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Children's Hospital of Philadelphia (CHOP)
Center for Cellular and Molecular Therapeutics

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Meeting Request

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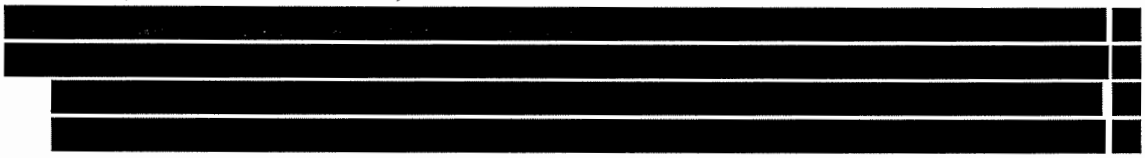

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1. PRODUCT NAME

CHOP- LNP1.PAH.ABE1

2. CHEMICAL NAME AND STRUCTURE

The drug product (DP) is a lipid nanoparticle (LNP) based editing therapeutic comprising lipid excipients, a messenger RNA (mRNA) drug substance (DS) encoding an adenine base editor (ABE), and a single guide RNA (gRNA) DS. The mRNA encodes an ABE that contains a common ABE8.8-m TadA deaminase domain and otherwise is >99% identical among all versions of the DP, with the ABE varying in its protospacer-adjacent motif (PAM) specificity. The gRNA DS is ≥80% identical among all versions of the DP.

gRNA Drug Substance

Each version of the LNP1.PAH.ABE1 DP comprises a gRNA DS, with each gRNA DS comprising (1) a distinct 20-nucleotide spacer sequence that corresponds to a protospacer DNA sequence matching the region of the *PAH* gene spanning the target variant, which includes the target adenosine nucleotide to be corrected by the DP, and (2) a common 80-nucleotide tracrRNA domain that complexes with a Cas9 nickase domain in an ABE.

The gRNA DS for the version of LNP1.PAH.ABE1 that corrects the *PAH* c.842C>T variant (p.Pro281Leu, or P281L), designated **PAH-001**, is a 100-mer oligonucleotide [redacted] as shown in **Table 1**.

The gRNA DS for the version of LNP1.PAH.ABE1 that corrects the *PAH* c.1222C>T variant (p.Arg408Trp, or R408W), designated **PAH-002**, is a 100-mer oligonucleotide [redacted] as shown in **Table 1**.

The gRNA DS for the version of LNP1.PAH.ABE1 that corrects the *PAH* [redacted] variant, designated **PAH-003**, is a 100-mer oligonucleotide [redacted] as shown in **Table 1**.

The gRNA DS for the version of LNP1.PAH.ABE1 that corrects the *PAH* [redacted] variant [redacted], designated **PAH-004**, is a 100-mer oligonucleotide [redacted] as shown in **Table 1**.

The gRNA DS for the version of LNP1.PAH.ABE1 that corrects the *PAH* [redacted] variant [redacted], designated **PAH-005**, is a 100-mer oligonucleotide [redacted] as shown in **Table 1**.

The gRNA DS for the version of LNP1.PAH.ABE1 that corrects the *PAH* [redacted] variant [redacted], designated **PAH-006**, is a 100-mer oligonucleotide [redacted] as shown in **Table 1**.

Table 1. gRNA DS sequence.

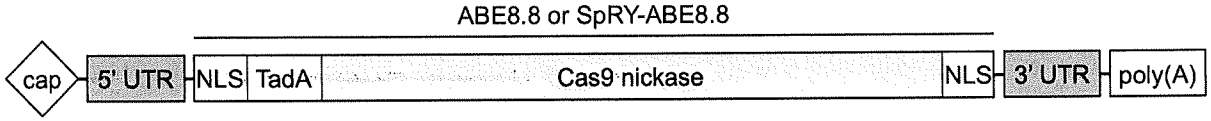
PAH-001	[redacted]
PAH-002	[redacted]
PAH-003	[redacted]
PAH-004	[redacted]
PAH-005	[redacted]
PAH-006	[redacted]

[redacted]

mRNA Drug Substance

Each version of the LNP1.PAH.ABE1 DP has one of two mRNA DSs. The first encodes the adenine base editor 8.8-m protein with a standard *Streptococcus pyogenes* Cas9 D10A nickase (hereafter referred to as ABE8.8), and the second encodes the adenine base editor 8.8-m protein with a SpRY variant of the *S. pyogenes* Cas9 D10A nickase (hereafter referred to as SpRY-ABE8.8). ABE8.8 and SpRY-ABE8.8 share the same TadA deaminase domain. Each mRNA comprises the same 5′ cap, 5′ untranslated region (UTR), 3′ UTR, and 3′ polyadenylate tail (**Figure 1**). In each mRNA, the ABE coding sequence is codon-optimized with uridine minimization and has substitution of all uridines with the modified nucleotide N1-methylpseudouridine. The mRNA sequences of ABE8.8 and SpRY-ABE8.8 are >99% identical. The two coding sequences and the limited number of positions that differ between the sequences are shown in **Table 2**.

Figure 1. Structure of mRNA DS.



Abbreviations: Cap = methylated 5′-Cap-1; UTR = untranslated region; NLS = nuclear localization signal; TadA = evolved tRNA-specific adenosine deaminase; Cas9 = clustered regularly interspaced short palindromic repeats (CRISPR)-associated protein 9; poly(A) = polyadenylic acid. Not to scale.

Table 2. ABE mRNA sequences, not including 5′ cap or 3′ polyadenylate tail, and protein sequences.

ABE8.8 mRNA	[redacted]

	[redacted]
SpRY-ABE8.8 protein	[redacted]

Underline = TadA domain; bold underline = differences between mRNAs/proteins.

Lipid Nanoparticle Drug Product

The LNP1.PAH.ABE1 DP comprises the mRNA and gRNA DSs and four lipid excipients: [redacted]

The structures of these components and the target composition of lipid excipients in the DP are provided in **Table 3** below. [redacted]

Table 3. Lipid excipients of the LNP formulation.

[redacted]

3. PROPOSED INDICATION FOR USE

LNP1.PAH.ABE1 is proposed for the reduction of blood phenylalanine (Phe) concentrations in adolescents and adults with phenylketonuria (PKU) who are homozygous or compound heterozygous for a variant in the phenylalanine hydroxylase (*PAH*) gene that can be efficiently corrected by an adenine base editor (ABE) with an ABE8.8-m TadA deaminase, such as c.842C>T, c.1222C>T, [redacted], [redacted], [redacted], and [redacted].

4. DOSAGE FORM, ROUTE OF ADMINISTRATION, AND DOSING REGIMEN

LNP1.PAH.ABE1 will be administered via an intravenous infusion at a dose based on body weight. There is the potential for repeat dosing to achieve the desired therapeutic effect.

