.63 (−)	9	43.2 (36)	626	1.52E+06	8.37
2001	3 (TAb−/TI+)	1.16 (−)	6	22.4 (22)	333	1.53E+06	8.41
4002	3 (TAb−/TI+)	0.58 (−)	5	26.1 (36)	278	1.10E+05	0.60
2002	4 (TAb+/TI+)	8.60 (+)	7	5.19 (22)	74.7	3.61E+05	1.99
2003	4 (TAb+/TI+)	188.22 (+)	59	27.7 (22)	428	9.41E+05	5.18
3001	4 (TAb+/TI+)	5.52 (+)	6	BLQ	0	1.62E+03	0.01
4003	4 (TAb+/TI+)	30.70 (+)	18	7.71 (22)	104	3.21E+04	0.18
4004	4 (TAb+/TI+)	137.94 (+)	51	BLQ	0	2.29E+04	0.13

BLQ, below the lower limit of quantitation. Animals with BLQ FVIII results at all visits were imputed as zero for AUC.
[a]The screening cutpoint for positivity of the S/N ratio is 1.70 for the AAV5 TAb assay. S/N values were included for information only and not used to assess the magnitude of the antibody response.

Because of the limited dataset (n = 5) and large biological variability, it was not possible to perform a regression analysis to obtain a more generalized biological threshold for tolerable TI titers. Nonetheless, the highest level of pre-existing neutralizing AAV5 antibodies for which at least some plasma FVIII-SQ was detectable had a baseline TI titer of 37 (4003).

hFVIII-SQ Transgene DNA in Monkey Liver

Liver samples for transgene DNA analysis were collected at necropsy (day 56) from the quadrate, left lateral, right lateral, left medial, and right medial lobes from each animal. Total liver DNA was extracted, and the copy number of FVIII-SQ vector genomes (vg) was determined using quantitative real-time PCR. Results were back-calculated to vector genomes per microgram total DNA or vector genomes per cell and averaged across lobes for individual animals (Figures 4A and 4B), and group means were calculated from these averages (Figures 4C and 4D).

All BMN 270-treated monkeys had measurable hFVIII-SQ transgene DNA in the liver on day 56, but levels varied by animal and study group. Similar amounts of transgene DNA were observed in liver tissue from non-immune control animals (group 1, 0.97–12.9 vg/cell) and animals with different levels of non-antibody transduction inhibitors (group 2, 1.88–15.74 vg/cell; group 3, 0.6–8.41 vg/cell). Lower mean quantities of vector genomes were, however, detected in animals with pre-existing neutralizing AAV5 antibodies (group 4, 0.01–5.18 vg/cell), suggesting decreased liver transduction of BMN 270 (Figure 4). Livers from two individual animals in group 4 (2002 and 2003) contained FVIII-SQ transgene DNA levels comparable with those in group 1 animals, indicating that liver transduction of BMN 270 may proceed normally in some animals, even in the presence of detectable neutralizing anti-AAV5 antibodies.

To further substantiate that FVIII-SQ concentrations in plasma reflected the variegated transduction of liver cells, a regression analysis was performed for individual FVIII-SQ AUC values, and vector genome levels were expressed as both vector genomes per microgram total DNA and vector genomes per cell (Figures 4E and 4F). Although the relationship was not strictly linear (Pearson $R^2 = 0.72$), the data were positively correlated ($p < 0.0001$), confirming that FVIII-SQ plasma levels are indicative of the relative efficiency of liver transduction by BMN 270.

hFVIII Antibodies after BMN 270 Dosing

Plasma samples were not reactive for anti-hFVIII antibodies at baseline or on day 29 (Table 3), demonstrating that FVIII-specific antibodies did not affect the measurement of plasma FVIII-SQ concentrations up to that time point. Therefore, differences in FVIII-SQ protein plasma levels between study groups that occur from days 8 through 29 cannot be explained by differences in the developing FVIII antibody response. On day 56, 3 of 3 animals in group 1, 3 of 4 animals in group 2, 1 of 3 animals in group 3, and 0 of 5 animals in group 4 tested positive for anti-hFVIII antibodies. These results demonstrate the immunogenicity of hFVIII-SQ in monkeys, leading to a presumably species-specific antibody response in a total of 7 of 15 (46.7%) cynomolgus monkeys at the end of the study. Consistent with the decreased transduction and low or undetectable plasma

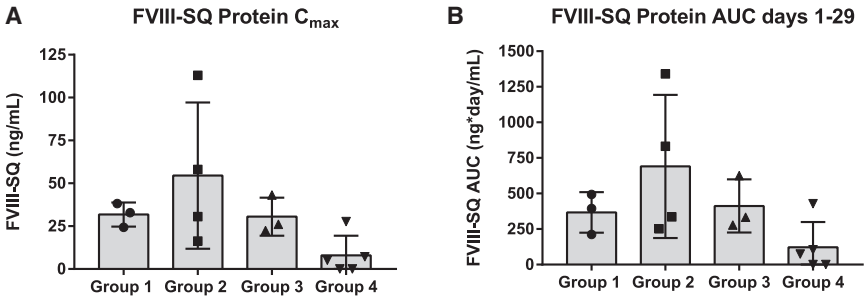

Figure 2. Maximal FVIII-SQ Protein Plasma Concentration and AUC by Study Group

(A) The maximal FVIII-SQ protein plasma concentration (C_{max}, ng*day/mL), which was observed for each animal from day 22 through day 36, is plotted as the mean (SD) by study group. (B) A truncated FVIII-SQ area under the concentration-time curve (AUC, ng*day/mL) from day 1 through day 29 was calculated for each animal and averaged by study group. Group 1, non-immune control; groups 2 and 3, non-antibody transduction inhibitors with low and moderate TI titers, respectively; group 4, neutralizing AAV5 antibodies.

concentration of FVIII-SQ as a stimulating antigen, FVIII-specific antibodies were not detected in any group 4 animals.

AAV5 Antibodies after BMN 270 Dosing

To evaluate the kinetics of the post-dose antibody response to the AAV5 capsid, plasma samples from all animals were tested in the AAV5 TAb assay at baseline and post-treatment on days 8, 15, 22, 29, and 56 (Table 3). With the exception of group 4 animals, which were AAV5 antibody-positive prior to BMN 270 infusion, all other animals were AAV5 antibody-negative at baseline, with normalized enhanced chemiluminescence unit (ECLU) values (signal-to-noise [S/N] ratio) below the assay cutpoint of 1.70 (Table 3). By day 8, all dosed animals were AAV5 antibody-positive, indicating that the onset of the AAV5 antibody response occurred rapidly after BMN 270 infusion and with similar kinetics across all four study groups, regardless of the presence of pre-existing AAV5 immunity. Plasma from all dosed animals remained positive for AAV5 antibodies throughout the study until day 56, without any apparent effect of pre-existing AAV5 immunity.