5. LIST OF SPONSOR ATTENDEES

Attendee	Affiliation	Title
[redacted]	[redacted]	[redacted]

6. HISTORY OF THE PROJECT/BACKGROUND

Introduction

Phenylketonuria (PKU) is an autosomal recessive disorder caused by mutations in the gene encoding phenylalanine hydroxylase (PAH), resulting in the accumulation of phenylalanine (Phe) to neurotoxic levels (Regier and Greene, 2017). PAH deficiency prevents the conversion of Phe to tyrosine (Tyr). Untreated PKU patients can have very high blood Phe levels of more than 600 µmol/L (normal Phe levels are less than 120 µmol/L), which can result in impaired cognitive development and a host of neuropsychiatric conditions including mood, attention, and anxiety disorders (Blau et al. 2010; Levy et al. 2018; Ashe et al., 2019).

Unmet clinical need

Although it was discovered in the 1950s that restriction of Phe intake from diet can mitigate some aspects of disease (Bickel et al., 1953), unfortunately there is still no curative therapy for PKU. For dietary therapy to be beneficial, patients must strictly comply with a very difficult and expensive regimen of medical foods and a protein-restricted diet. The degree and duration of Phe elevation is strongly correlated with the degree of cognitive and neuropsychiatric impairment patients experience (Ashe et al., 2019; Brumm et al., 2010). **Real-world compliance is very poor, with 50-80% of adolescent and adult patients regularly experiencing Phe levels in the neurotoxic range** (Cazzorla et al., 2018; MacDonald et al., 2010). This is associated with a significantly increased risk of anxiety, depression, and attention disorders (Didycz et al., 2017; Altman et al., 2021). **Thus, new definitive, durable Phe-lowering therapies are needed for PKU.**

The Sponsor undertook a study (Brooks et al., 2023b) to better define the unmet need of PKU patients due to the *PAH* R408W variant , which is targeted by one version of the LNP1.PAH.ABE1 DP. Adults and children with PKU born prior to January 1, 2021, and followed in the Children's Hospital of Philadelphia (CHOP) Metabolism Clinic were considered for inclusion in the study. All available genotype information was reviewed, and subjects who were either homozygous or compound heterozygous for the *PAH* R408W variant were included in the final study cohort. Among 129 patients with PKU, 32 (25%) were found to be compound heterozygous for the *PAH* R408W variant, while 4 (3%) were homozygous for the variant. (This may be an underestimate of true R408W prevalence, as genotype information was not available for some older individuals with PKU.)

In this study cohort, 33 of 36 (92%) treated individuals with R408W PKU had at least a single Phe level above 360 µmol/L, and 25 of 36 (69%) had at least a single level above 600 µmol/L (Brooks et al., 2023b) (**Figure 2**). Furthermore, 10 of 36 (28%) had lifetime average Phe levels above 360 µmol/L. The four individuals homozygous for the R408W allele demonstrated especially poor metabolic control. In addition to high Phe levels, individuals with R408W variants demonstrated poor adherence to Phe monitoring schedules (Brooks et al., 2023b). Collectively, these data demonstrate that most individuals with the *PAH* R408W variant have poor metabolic control and are at risk for chronic neurologic damage. The *PAH* P281L variant, which is also targeted by one version of the LNP1.PAH.ABE1 DP, is an equally severe variant as R408W (Himmelreich et al., 2018) and would be expected to be associated with a similar unmet clinical need.

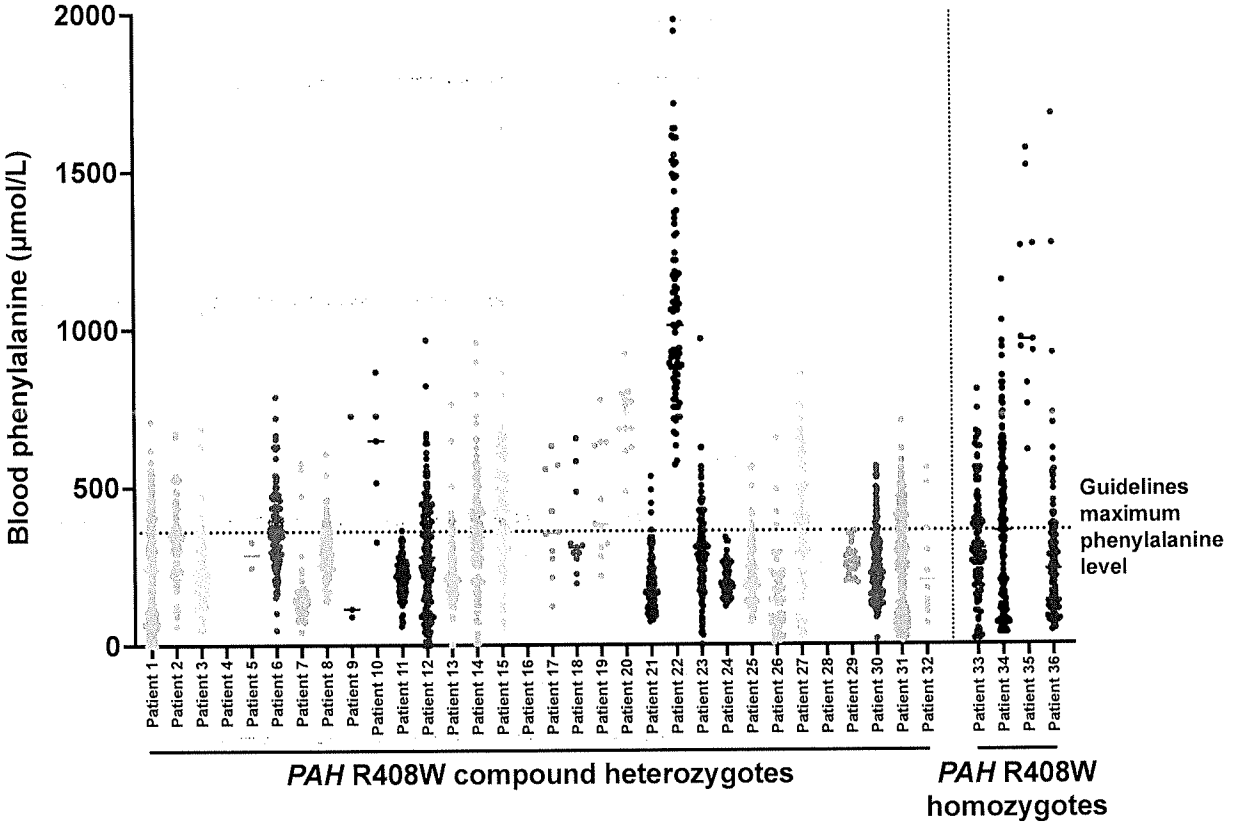


Figure 2. Phe levels in PKU patients. Most patients with PKU due to the *PAH* R408W variant have frequent Phe levels in the neurotoxic range. All available Phe levels from individuals with compound heterozygous (left) or homozygous (right) R408W variants were recorded as individual points. In total, 33 of 36 individuals had at least one level above the guidelines maximum level of 360 µmol/L (indicated by the horizontal dotted line). Adapted from Brooks et al., 2023b.

In addition to having neurocognitive and neuropsychiatric morbidity, pregnant women with uncontrolled PKU are at high risk for adverse fetal outcomes. Women with high Phe levels (>360 µmol/L) during pregnancy have an increased risk of spontaneous abortion and abnormal findings (developmental delay, intellectual disability, microcephaly, congenital heart defects, and low birth weight) in their offspring—so-called **maternal PKU syndrome**—with a dose-response relationship between maternal blood Phe levels and these abnormalities (Adams et al., 2023).

Current therapeutic approach

A strict low-Phe diet is the mainstay of treatment for PKU patients, with the goal of maintaining Phe levels of 120-360 µmol/L, which still exceed the physiologic range (Vockley et al., 2014; Smith et al., 2025). However, many PKU patients find it challenging to adhere to the unpalatable and cost-prohibitive diet. There are only two approved medical therapies. The first is sapropterin, an oral medication that serves as a cofactor of the PAH protein and can moderately improve the activity of some mutant forms of PAH. However, most variants that cause severe PKU, including R408W and P281L, are not responsive to sapropterin. The second is pegvaliase, an injectable enzyme that acts directly to catabolize Phe. However, pegvaliase carries a substantial risk of

anaphylaxis and has a black box warning from the U.S. Food and Drug Administration (FDA) on its label for that reason. In addition, the dosing regimen is complex, starting with once-weekly injections and slowly up-titrating to once-daily injections, and it is available in the U.S. only through a Risk Evaluation and Mitigation Strategy program. In a long-term study, patients on pegvaliase achieved a mean 51% Phe reduction at one year after initiation (1233 µmol/L to 565 µmol/L) (Burton et al., 2020).

As the Sponsor's PKU study data show (**Figure 2**), only 3 out of a total cohort of 36 patients consistently maintained non-neurotoxic levels of Phe with currently available therapeutic options, with the others vulnerable to long-term morbidity from PKU. Thus, a safe one-time therapy that would durably, even permanently, normalize blood Phe levels would be a superior treatment option over existing alternatives.

Population with potential for benefit

Out of the more than 1,000 *PAH* variants that have been cataloged in PKU patients, many of the most frequent pathogenic variants linked to classic PKU are transition mutations, specifically G>A or C>T variants on the sense strand (Hillert et al., 2020). As such, each of these variants is potentially amenable to correction by adenine base editing, which can drive site-specific A>G changes on either DNA strand (Gaudelli et al., 2017; Gaudelli et al., 2020). Among the most frequently occurring pathogenic *PAH* variants worldwide (Hillert et al., 2020) are:

- The **R408W** (c.1222C>T, p.Arg408Trp) variant, with its highest prevalence in European populations (e.g., present in 98.9% of PKU patients in Estonia, 89.2% in Poland, 75.7% in Russia, 43.6% in Sweden, and 35.7% in Germany), Australia (34.7%), and the United States (32.9%).
- [redacted]
- The **P281L** (c.842C>T, p.Pro281Leu) variant, with its highest prevalence in populations in the Middle East, Europe, and Russia (e.g., present in 14.8% of PKU patients in the Netherlands, 11.2% in Turkey, 10.8% in Portugal, 10.3% in Italy, and 9.4% in Germany).
- [redacted]
- [redacted]

In general, these variants are not responsive to sapropterin (Leuders et al., 2014), limiting the treatment options.

The Sponsor is proposing to treat adolescent and adult PKU patients harboring at least one copy of the *PAH* P281L variant, the *PAH* R408W variant, the *PAH* [redacted] variant, the *PAH* [redacted] variant, the *PAH* [redacted] variant, and/or the *PAH* [redacted] variant and in whom currently available interventions are not achieving guidelines-recommended Phe goals. Recently published data support that this age group would benefit from a reduction in Phe levels, even after several years of poor metabolic control. Specifically, in a study of women with PKU aiming to lower Phe levels prior to pregnancy, subjects had significant, durable improvement in several

neurocognitive domains and IQ after achieving blood Phe levels <240 µmol/L (Manti et al., 2023). These data suggest that at least some aspects of the neuropsychiatric phenotype observed in untreated adolescent and adult PKU patients are reversible.

Therapeutic Rationale

Rationale for the liver as the target organ

Although the liver is spared from toxicity in PKU, the *PAH* gene is largely expressed in hepatocytes, and correction of the primary genetic defect solely within the liver is curative in PKU patients (Vajro et al., 1993). *PAH* is unequivocally the causal gene responsible for PKU, inherited in a recessive manner. Besides the unambiguous human genetic evidence, there is a plethora of animal data documenting the causal role of mutations in the orthologous gene in PKU disease pathogenesis, i.e., mice and pigs (Koppes et al., 2020; Kaiser et al., 2021). A variety of studies in a mouse model of PKU, caused by the *N*-ethyl-*N*-nitrosourea-induced c.835T>C mutation in *Pah* (known as the Pah^{enu2} model), have indicated that replacement of ≈10% of PAH enzyme activity in the liver—regardless of whether the method is cellular replacement, virally mediated gene replacement, or corrective genome editing—is sufficient to substantially reduce blood Phe levels, even to the normal range (Fang et al., 1994; Hamman et al., 2005; Ding et al., 2006; Böck et al., 2022). By extrapolation, correction of ≈10% of the *PAH* alleles in the hepatocytes of PKU patients should ameliorate disease phenotypes in these patients.

Rationale for a base editing strategy

With the LNP1.PAH.ABE1 DP, the Sponsor plans to use corrective adenine base editing that introduces an A>G change in the genome, in a highly specific manner, at the site of any of a collection of variants (P281L, R408W[redacted]) in the *PAH* gene. The effect of reverting one of these variants to wild-type would be to restore functionality to the PAH protein product, durably reducing and even normalizing blood Phe levels in a PKU patient with at least one copy of the variant in question. The DP will comprise LNPs encapsulating an mRNA encoding an adenine base editor (ABE) and a single guide RNA (gRNA) targeting the site of the target *PAH* variant, in liquid form for intravenous (IV) infusion and delivery to the hepatocytes in the liver:

- The LNP1.PAH.ABE1 DP will contain ABE8.8 or SpRY-ABE8.8 mRNA, as well as a gRNA (designated PAH-001, PAH-002, PAH-003, PAH-004, PAH-005, or PAH-006) matched to the target *PAH* variant to be corrected.
- Each version of the investigational DP will otherwise contain **identical excipients** and will be formulated in the same way. Thus, only the gRNA DS and, in one case, the mRNA DS will vary.
- The gRNA DS varies in its sequence in only 20 of 100 positions at most.
- The mRNA DS varies in its sequence in only 27 of ≈5100 positions at most, i.e., 99.5% identity.