Additional Animal and Laboratory Observations

Overall, the administration of BMN 270 by a single intravenous bolus injection was well-tolerated in cynomolgus monkeys, regardless of their pre-dose TI titer or AAV5 antibody status. There were no BMN 270-related unscheduled mortalities; changes in clinical observations, food consumption, body weight, coagulation, and clinical chemistry parameters; or gross necropsy or histopathology findings. Non-treatment-related observations in study animals included localized abrasions, bruises, and rectal prolapse. A minor but statistically significant BMN 270-related increase in mean (SD) lymphocyte count occurred in group 4 (11.11 [0.73] $\times$ $10^3/\mu L$) on day 56, which was 1.5 times that of group 1 (7.51 [0.82] $\times$ $10^3/\mu L$). All other hematology parameters were normal. No BMN 270-related microscopic findings were observed in any group. ALT levels remained within the reference range of the colony for all animals over the course of the study (Figure 5), indicating that no adverse events occurred in the liver, even when BMN 270 was administered to animals with pre-existing AAV5 immunity (groups 2–4).

DISCUSSION

Previous non-clinical studies in non-human primates as well as clinical studies of AAV-mediated gene therapies, have shown that pre-existing neutralizing AAV antibodies can interfere with vector transduction *in vivo* and limit therapeutic efficacy.[3,26–30] Pre-existing immunity in these studies has variously been referred to as NAbs, neutralizing titers, neutralizing factors, or inhibitors of transduction, and all reflect different assay methodologies, sensitivities, and cut-points, which limits the utility of comparing results across studies. Nevertheless, neutralizing titers as low as 1:1 have been described as having an inhibitory effect on AAV vector transduction.[30] In some cases, these titers have resulted in decreased therapeutic efficacy compared with patients with no evidence of pre-existing immunity,[3,30] and in other cases, titers as low as 1:5 have completely blocked liver transduction.[27]

Neutralizing activity has been described as both AAV serotype-specific in some instances[26,29] and cross-reactive between serotypes in others.[10] In the former instance, efficient transduction could be achieved *in vivo* by using AAV serotypes that differ from the serotype to which immunity could be detected.[26,29] Further, capsid-specific neutralizing immunity may not always fully block vector transduction and therapeutic efficacy; in an ongoing clinical trial of AAV5-FIX (AMT-060) in hemophilia-B patients, pre-treatment sera were retrospectively re-analyzed in a more sensitive NAb assay than used for enrollment exclusion (A. Majowicz et al., 2018, ASGCT, conference). Three of 10 patients had detectable NAb titers in the more sensitive assay, but there was no relationship with therapeutic efficacy because all three patients had detectable but low post-dose FIX activity levels similar to those in NAb-negative patients.

Further clouding the issue is whether neutralizing immunity to various AAV serotypes equally limits therapeutic efficacy. This question may relate to the species-specific origin of different AAVs because environmental prevalence may determine the extent of natural exposure and the magnitude of pre-existing immunity in humans. In particular, AAV5 has a high degree of sequence identity with caprine (goat)-isolated AAV-Go.1, whereas many other AAVs commonly used as gene therapy vectors, such as AAV2 and AAV8, were cloned from primate species.[31,32] A non-primate origin of AAV5 may partially explain both the reduced seroprevalence of pre-existing AAV5 antibodies in human populations[4] and their variable neutralizing capacity.

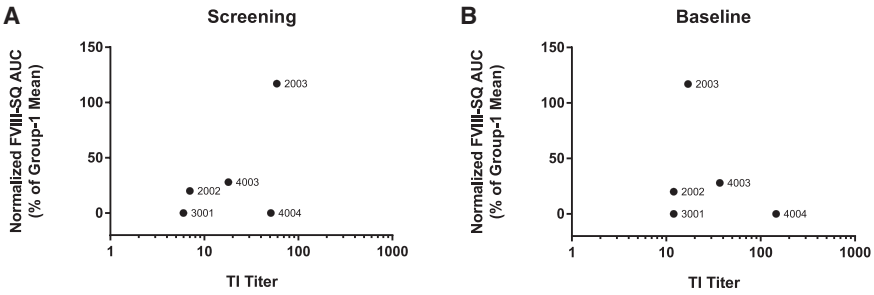

Figure 3. FVIII-SQ Expression Compared with TI Titers of Pre-existing Neutralizing AAV5 Antibodies

(A and B) The FVIII-SQ AUCs of five individual group 4 animals (2002, 2003, 3001, 4003, and 4004) with neutralizing AAV5 antibodies were normalized as a percentage of the mean FVIII-SQ AUC of group 1 non-immune control animals. The normalized FVIII-SQ AUC of individual group 4 animals was plotted versus the TI titers of neutralizing AAV5 antibodies measured at (A) screening (~6 weeks before BMN 270 dosing) or (B) baseline (7 days before BMN 270 dosing).

The current study analyzed the pharmacodynamics of AAV5-mediated gene transfer and FVIII-SQ transgene expression following a single infusion of BMN 270 into cynomolgus monkeys with detectable pre-dose levels of neutralizing AAV5 antibodies or non-antibody transduction inhibitors. Administration of BMN 270 in the presence of these various forms of pre-existing immunity did not result in any drug-related toxicological changes. The only change noted was a minor increase in lymphocytes in the peripheral blood of some animals. The presence of neutralizing anti-AAV5 antibodies was associated with reduced liver transduction and a mean decrease of ~75% in FVIII-SQ C_{max} plasma concentration, whereas no reduction was observed in animals with non-antibody transduction inhibitors. This suggests that pre-existing AAV5 antibodies, as opposed to non-antibody transduction inhibitors, are the key neutralizing factors in plasma that interfere with AAV5 gene therapy *in vivo*. Therefore, animals in this study could have been pre-screened and enrolled based solely on the AAV5 TAb assay. It will be interesting to evaluate, in future studies, whether this conclusion holds true in the clinic for different AAV serotypes and for different expressed transgenes.

One potential limitation of this interpretation is that all animals with non-antibody transduction inhibitors (groups 2 and 3) had TI titers of less than 10, raising the question of whether the lack of effect observed for non-antibody transduction inhibitors was due to their lower TI titers. This seems unlikely because neutralizing anti-AAV5 antibodies with TI titers of less than 10 were still effective. For example, animals 2002 and 3001 had neutralizing AAV5 antibodies with TI titers of 7 and 6 (at pre-screening), respectively, and still showed reduced or blunted FVIII-SQ transgene expression in plasma. It therefore follows that the different nature of AAV5 neutralizing factors in plasma (antibodies versus non-antibodies), rather than a difference in their TI titers, determines the effect on AAV5 transduction *in vivo*.