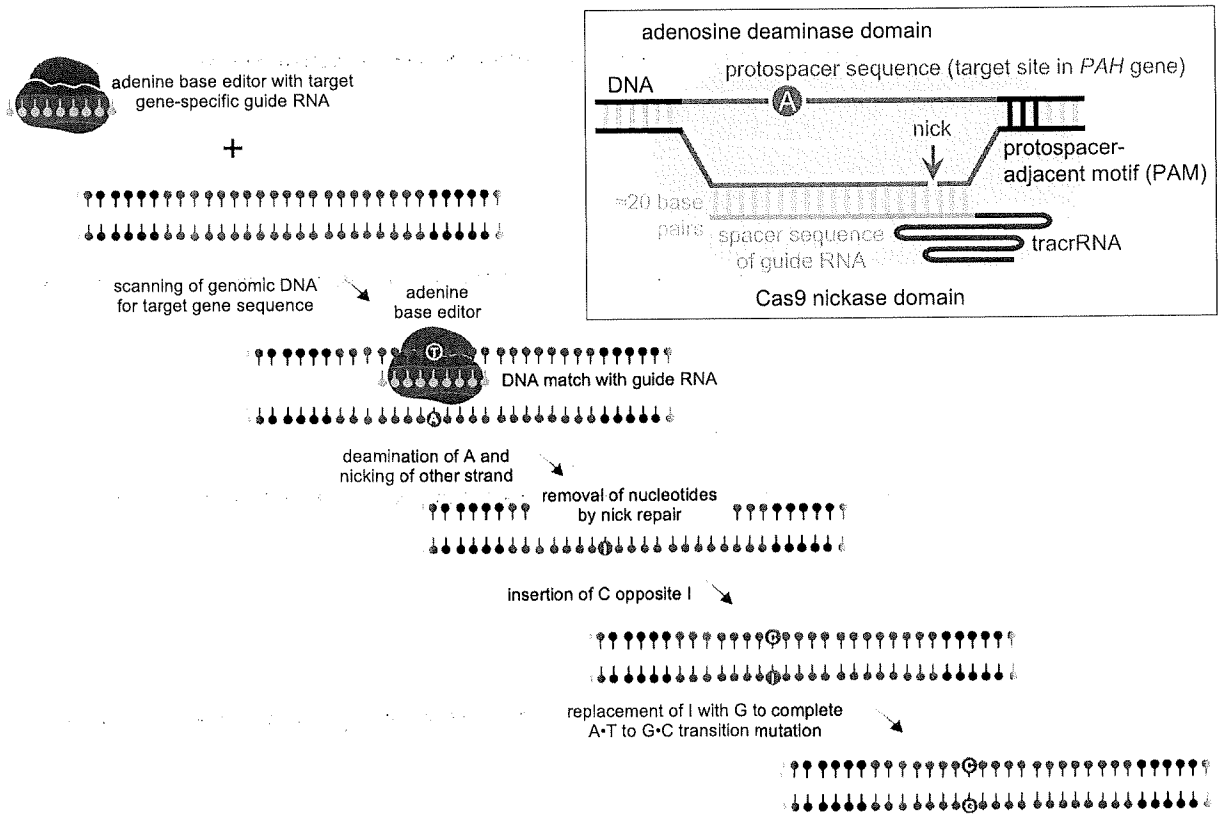


Figure 3. Schematic of the stepwise mechanism of adenine base editing. The adenine base editor (ABE) combines with a guide RNA (gRNA), engages with double-strand genomic DNA, and scans through the DNA to find the unique site in the genome that matches the spacer sequence (first 20 bases) of the gRNA. Deamination of the target adenine base on one strand, nicking of the other strand, and the ensuing cellular DNA repair process results in conversion of the original adenine base to guanine. The inset shows a close-up of the ABE in a complex with the gRNA and the specific target gene sequence.

The mechanism of action of the DP is as follows:

(1) The LNPs will be internalized by the hepatocytes following binding of endogenous apolipoprotein E (apoE) to the LNP, leading to engagement of low-density lipoprotein receptor (LDLR) on the plasma membrane and subsequent receptor-mediated endocytosis of the LNP carrying the mRNA and gRNA. Comprehensive nonclinical data have shown that **the LNP characteristics of the DP drive the biodistribution profile of the DP**.

(2) After LNP-bound apoE engages with LDLR, endocytosis of the LNP occurs. The subsequent reduction in endosomal pH results in a charge-based interaction of the ionizable lipid with the endosomal membrane, which ultimately results in the release of the mRNA and gRNA cargoes into the cytoplasm (Akinc et al., 2010; Kulkarni et al, 2018).

(3) The mRNA will be translated into an ABE protein, which comprises a catalytically impaired clustered regularly interspaced short palindromic repeats (CRISPR)-associated protein 9 (Cas9) nuclease domain (that functions as a single-strand nickase and minimizes the production of double-strand breaks) fused with an adenosine deaminase

domain (Gaudelli et al., 2017; Gaudelli et al., 2020) (**Figure 3**). The gRNA comprises a tracrRNA domain that complexes with the Cas9 nickase domain and a spacer sequence that corresponds to a protospacer DNA sequence matching the region of the *PAH* gene spanning the targeted *PAH* variant, which includes the target adenosine nucleotide to be corrected. The protospacer is located immediately upstream of the protospacer-adjacent motif (PAM), which is required for ABE activity. **The protospacer is chosen to be unique in the genome, enabling a highly specific gRNA that would not bind efficiently elsewhere in the genome and that would thus minimize off-target editing.**

(4) Base pairing between the gRNA and the target DNA sequence will result in displacement of the PAM-containing genomic DNA strand to form a single-stranded DNA R-loop, i.e., an editing window, that exposes the target adenosine nucleotide to the deaminase activity of the ABE (**Figure 3**).

(5) Deamination of adenosine will produce inosine, which is read as guanosine by DNA polymerase during DNA repair.

(6) To increase efficiency of the DNA repair process, the Cas9 domain will nick the unedited DNA strand to induce a DNA repair mechanism that uses the edited strand as the template for DNA repair, resulting in an adenine to guanine substitution. For a G>A variant, this substitution on the *PAH* sense strand will revert the variant to wild-type. For a C>T variant, this substitution on the *PAH* antisense strand will result in a thymine to cytosine substitution on the sense strand, reverting the variant to wild-type.

(7) Corrected alleles will produce functional PAH protein, which will convert Phe to Try and would be expected to reduce Phe levels in the blood.