In addition, non-antibody transduction inhibitors with TI titers of more than 10 are not frequently detected. In this non-clinical study, the strongest non-antibody transduction inhibitor identified during pre-screening of 60 monkeys had a TI titer of 9 (Table 1; Figure S2). Similar results were observed previously in human plasma,[16] where non-antibody transduction inhibitors were detected in 24 of 100 normal human plasma samples; 22 of these 24 samples had a TI titer of 10 or less, utilizing the same TI assay format as in this non-clinical study. This amounts to an estimated total seroprevalence of these possibly more potent non-antibody transduction inhibitors (i.e., with a TI titer of more than 10), of about 2 in 100 human subjects (2%), a frequency so low that it does not restrict the general utility of the AAV5 TAb assay for pre-screening of likely responders. Clinically, these data suggest a reasonable likelihood that most hemophilia A patients with non-antibody inhibitors will respond to BMN 270 gene therapy, similar to their non-immune counterparts.[33] This is being confirmed in ongoing clinical trials for BMN 270 by collecting the same data as described here while relaxing the exclusion criteria to only the AAV5 TAb assay, rather than using both the AAV5 TAb and TI assays for enrollment decisions.

Interestingly, several animals with pre-existing neutralizing AAV5 antibodies remained responsive to BMN 270 treatment, displaying varying levels of transgene expression and vector DNA, sometimes comparable with those observed for non-immune control animals. This indicates that hepatocyte transduction of BMN 270 can occur under certain circumstances in individual subjects, even when pre-existing AAV5 immunity is detected in both TAb and TI assays. The detection cutoff in these two non-clinical assays was based on empirically verified "cutpoints" derived by using statistical methods, as recommended by industry white papers and Food and Drug Administration (FDA) guidance for immunogenicity assays.[34–36] Because statistical cutpoints are determined in reference to the antibody-negative population by using 30–50 individual naive donor samples, the resulting assay sensitivity is typically very high and able to identify even low-positive samples but without any clear indication as to whether the detected antibody levels are likely to be effective *in vivo*. The data reported here suggest that there is a second, biological threshold of tolerable AAV5 antibody levels below which vector transduction and therapeutic efficacy may be achievable. This is currently being evaluated clinically for BMN 270 by enrolling hemophilia A patients presenting with detectable levels of anti-AAV5 antibodies in a titer escalation trial (BMN 270-203).

There was no strictly linear correlation between the neutralizing TI titer of pre-existing AAV5 antibodies and achieved FVIII-SQ expression levels (Figure 3), and in the low-to-medium TI titer range (6–30), conflicting outcomes were obtained (e.g., animals 2003 and 3001) to conclude that variable thresholds for tolerable

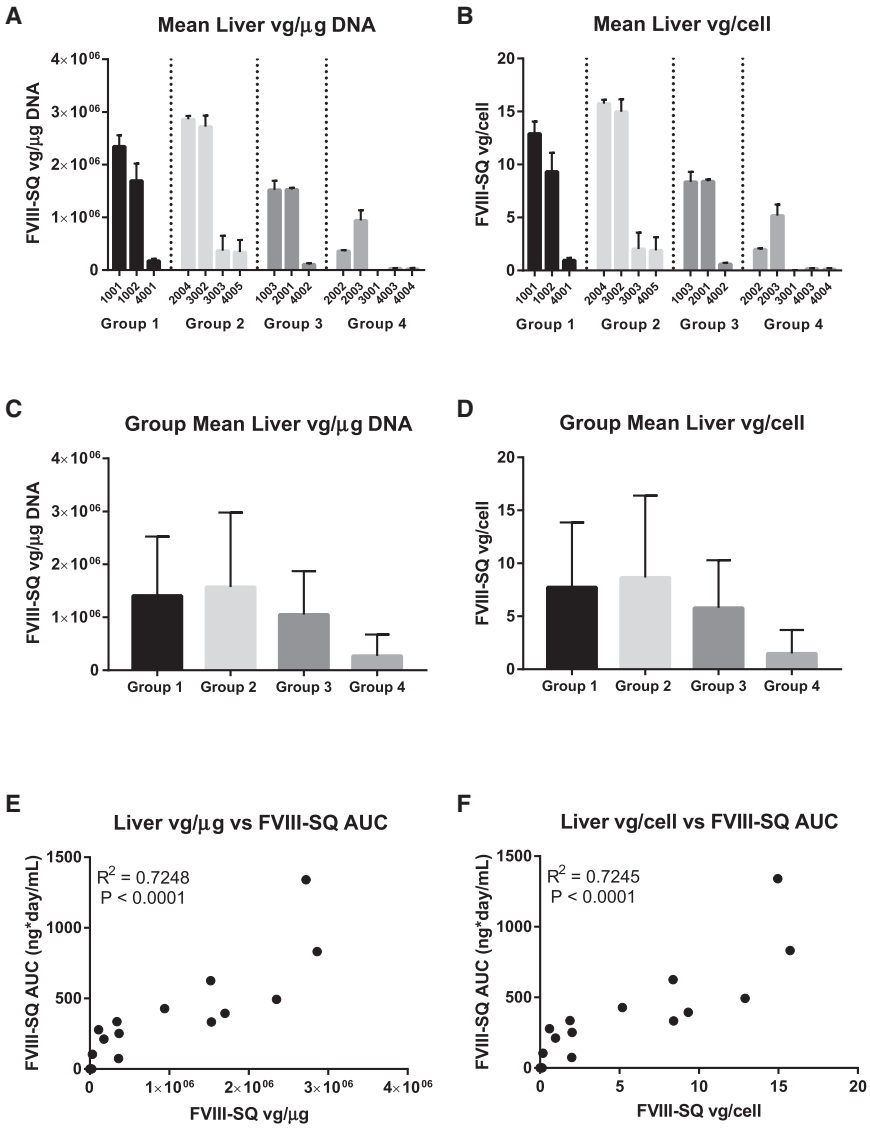

Figure 4. FVIII-SQ Vector Genomes in Cynomolgus Monkey Liver

Liver samples for DNA analysis were collected at the end of study (day 56), and copies of FVIII-SQ vector genomes were determined by qPCR. (A and B) Mean copies of FVIII-SQ vector genomes across all liver quadrants in individual animals were normalized per microgram input DNA (A, vector genomes per microgram) or per diploid cell (B, vector genomes per cell). (C and D) Mean copies of FVIII-SQ vector genomes by study group were normalized per microgram input DNA (C, vector genomes per microgram) or per diploid cell (D, vector genomes per cell). The results for one animal in group 4 were below the limit of quantitation (50 vg/reaction) and were imputed as 25 vg/reaction for calculation of group means. (E and F) Mean copies of FVIII-SQ vector genomes across all liver quadrants in individual animals, either normalized per microgram input DNA (E, vector genomes per microgram) or per diploid cell (F, vector genomes per cell), were correlated with FVIII-SQ AUC in plasma from day 1 through day 29. Group 1, non-immune control; groups 2 and 3, non-antibody transduction inhibitors with low and moderate TI titers, respectively; group 4, neutralizing AAV5 antibodies. All error bars show SD of the mean.

AAV5 antibody levels may exist in individual animals. In this study, no FVIII-SQ expression was observed in animals with pre-existing AAV5 antibodies and a baseline TI titer of more than 37; however, only one animal showed a TI titer above that level (4004; TI, 146). From this limited dataset, no firm conclusion regarding the physiological TI titer threshold can be reached. Further, if a larger non-clinical study with higher statistical power were to determine the maximally tolerable TI titer for AAV5 antibodies for the general monkey population, above which no FVIII-SQ protein expression is observed, it is unclear whether this TI titer threshold could be translated directly to clinical patients.

Nonetheless, the testing conditions of the non-clinical TI assay, which are highly similar to those used in BioMarin Pharmaceutical's clinical TI assay,[16,24] can be utilized to theoretically model this threshold by scaling the amount of plasma and AAV5 vector used in the assay to the total plasma volume and total AAV5 vector dose of an average monkey (refer to the Supplemental Materials and Methods), yielding theoretical *in vivo* dose-neutralization curves. The predicted TI titer threshold for complete BMN 270 dose neutralization in monkeys falls between 50 and 60 for a vector dose level of 6×10^{13} vg/kg (Figure S3). Accordingly, lower TI titer thresholds are predicted for lower dose levels. Intriguingly, the data obtained in this study are consistent with a predicted TI titer threshold of 50–60, confirming that it is possible to obtain (at least partial) FVIII-SQ expression as long as the TI titer of pre-existing neutralizing AAV5 antibodies remains below this theoretical biological cutoff.

Given the variable responses in animals with similar AAV5 antibody TI titers (e.g., 2003 and 3001), the question arises why the *in vitro* TI titers were not linearly correlated with FVIII-SQ expression. There are at least four possible explanations that we can imagine: first, the precision of titer measurements is rather limited. Titer determinations are intrinsically linked to the sample dilution scheme used because the same sample may easily test one titer level up or down because of analytical variability. In this study, we used a 1:2 serial dilution scheme where up to a 2-fold difference in TI titers would be considered analytically equivalent. Second, there could be different AAV5 vector uptake receptors on human HEK293T/17 cells used

Table 3. Summary of AAV5 and FVIII Antibody Responses following BMN 270 Administration

	AAV5 Antibodies ECLU S/N (Result +/−)						FVIII Antibodies ECLU S/N (Result +/−)		
Animal (Group)	Baseline	Day 8	Day 15	Day 22	Day 29	Day 56	Baseline	Day 29	Day 56
1001 (1)	0.54 (−)	101.55 (+)	246.58 (+)	224.48 (+)	363.71 (+)	300.68 (+)	0.96 (−)	0.96 (−)	75.20 (+)
1002 (1)	0.67 (−)	165.91 (+)	341.60 (+)	410.53 (+)	433.58 (+)	333.26 (+)	0.90 (−)	0.92 (−)	1.06 (+)
4001 (1)	0.50 (−)	43.08 (+)	348.32 (+)	416.66 (+)	476.44 (+)	333.35 (+)	0.80 (−)	0.86 (−)	5.41 (+)
2004 (2)	0.80 (−)	83.40 (+)	290.46 (+)	366.84 (+)	513.79 (+)	349.46 (+)	0.82 (−)	0.97 (−)	18.90 (+)
3002 (2)	0.60 (−)	249.74 (+)	423.83 (+)	429.40 (+)	476.18 (+)	312.11 (+)	0.83 (−)	0.83 (−)	35.00 (+)
3003 (2)	0.86 (−)	190.93 (+)	466.35 (+)	441.44 (+)	454.98 (+)	325.12 (+)	0.81 (−)	0.80 (−)	0.82 (−)
4005 (2)	0.84 (−)	222.57 (+)	452.15 (+)	462.49 (+)	478.57 (+)	349.18 (+)	0.86 (−)	0.87 (−)	8.47 (+)
1003 (3)	0.52 (−)	260.10 (+)	453.08 (+)	441.71 (+)	433.17 (+)	335.19 (+)	0.90 (−)	0.87 (−)	0.89 (−)
2001 (3)	1.37 (−)	351.15 (+)	441.78 (+)	400.86 (+)	480.79 (+)	349.93 (+)	0.91 (−)	0.91 (−)	8.43 (+)
4002 (3)	0.42 (−)	135.92 (+)	361.74 (+)	426.45 (+)	426.82 (+)	312.56 (+)	0.85 (−)	0.83 (−)	0.81 (−)
2002 (4)	9.23 (+)	154.07 (+)	454.88 (+)	415.22 (+)	493.55 (+)	337.73 (+)	0.89 (−)	0.90 (−)	0.89 (−)
2003 (4)	25.34 (+)	302.80 (+)	417.64 (+)	453.37 (+)	514.90 (+)	348.81 (+)	0.83 (−)	0.85 (−)	0.89 (−)
3001 (4)	22.31 (+)	169.90 (+)	269.82 (+)	384.10 (+)	483.70 (+)	349.72 (+)	0.82 (−)	0.79 (−)	0.80 (−)
4003 (4)	55.23 (+)	350.55 (+)	371.71 (+)	383.87 (+)	432.21 (+)	352.12 (+)	0.86 (−)	0.88 (−)	0.89 (−)
4004 (4)	244.52 (+)	395.27 (+)	370.81 (+)	359.96 (+)	365.57 (+)	296.31 (+)	0.85 (−)	0.89 (−)	0.85 (−)

The screening cutpoints for positivity of the S/N ratio are 1.70 in the AAV5 TAb assay and 1.05 in the FVIII TAb assay. S/N values were included for information only and not used to assess the magnitude of the antibody response.

in the TI assay versus those on primary hepatocytes in monkeys, with different binding sites on the AAV5 capsid. Differences in AAV uptake have been described, for example, between Chinese hamster ovary (CHO) and HepG2 cells.[37] Thus, AAV5 antibodies targeting a particular capsid epitope may block transduction of HEK293T/17 cells but may be less effective for blocking transduction of hepatocytes or vice versa. Third, any additional non-specific effect a plasma sample might have on cell viability or functionality could potentially lead to an overestimation of TI titers in this cell-based assay. Cell viability was checked visually, but this may not always reveal all types of non-functional cellular states. Fourth, the AAV5 luciferase reporter vector used in the TI assay differs from BMN 270, for example, in the use of a cytomegalovirus (CMV) promoter instead of a liver-specific promoter to express the luciferase gene.[16] Therefore, any direct effect of the plasma sample on signaling or transcription factors specific to the CMV promoter may suppress luciferase gene expression and may inflate the interpretation of actually existing AAV5 antibody TI titers.