Rationale for clinical trial design

As the study design and endpoints would be identical for an individual trial of any of the versions of the LNP1.PAH.ABE1 DP, regardless of the *PAH* variant targeted for correction, the Sponsor is proposing an umbrella clinical trial design (Woodcock and LaVange, 2017) with the LNP1.PAH.ABE1 DP. Specifically, the Sponsor's proposed Phase I/II first-in-human (FIH) clinical study plans to enroll PKU patients with at least one copy of one of the targetable *PAH* variants (P281L, R408W[redacted]) and with elevated blood Phe levels (i.e., ≥600 µmol/L) in an umbrella trial design. The proposed design is provided in **Section 12, Clinical Program Overview**.

Status of Product Development

The Sponsor has established that certain combinations of an ABE and a gRNA can efficiently and specifically correct each of the *PAH* variants (P281L, R408W[redacted]) to wild-type in human hepatocytes *in vitro* and, in some cases, variant-humanized mice *in vivo*. A description of the studies performed to date are provided in this section, below. The design of the proposed IND-enabling studies, including the definitive pharmacology/proof-of-concept (POC), biodistribution, and toxicology animal studies and the off-target analyses, are provided in **Section 10, Proposed Nonclinical Studies**. The planned chemistry, manufacturing, and controls (CMC) are described in **Section 11, Chemistry, Manufacturing, and Controls**. The proposed clinical study design is described in **Section 12, Clinical Program Overview.**

Adenine base editor and guide RNA screening in cell models for six* PAH *variants causative of classic phenylketonuria

The most frequently recurrent *PAH* variants in classic PKU (Hillert et al., 2020) are indicated in **Figure 4A**. The Sponsor initially focused on: (1) the P281L variant, because of the availability of candidate gRNAs with PAM sequences matching the NGG motif (where N is any nucleotide) that is used by *Streptococcus pyogenes* Cas9 (SpCas9) (**Figure 4B**); and (2) the R408W variant, because it is the single-most frequently recurrent *PAH* variant (**Figure 4C**).

A challenge in developing corrective base editing therapies is the lack of readily available *in vitro* models harboring rare patient-specific variants in which to test the efficacy of drug candidates. Accordingly, the Sponsor generated human hepatocyte cell lines and humanized mouse models each bearing the *PAH* P281L variant or the *PAH* R408W variant. Prime editing was used to introduce each variant into HuH-7 human hepatoma cells, a commonly used proxy for human hepatocytes, resulting in a P281L homozygous HuH-7 cell line and a R408W homozygous HuH-7 cell line.

The Sponsor wished to identify optimal ABE/gRNA sets for corrective editing of the *PAH* P281L variant or the *PAH* R408W variant. The homozygous HuH-7 cell lines were used to screen a variety of ABEs in combination with individual candidate gRNAs in plasmid transfection experiments.

For the *PAH* P281L variant, two gRNAs (designated gRNA4 and gRNA5, because the P281L variant adenine is in the fourth or fifth position of the protospacer, respectively) with NGG PAM sequences were tested in combination with various ABEs (**Figures 4B and 5A**) in P281L homozygous HuH-7 cells via **plasmid transfection**. Each of the gRNAs, in combination with an ABE, has an editing window spanning the site of the on-target (variant) adenine but spanning other adenines, which also have the potential to undergo editing to guanine, i.e., bystander editing. Although many ABE/gRNA sets produced a substantial level of the desired corrective editing of the target variant, there was also bystander editing that resulted in nonsynonymous, splice site, or intronic changes (in red in **Figures 4** and **5**). For the *PAH* P281L variant, the most favorable combination of maximal on-target corrective editing and minimal bystander editing (i.e., a few percent of total edited alleles) was produced by ABE8.8 with gRNA5, designated the "**PAH1**" gRNA (Brooks et al., 2023a) (**Figure 5A**).

For the *PAH* R408W variant, there were no gRNAs with NGG PAM sequences that placed the on-target adenine within the ABE editing window (**Figure 4C**). Various ABEs with altered PAM preferences—i.e., containing engineered Cas9 nickases that recognize PAMs other than NGG—were tested in combination with up to six gRNAs (designated gRNA3 through gRNA8, reflecting the positions of the R408W variant adenine in each gRNA's protospacer sequence) with non-NGG PAM sequences in R408W homozygous HuH-7 cells (**Figures 4C and 5B**). Many ABE/gRNA sets produced a substantial level of the desired corrective editing but also had substantial nonsynonymous bystander editing. The most favorable combination of maximal on-target corrective editing and minimal bystander editing was produced by SpRY-ABE8.8 (Walton et al. 2020) with gRNA5, designated the "**PAH4**" gRNA (Brooks et al., 2024) (**Figure 5**).

[Please note that the "**PAH4**" gRNA, which corrects the *PAH* R408W variant, was so-named for historical reasons, and it is distinct from the **PAH-004** gRNA DS, which corrects the *PAH* [redacted] variant. ("**PAH4**" is analogous to the **PAH-002** gRNA DS, both correcting the R408W variant.) The "**PAH1**" gRNA and **PAH-001** gRNA DS both correct the *PAH* P281L variant.]