Another noteworthy observation was the exceptionally high FVIII-SQ plasma concentration in one group 2 animal (3002). Despite the detectable presence of non-antibody transduction inhibitors in this animal, the plasma FVIII-SQ C_{max} concentration was at least approximately 2 times higher than those in all other animals in this study (Figure 1; Table 2). It could be speculated that certain transduction-enhancing factors in animal 3002 created a highly favorable environment for AAV5 transduction at the time of BMN 270 dosing, which may have not been adequately captured in the TI assay. For example, serum albumin can directly interact with the AAV capsid and augment transduction *in vivo*, likely by increasing AAV binding to target cells.[38] Because total vector genome levels in the liver of animal 3002 were comparable with those in animal 2004, which showed an approximately 2 times lower FVIII-SQ C_{max} plasma concentration, it may be more plausible to assume that the transduction-enhancing effects occurred during DNA repair synthesis or transgene expression. One potential mechanism could be adenovirus co-infection, either at the time of BMN 270 dosing or sometime after gene transfer (A.M. Davidoff, et al., 2003, Am. Soc. Gene Ther., abstract).[39]

This study did not address the potential effect of non-neutralizing AAV5 antibodies on BMN 270 gene transfer; i.e., it did not include animals that were positive in the AAV5 TAb assay but negative in the TI assay (Figure S1). This decision was made mainly because the clinical seroprevalence of non-neutralizing AAV5 antibodies is rather low, occurring in only about 5% of the normal human population.[16] In addition, the presence of AAV5 antibodies, regardless of their neutralizing capacity, is an indicator of previous exposure to wild-type AAVs. Depending on the course of this natural, non-pathogenic infection, human individuals might have also developed other forms of AAV immunity, such as memory T cells that could antagonize transduced hepatocytes upon re-challenge with AAV5 gene therapy.[3,40] Hence, human subjects with non-neutralizing AAV5 antibodies may not be optimal responders. Such cellular AAV5 immunity, however, does not appear to play a role in monkeys.[2] Therefore, the question of whether the distinction between neutralizing and non-neutralizing AAV5 antibodies carries any weight with regard to gene therapy outcomes may be best assessed directly in the clinic.

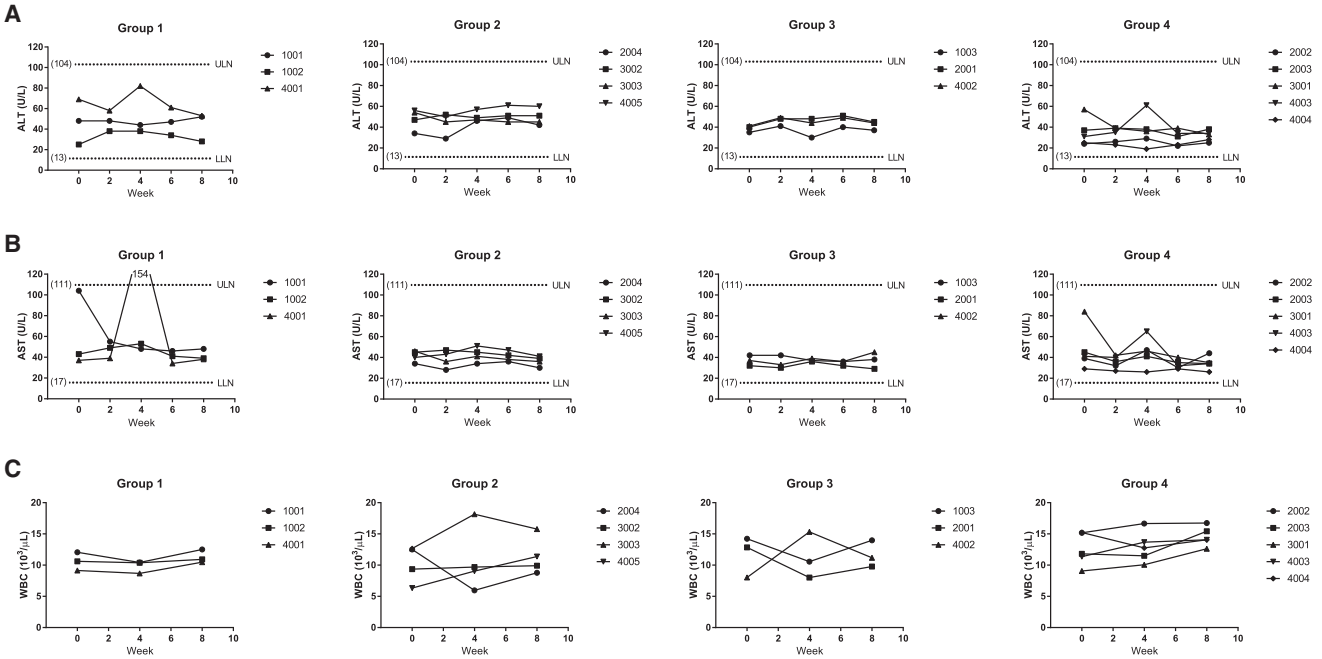

Figure 5. Plasma Levels of Liver Transaminases and White Blood Cell Counts

(A and B) Plasma samples were collected at baseline and then every 2 weeks for measurement of alanine aminotransferase (ALT) activity (A) and aspartate aminotransferase (AST) activity (B). ULN, upper limit of normal range; LLN, lower limit of normal range. (C) Whole blood was collected at baseline, week 4, and week 8 for enumeration of white blood cells (WBCs). Group 1, non-immune control; groups 2 and 3, non-antibody transduction inhibitors with low and moderate TI titers, respectively; group 4, neutralizing AAV5 antibodies.

MATERIALS AND METHODS

Test Material

BMN 270 (AAV5-Proto1-UCL-FVIII-SQ), lot number 16-097W (4.17×10^{13} vg/mL) was provided by BioMarin Pharmaceutical and stored at −80°C until use. BMN 270 was equilibrated at room temperature for approximately 1 h on the day of use, prepared for administration under a laminar flow hood, and administered within 5 h after preparation. BMN 270 was administered as a single slow intravenous bolus injection on study day 1.

Study Animals

Cynomolgus monkeys were chosen as the animal model for this study because it is a non-rodent species for preclinical toxicity testing acceptable to regulatory agencies. The number of animals and animal groups used was considered to be the minimum required to properly characterize the effects of the test article and was designed so that it did not require an unnecessary number of animals to accomplish its objectives. Studies using laboratory animals provide the best available basis for extrapolation to humans and are required to support regulatory submissions. Acceptable models that do not use live animals do not currently exist. The dose of BMN 270 used was based on previous work (unpublished data, BioMarin Pharmaceutical) and known to produce FVIII protein expression. No toxicity was noted at this dose in previous studies. Male cynomolgus monkeys were 2.5 to 4.4 years old and weighed 2.4 to 3.3 kg on the day prior to BMN 270 dosing. Ambient temperature was maintained at 64–84°F (18–29°C) with a relative humidity of 30%–70%, 10 or more fresh air exchanges per hour, and a 12-h light-dark cycle. The animal diet consisted of daily Certified Primate Chow No. 5048 (PMI Nutrition International, St. Louis, MO) and was supplemented with fruits or vegetables at least 2 to 3 times weekly. Municipal tap water treated by reverse osmosis and UV irradiation was freely available to each animal via automatic watering systems. Animals were acclimated to laboratory housing before BMN 270 dosing and permitted to socialize in groups of up to three per dosing group in stainless steel cages. Primary enclosures were as specified in the United States Department of Agriculture (USDA) Animal Welfare Act (9 Code of Federal Regulations [CFR], parts 1, 2, and 3) and as described in the Guide for the Care and Use of Laboratory Animals.[41] Cage-side observations for general health were performed once daily in the morning beginning on day −7 and continued throughout the study. Animals were removed from cages weekly, and a detailed clinical observation was performed, which also included body weight measurement and food consumption evaluation.

This study complied with all applicable sections of the Final Rules of the Animal Welfare Act regulations (CFR, Title 9), the Public Health Service Policy on Humane Care and Use of Laboratory Animals from the Office of Laboratory Animal Welfare, and the Guide for the Care

and Use of Laboratory Animals from the National Research Council.[41] The protocol and any amendments or procedures involving the care or use of animals in this study were reviewed and approved by the Testing Facility Institutional Animal Care and Use Committee before the initiation of such procedures.

Sample Collection for Standard Laboratory Evaluations

Blood was obtained by venipuncture for hematology studies (red blood cell count, hemoglobin concentration, hematocrit, mean corpuscular volume, red blood cell distribution width, mean corpuscular hemoglobin concentration, mean corpuscular hemoglobin, reticulocyte count [absolute], platelet count, white blood cell count, neutrophil count [absolute], lymphocyte count [absolute], monocyte count [absolute], eosinophil count [absolute], basophil count [absolute], and large unstained cells) at week −1 and on days 29 and 56. Coagulation studies (activated partial thromboplastin time and fibrinogen and prothrombin time) were performed at week −1 and on days 29 and 56, and fasting clinical chemistry studies (alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, gamma-glutamyl transferase, lactate dehydrogenase, total bilirubin, urea nitrogen, creatinine, calcium, phosphorus, total protein, albumin, globulin, albumin/globulin ratio, glucose, cholesterol, triglycerides, sodium, potassium, and chloride) were performed at week −1 and on days 15, 29, 43, and 56.

Sample Collection for FVIII-SQ Protein, FVIII TAb, and AAV5 Tab/TI Assays

Blood was obtained by venipuncture, and samples were collected into 3.2% sodium-citrated tubes that were gently mixed and then centrifuged. Time points included pre-treatment and days 8, 15, 22, 29, 36, 43, 50, and 56 (FVIII-SQ protein), pre-treatment and days 29 and 56 (anti-FVIII TAb and anti-AAV5 TAb), and pre-treatment only for the AAV5 TI assay. Each plasma sample was divided into two 100-μL aliquots (in-life) or four 100-μL aliquots (at termination) in polypropylene tubes and immediately frozen at −60°C or below. Any leftover plasma was divided into 100-μL aliquots in polypropylene tubes and immediately frozen at −60°C or below. Two of these leftover aliquots were designated for potential additional anti-FVIII TAb, and two were designated for potential anti-capsid TAb. Plasma samples were shipped on dry ice within 7 business days after day 29 and termination collections to Precision for Medicine (PFM; Redwood City, CA) (anti-AAV5 Tab) or BioMarin Pharmaceutical (San Rafael, CA) (FVIII protein and anti-FVIII TAb). Upon receipt, samples collected at protocol-specified time points were stored at −60°C or below and subsequently analyzed for FVIII-SQ protein, anti-FVIII TAb (BioMarin Pharmaceutical), and anti-AAV5 TAb/TI (PFM).

Cell-Based AAV5 TI Assay

The AAV5 TI assay used HEK293T/17 cells seeded at 40,000 cells per well cultured overnight at 37°C with 5% CO_2 in a white clear-bottom 96-well plate with high glucose DMEM containing fetal bovine serum (FBS). On the next day, a titer quality control (TQC) prepared with a mouse monoclonal anti-AAV5 antibody (LifeSpan Bioscience, catalog number 200914, clone ADK5b) at 3,000 ng/mL in pooled cynomolgus cutpoint control (CC) plasma was serially diluted 1:2 with the CC in a total of eight dilution steps. Test samples, diluted TQC, a low-QC (LQC) at 120 ng/mL anti-AAV5, and the blank CC were incubated 1+1 with an AAV5 vector containing a firefly luciferase reporter gene, a 61-kDa monomer that catalyzes the mono-oxygenation of beetle luciferin, 30 min prior to transduction in transduction medium (DMEM containing 1% BSA and a 25,000 MOI AAV5 vector). The mixtures were added to the cells, and after approximately 1 h of incubation, 20 mM etoposide was added. Two days later, cells were lysed in buffer containing the luciferase substrate (luciferin) and read for luminescence signal. Cells transduced with the AAV5-luciferase virus in CC plasma showed greater relative luminescence units (RLUs) than cells exposed to the AAV5 transduction inhibitory activity. Thus, the transduction observed for test samples, TQC, and LQC was normalized as a percentage of the transduction observed for the blank CC. All plasma samples were initially analyzed in a screening assay to identify the presence or absence of AAV5 neutralizing activity in the samples. If the normalized transduction signals for a given plasma sample were below the screening cutpoint (SCP = 44.1% transduction), the sample was considered positive. The positive samples were then analyzed with the titer method to determine the relative magnitude of AAV5 TI (TI titer). In the TI titer assay, samples were subjected to a serial dilution in CC plasma (negative control plasma pool), similar to TQC, and all dilutions of the samples were assayed for luciferase activity. The reciprocal sample dilution factor (DF) at which the dilution curve crossed the titer cutpoint was interpolated and reported as the titer of the unknown sample. The lower limit of detection for the TI assay was 92.9 ng/mL of the monoclonal positive-control anti-AAV5 antibody.