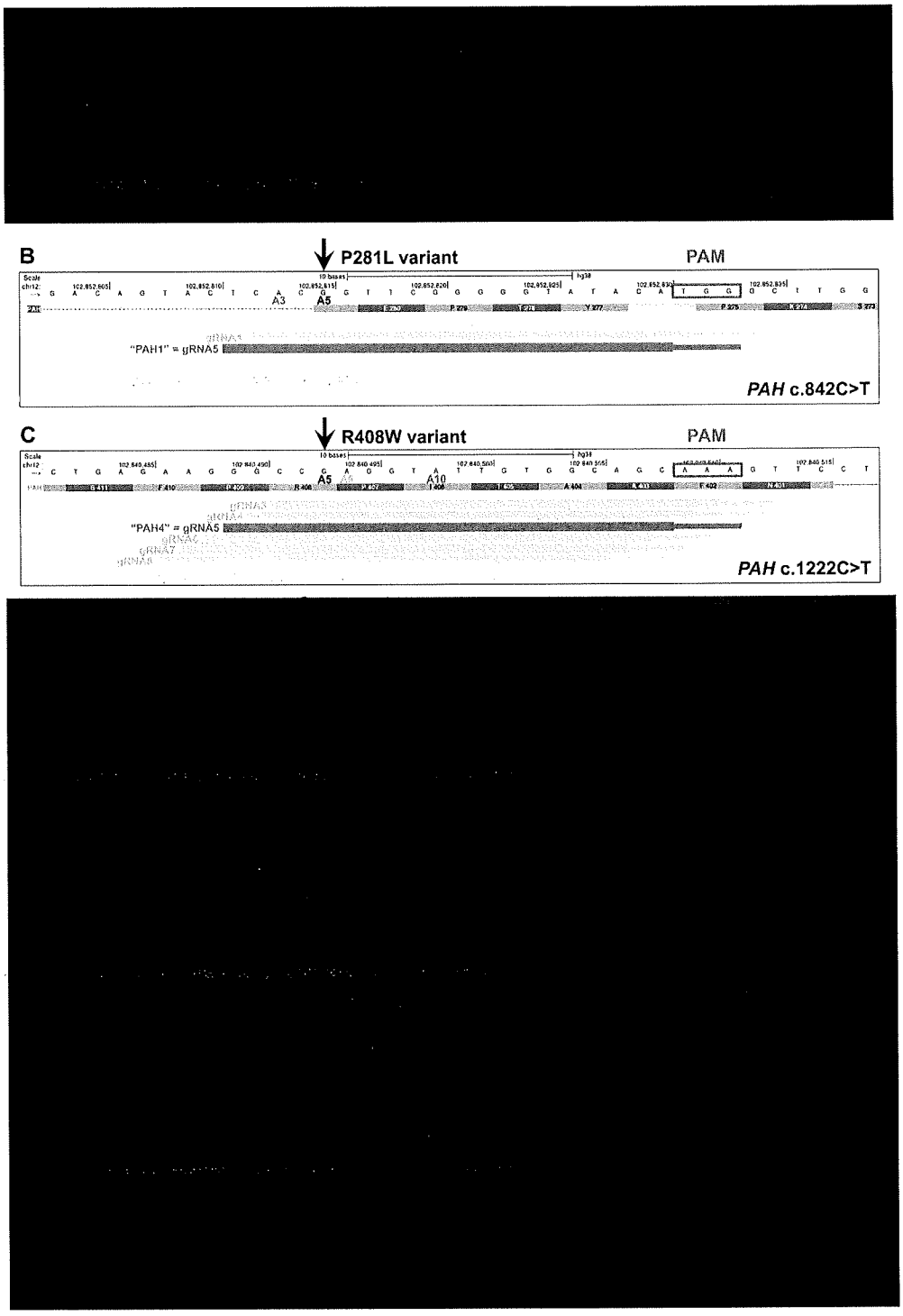


Figure 4. Target *PAH* loci. (A) A schematic of the genomic *PAH* locus with the locations of the most frequently recurrent classic PKU variants (allele frequencies in PKU patients indicated in parentheses), with the DP targets indicated by blue boxes. **(B–G)** Schematics of the genomic sites of *PAH* variants, adapted from the UCSC Genome Browser (GRCh38/hg38). In each schematic, the on-target variant adenine is indicated with a black arrow and black text, nearby potential bystander adenines causing nonsynonymous/splice site/intronic changes with red text, and nearby potential bystander adenines causing synonymous changes with grey text. The protospacer sequences of the lead gRNAs used in the DP are indicated with thick blue bars and PAM sequences with thin blue bars. Adenine positions are numbered according to the lead gRNA protospacer.

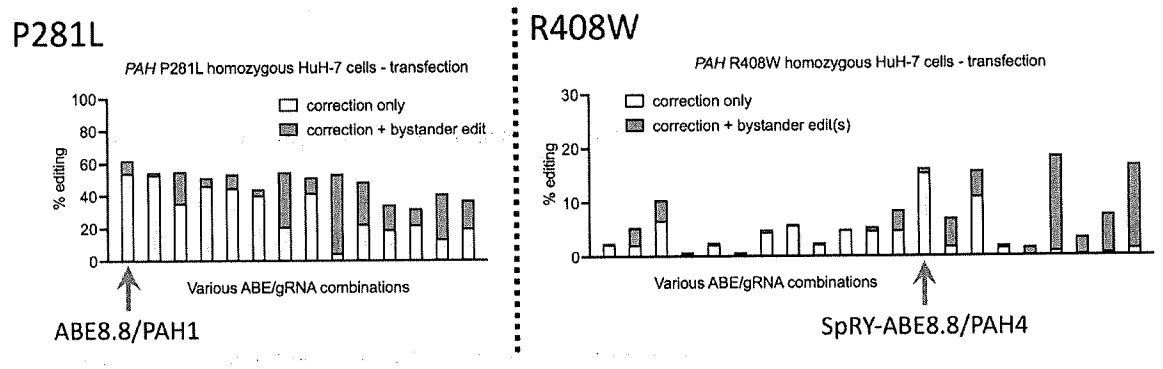


Figure 5. Screening for editing of P281L and R408W variants. On-target corrective editing and bystander editing [by next-generation sequencing (NGS)] by ABE/gRNA sets via plasmid transfection in homozygous variant HuH-7 cells. The candidates indicated by the red arrows had the most favorable combinations of maximal on-target corrective editing and minimal bystander nonsynonymous/splice site editing.

Of note, because the *PAH* P281L and R408W variants are severe loss-of-function alleles, bystander editing would not further reduce PAH function and would not worsen disease beyond a patient's baseline condition. Based on the data above and the data shown below, it is anticipated that at most only a small percentage of total edited alleles in treated patients will harbor bystander edits.

Although the Sponsor was able to generate P281L and R408W homozygous HuH-7 cell lines with prime editing, the Sponsor has found that prime editing does **not** reliably allow for generation of such cell lines for all variants. Moreover, a disadvantage of using clonal cell lines derived from single cells of a transformed cell line like HuH-7 is that there can be substantial heterogeneity among different clonal cell lines in various characteristics, including transfectability with either plasmids or LNPs. Thus, the ability to make head-to-head comparative assessments of editing efficiencies of different variants, across different cell lines (e.g., ABE8.8/PAH1 for P281L versus SpRY-ABE8.8/PAH4 for R408W in the two different homozygous HuH-7 cell lines), is compromised. Accordingly, the Sponsor has adopted an alternative approach in which multiple variants are introduced into the same HuH-7 cells. This is achieved using a lentiviral vector with genomic sequences spanning individual variants. For example, the lentiviral vector might have a ≈100-bp *PAH* genomic sequence spanning the P281L variant, a ≈100-bp *PAH* genomic sequence spanning the R408W variant, and ≈100-bp *PAH* genomic sequences spanning third, fourth, fifth, and sixth variants (**Figure 6**); the lentivirus is used to transduce HuH-7 cells.

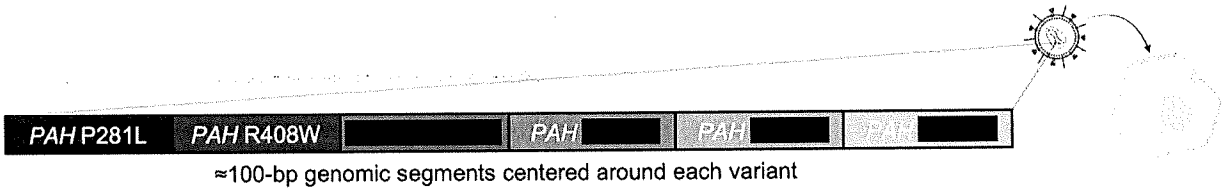


Figure 6. Lentivirus-transduced HuH-7 cell line. Schematic showing how a lentiviral vector is used to transduce human HuH-7 hepatoma cells with a cassette comprising six adjacent 100-bp genomic segments with six *PAH* variants, with two of the variants (P281L and R408W) serving as reference controls.