AAV5 TAb Assay

Antibodies against AAV5 were detected in cynomolgus monkey plasma using a sandwich ECL assay on the Meso Scale Discovery (MSD) platform. All plate incubation steps were performed for 1 h with shaking at ambient temperature, followed by washing with Tris-buffered saline with Tween 20 (TBST; DBPS and 0.1% Tween 20). First, bare standard-bind multi-array MSD plates were coated with 5.0 10^{11} vg/mL AAV5 in PBS and blocked with TBS with 1% casein (TBS-C) (1% casein in 20 mM Tris and 500 mM NaCl [pH 7.4]). Low and high QCs were prepared at 5 and 1,000 ng/mL by diluting a rabbit polyclonal anti-AAV5 antibody (Fitzgerald Industries, catalog number 20R-2587) in 100% pooled cynomolgus CC plasma. CC plasma without antibody was also tested as a negative control. QCs and test samples were diluted at the minimum required dilution (MRD) of 1:20 in TBS-C and added to the plate in duplicate. For detection, 0.1 μg/mL ruthenium-labeled protein A/G/L in TBS-C was added, electrochemiluminescence was detected by adding 1× MSD read buffer containing the substrate tripropylamine by the MSD 1300 MESO QuickPlex SQ 120, and the signal was expressed in relative ECLUs. Sample results were reported as a normalized signal-to-noise (S/N) value, calculated by dividing the mean ECLU of a test sample by the mean ECLU of the CC plasma. All plasma samples were analyzed in the screening assay to determine the presence or

absence of anti-AAV5 antibodies in the samples. If the normalized S/N value for a given plasma sample was greater or equal to the SCP (SCP = 1.70), the sample was considered positive, all other results were considered negative. No titrations were performed to generate antibody titer values for each sample. S/N values are reported for informational purposes only and were not used to assess the magnitude of the antibody response. The limit of detection of the assay was 4.34 ng/mL of the polyclonal positive-control anti-AAV5 antibody, which corresponds to 61.2 ng/mL if the mass concentration is expressed as monoclonal anti-AAV5 (clone ADK5a) equivalents.

It should be noted that BioMarin Pharmaceutical's clinical AAV5 TAb assay[16,24] is different from the above-described non-clinical AAV5 TAb assay and uses a bridging ECL immunoassay format in which antibodies are captured by a passively coated AAV5 capsid and detected using a ruthenylated AAV5 capsid irrespective of antibody class but requiring some degree of multivalence for the antigen-binding sites. The clinical assay also has a different SCP and reports semiquantitative AAV5 antibody binding titers rather than normalized ECLU (S/N) values. Therefore, a direct comparison of test results between the non-clinical and clinical AAV5 TAb assays cannot be made.

FVIII-SQ Protein Assay

A sandwich ECL assay was performed on the MSD QuickPlex SQ 120 Imager (QuickPlex Imager) to measure the concentration of hFVIII in sodium-citrated normal pooled cynomolgus monkey plasma. A monoclonal antibody to the A2 domain of hFVIII was conjugated to an extra-long-chain amine-reactive *N*-hydroxysulfosuccinimide (LC-LC-NHS) biotin, and a sheep polyclonal antibody to hFVIII was conjugated to a ruthenium NHS (Sulfo-NHS) tag. Standard calibrators and QCs were prepared with Xyntha (clinical-grade hFVIII-SQ) in 100% pooled cynomolgus monkey plasma, ranging from 301 to 0.294 ng/mL (a total of 11 points), with the bottom three designated as anchor points, making the range of quantitation (ROQ) from 301 to 2.35 ng/mL FVIII-SQ. High-, mid-, and two LQC samples were tested at concentrations of 226, 60.3, 5.00, and 3.00 ng/mL FVIII-SQ, respectively. The standard calibrators, QCs, and study samples were diluted to an MRD factor of 1:10 in assay diluent (Cedarlane, catalog number CL2003SK-DILUENT) prior to addition to the MSD plate wells. Samples and combined labeled anti-FVIII detection reagents were incubated for 2 h at room temperature with shaking. Simultaneously, an MSD plate coated with streptavidin was blocked with 6% BSA in TBST for 2 h at room temperature with shaking. At the end of the blocking step, the blocking buffer in the wells of the MSD plate was removed without washing. The mixture of the labeled antibodies and plasma samples was then incubated for 1 h at room temperature with shaking on the blocked MSD plate to capture the FVIII-SQ-antibody complexes via the biotin label. After a triple wash with TBST, 1× MSD read buffer containing the substrate tripropylamine (TPA) was added to react chemically with ruthenium in the presence of applied voltage. The FVIII-SQ complexes bound to both the biotinylated and ruthenylated antibodies generate an ECL signal detected by the QuickPlex Imager. Standard curve regression analysis using a 4-parameter logistic algorithm with 1/Y weighting in Watson Laboratory Information Management System (LIMS) version 7.4.2 was used to report concentrations, percent coefficient of variation (CV), and percent relative error (RE) of back-calculated unknown QC and study samples. The lower limit of quantitation for the assay was 2.35 ng/mL FVIII-SQ.

FVIII TAb Assay

Antibodies against hFVIII were measured in cynomolgus monkey plasma using a bridging ECL assay on the MSD QuickPlex Imager. B domain-deleted (BDD) recombinant hFVIII-SQ (Xyntha) was either conjugated with an LC-LC biotin tag (FVIII-Bio) or a Sulfo-NHS tag (FVIII-Ru). TQCs were prepared using a mouse monoclonal anti- hFVIII (Green Mountain Antibodies, catalog number GMA-8012) spiked into pooled cynomolgus CC plasma at 8,000 ng/mL and serially diluted 1:4 a total of six times to 1.95 ng/mL. LQCs at 40.0 ng/mL, a separate naive pool used as a blank negative QC (NQC), and the CC plasma were tested on all plates. Samples and QCs were heat-treated for 30 min to dissociate any antibody complexes with endogenous plasma FVIII and then placed on ice. Next, samples and QCs were diluted to the MRD of 1:20 in diluent buffer (TBS with 1% casein). The conjugated reagents were combined at equimolar ratios (0.5 μg/mL each) and incubated 1:1 with diluted samples and QCs. During the incubation, anti-hFVIII antibodies in plasma formed complexes with the conjugated FVIII reagents. The mixture was transferred to a streptavidin-coated polypropylene MSD plate to capture the hFVIII-antibody complexes. Plates were washed with Dulbecco's PBS (DPBS) containing 0.1% Tween 20 and 0.05% Proclin300 before addition of MSD read buffer T (1× concentration) containing the substrate TPA, which reacts chemically with ruthenium (FVIII-Ru) in the presence of voltage applied to each well by the MSD QuickPlex Imager plate reader. Samples that contained human anti-FVIII antibodies bound to both the FVIII-Bio and FVIII-Ru generated ECL signals that were detected by the MSD Imager. All plasma samples were analyzed in a screening assay to identify the presence or absence of anti-FVIII antibodies in the samples. Mean sample ECLU values were normalized as S/N values by dividing them by the mean ECLU value of the CC plasma that was run on the same plate. If the normalized ECLU signal for a given plasma sample was greater or equal to the plate-specific SCP (mean CC signal × SCP factor) (SCP factor [SCPF] = 1.05), the sample was considered positive; otherwise, the sample was considered negative. The limit of detection (LOD) for the assay was 1.93 ng/mL of the monoclonal positive-control anti-FVIII antibody.

Terminal Procedures

All animals survived until day 56, when they were weighed, anesthetized, and euthanized by exsanguination, followed by a complete necropsy examination under the supervision of a veterinary pathologist. The brain, thyroid, heart, kidneys, liver, lungs, spleen, and testes were weighed (the lungs, kidneys, and testes were weighed together), and relative organ weight as a percentage of body weight (using the terminal body weight) and as a percentage of brain weight was calculated.

Liver Tissue Collection for the qPCR Assay

At termination, segments of liver tissue were obtained from each lobe (right lateral, right medial, quadrate, left medial, and left lateral samples) and collected as quickly as possible using new sterile instruments cleaned with RNA wipes for each tissue. Segments (approximately 0.5 to 1.0 g) were placed into separate cryo-vials, immediately frozen in liquid nitrogen, and maintained at −80°C until shipment on dry ice for sample testing.

FVIII-SQ Vector Genome Analysis

Total liver DNA (including hFVIII-SQ vector genomes and monkey genomic DNA) was extracted from approximately 25 mg of monkey liver segments using the DNA/RNA AllPrep kit (QIAGEN) following the manufacturer's instructions. The DNA concentration in the eluate was quantified by Nanodrop 8000. The extracted DNA was diluted to 20 ng/μL in elution buffer (EB). Copies of FVIII-SQ vector genomes present in monkey liver were quantified using real-time qPCR assays with absolute quantification against a standard curve. TaqMan DNA probes were chemically labeled with a fluorescent dye to enable detection and quantification of a specific qPCR product. The probe set was specific to the codon-optimized sequence of the hFVIII-SQ transgene. 20-μL qPCR reactions were set up with 2× TaqMan Environmental Mix 2.0 (Thermo Fisher Scientific), 20 μM (final concentration) of forward and reverse primers, 5 μM (final concentration) of fluorescent probes targeting the FVIII-SQ transgene, and approximately 100 ng of extracted total liver DNA (in 5 μL). The reaction volume was brought to 20 μL with DNase/RNase-free water (Invitrogen), loaded into white 384-well PCR plates, and run on a Roche Light Cycler 480 II. Thermal cycling at 95°C for 3 s and 60°C for 1 min was performed for 40 cycles with an initial denaturation step at 95°C for 10 min. Filters detecting fluorescence at 465–510 nm were used to detect amplicons corresponding to the FVIII-SQ transgene. Cycle crosspoint (Cp) values, defined as the number of PCR cycles required for the fluorescent signal to cross the threshold (i.e., exceeds the background level), were calculated for FVIII-SQ amplification by Light Cycler 480 software version 1.5.1. Sample Cp values were compared with those of the standard curve and interpolated to vector genomes per reaction. The quantities of FVIII-SQ vector determined for samples were then further back-calculated and normalized to vector genomes per microgram total input DNA and vector genomes per cell, whereby one diploid cell corresponded to 5.5 pg total DNA.

Statistical Evaluations

Correlation analysis of vector genome copy number in liver with FVIII-SQ AUC was performed using GraphPad Prism version 7. A two-tailed Pearson correlation was performed at the 95% confidence interval (alpha = 0.05). Statistical analysis was performed by the testing facility on all numerical data. As appropriate (i.e., when datasets contained more than two animals), further statistical analyses of the above mentioned parameters were performed using SAS (SAS/STAT User Guide, version 8; SAS Institute, Cary, NC). Significant intergroup differences were evaluated by use of ANOVA, followed by a multiple comparisons test. The assumptions that permitted use of a parametric ANOVA were verified using the Shapiro-Wilkes test for normality of the data and Levene's test for homogeneity of variance, with a $p \leq 0.001$ level of significance required for either test to reject the assumptions. If both assumptions were fulfilled, a single-factor ANOVA was applied, with animal grouping as the factor, utilizing a $p \leq 0.05$ level of significance. If the parametric ANOVA was significant at $p \leq 0.05$, then Dunnett's test was used to identify statistically significant differences between the control group and each test article-dosed group at the 0.05 level of significance. If either of the parametric assumptions was not satisfied, then the Kruskal-Wallis non-parametric ANOVA procedure was used to evaluate intergroup differences ($p \leq 0.05$). Dunn's multiple comparison test was applied when this ANOVA was significant, again utilizing a significance level of $p \leq 0.05$.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.omtm.2019.03.006.

AUTHOR CONTRIBUTIONS

C.V. and B.L. conceived the study and experiments, wrote the manuscript, and secured funding. K.S., J.H., and L.C. monitored the study and performed experiments. J.A. wrote the study protocol and monitored study conduct. S.Z., B.S., G.H., L.S.T., C.A.O., and C.F. provided expertise and feedback.

CONFLICTS OF INTEREST

B.L., K.S., J.H., L.C., G.H., J.A., C.F., B.S., C.A.O.N., S.Z., and C.V. are employees of BioMarin Pharmaceutical Inc. L.S.T. was an employee of BioMarin Pharmaceutical Inc. at the time of this study.

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