[redacted]

Using a lentivirus-transduced HuH-7 cell line, the Sponsor was able to directly compare editing efficiencies of the *PAH* P281L, R408W, and [redacted] variants (**Figure 7**). With **plasmid transfection** of ABE8.8/PAH1 for P281L, SpRY-ABE8.8/PAH4 for R408W, and a variety of candidate ABE/gRNA sets for [redacted], the Sponsor found that the most favorable combination of maximal on-target corrective editing and minimal bystander editing for [redacted]

[redacted]

[redacted] was produced by [redacted] with similar efficiency to ABE8.8/PAH1 for corrective editing of P281L, and higher efficiency than SpRY-ABE8.8/PAH4 for corrective editing of R408W. [redacted]

The Sponsor performed similar transfection-based analyses for the *PAH* [redacted], [redacted], and [redacted] variants and identified optimal combinations of SpRY-ABE8.8 and gRNAs for each variant (data not shown) that had intermediate editing efficiency between the efficiency of ABE8.8/PAH1 for corrective editing of P281L and the efficiency of SpRY-ABE8.8/PAH4 for corrective editing of R408W. Because SpRY-ABE8.8 was the common ABE showing favorable corrective editing efficiency for the *PAH* R408W, [redacted], [redacted], [redacted], and [redacted] variants, the Sponsor then performed analyses in multiple lentivirus-transduced HuH-7 cell lines, co-transfecting ***in vitro* transcribed** SpRY-ABE8.8 mRNA and chemically **synthesized** versions of the best-performing gRNAs (i.e., **RNA transfection**) for each of the aforementioned variants, as well as for additional *PAH* variants with non-efficient SpRY-ABE8.8/gRNA sets (**Figure 8**).

In parallel, the Sponsor has found that substituting DNA nucleotides for RNA nucleotides in certain empirically determined positions in the spacer sequence of a gRNA ("hybrid" gRNA) can (1) substantially reduce or even eliminate detectable off-target editing, (2) reduce bystander editing at the on-target site, and (3) increase the desired on-target corrective editing, both *in vitro* and *in vivo* (Whittaker et al., 2025). For example, the combination of ABE8.8 mRNA and "standard" PAH1 gRNA was found to have 7 verified sites of off-target editing; the hybrid configuration of the PAH1 gRNA, "hyb24", in which spacer positions 3, 4, 5, 9, 10, and 11 are substituted with DNA nucleotides, eliminated the off-target editing at all 7 sites (**Figure 9**). [As described in the next subsection, the PAH1_hyb24 gRNA also eliminated off-target editing *in vivo* in the liver in humanized mice, as well as reducing bystander editing and increasing on-target corrective editing of the *PAH* P281L variant (see **Figure 17**).]

[redacted]

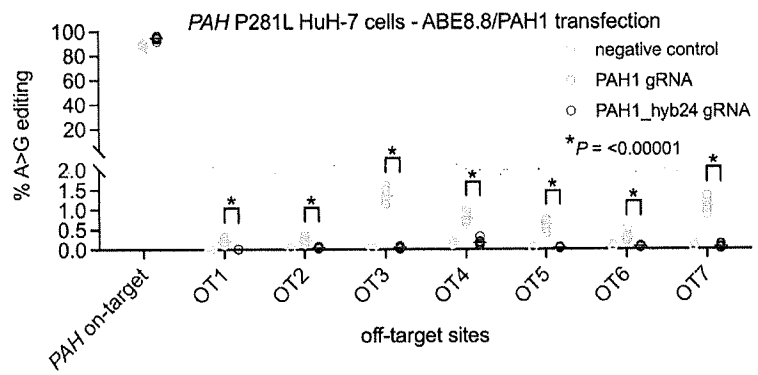


Figure 9. Reduction of off-target editing by hybrid gRNA. Total A-to-G editing (by NGS) of *PAH* P281L on-target site or any of seven verified PAH1 human off-target sites in untreated vs. treated *PAH* P281L homozygous HuH-7 cells with ABE8.8 mRNA in combination with standard PAH1 gRNA or PAH1_hyb24 gRNA (n = 11–12 biological replicates per condition).

[redacted]

Based on these data, the Sponsor has provisionally chosen the following configurations for the gRNA DSs to be used in the LNP1.PAH.ABE1 DP (correlating with the sequences shown in **Table 1** and the schematics shown in **Figure 4**):

- P281L variant: **PAH-001** = [redacted]
- R408W variant: **PAH-002** = [redacted]
- [redacted] variant: **PAH-003** = [redacted]
- [redacted] variant: **PAH-004** = [redacted]
- [redacted] variant: **PAH-005** = [redacted]
- [redacted] variant: **PAH-006** = [redacted]

(Of note, the [redacted] variant is **not** being included in the initial set of *PAH* variants proposed for treatment with the LNP1.PAH.ABE1 DP, due to its being a partial loss-of-function variant that is responsive to sapropterin treatment—i.e., not in the same class as the other variants, which are full loss-of-function of variants that are not responsive to sapropterin treatment.)

Generation and validation of clinically relevant phenotypes of humanized mouse models

The Sponsor wished to generate humanized PKU mice with the *PAH* P281L variant or the *PAH* R408W variant so that the lead ABE/gRNA sets and, ultimately, **the investigational DP can be directly tested *in vivo***, rather than using mouse-specific surrogate products.

The Sponsor generated a humanized PKU mouse model in the C57BL/6 background in which the *PAH* P281L variant was introduced into the orthologous position in endogenous mouse *Pah* exon 7, with the surrounding sequence also replaced by human sequence in order to match the

PAH1 protospacer/PAM sequences. Specifically, this mouse model was generated using *in vitro* transcribed Cas9 mRNA, a synthetic gRNA (spacer sequence 5'-UAGCUGAAGAAUGAUACU UA-3') (Integrated DNA Technologies), and a synthetic single-strand DNA oligonucleotide (Integrated DNA Technologies) with homology arms matching the target site and harboring the P281L variant and synonymous variants (bold with underline): 5'-TGCTGGCTTACTGTCGTC TCGAGATTTCTTGGGTGGCCTGGCCTTCCGAGTCTTCCACTGCACACAGTACATTAG GCATGGATCTAAGCCCATGTA**T**AC**C**CC**C**GAAC**T**GT**G**A**G**TATCATTCTTCAGCTACCC CTGCCAACCACAATGGATGCTCAAAGAATGCTGATCAGGCTCATTGCAGGCTGGTCC CCATGATCCAC-3'. The mixture of the 3 components was injected into cytoplasm of fertilized oocytes from C57BL/6J mice at the Penn Vet Transgenic Mouse Core (https://www.vet.upenn.edu/research/core-resources-facilities/transgenic-mouse-core). Genomic DNA samples from founders were screened for knock-in of the desired sequence in the *Pah* locus via homology-directed repair (**Figure 11**). Founders with the humanized P281L allele were bred through two generations to obtain homozygous mice (PAH^{P281L}/PAH^{P281L} mice).

The Sponsor also generated a humanized PKU mouse model in the C57BL/6 background in which the *PAH* R408W variant was introduced into the orthologous position in endogenous mouse *Pah* exon 12, with the surrounding sequence also replaced by human sequence in order to match the PAH4 protospacer/PAM sequences. Specifically, this mouse model was generated using *in vitro* transcribed Cas9 mRNA, a synthetic gRNA (spacer sequence 5'-AGCGAACGGA GAAGGGCCGG-3') (Synthego), and a synthetic single-strand DNA oligonucleotide (Integrated DNA Technologies) with homology arms matching the target site and harboring the R408W variant and synonymous variants (bold with underline): 5'-AAAAGC CACTTGGAACTCCTCCAG GATAACCTGTCTTTAAAT GGTGTCCTTCACTGGGGT CCTTGGTTTTGGTTTCAG GA**AC**TTTGCTGCCACAAT **A**CC**TT**GGCCCTTCTC**A**GTT CGCTA**C**GACCCCTACACT CAAAGGGTTGAGGTCCTG GACAATACTCAGCAGTTG AAGATTTTAGCTGACTCC ATTAATAGTAAGT-3'. The mixture of the 3 components was injected into cytoplasm of fertilized oocytes from C57BL/6J mice at the Penn Vet Transgenic Mouse Core. Genomic DNA samples from founders were screened for knock-in of the desired sequence in the *Pah* locus via

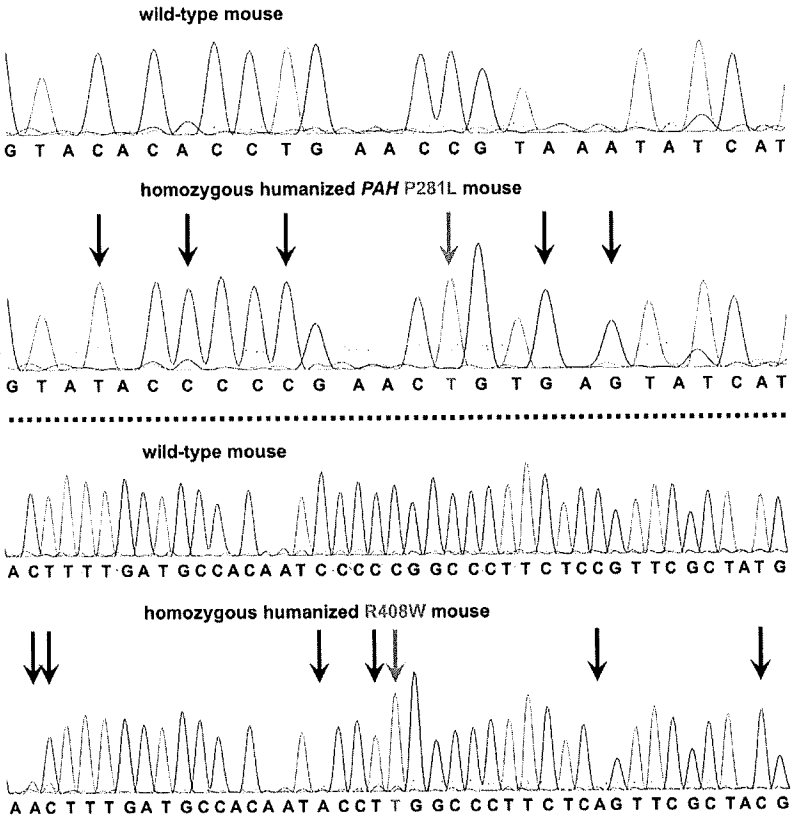


Figure 11. Generation of humanized PKU mice. Sanger sequencing chromatograms showing the humanized *PAH* P281L allele and the humanized *PAH* R408W allele in the respective knock-in mice.

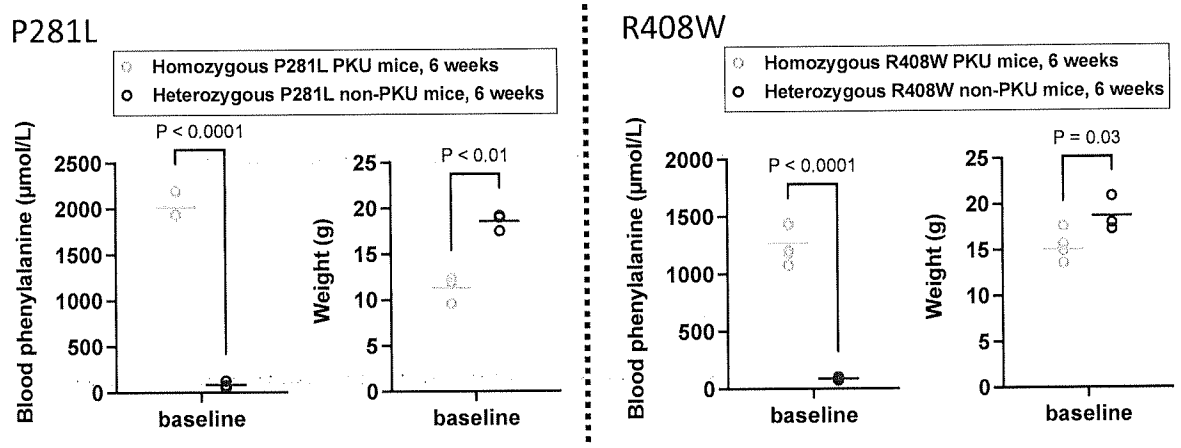


Figure 12. Elevated blood Phe levels and reduced body weight in humanized PKU mice. Shown are data for mice with the *PAH* P281L variant or the *PAH* R408W variant prior to any treatment.

homology-directed repair (**Figure 11**). Founders with the humanized R408W allele were bred through two generations to obtain homozygous mice (PAH^{R408W}/PAH^{R408W} mice) or bred with humanized *PAH* P281L mice to obtain compound heterozygous mice (PAH^{P281L}/PAH^{R408W} mice).

PAH^{P281L}/PAH^{P281L} mice have three phenotypes consistent with human PKU disease that emerge within several weeks of birth (**Figures 12 and 13**) and have lifespans of 1-2 years:

(1) Highly elevated blood Phe levels (typically in the 1500-2000 µmol/L range)

(2) Hypopigmentation of the fur (from reduced melanin synthesis due to decreased Tyr levels from loss of PAH function)

(3) Reduced weight

The same phenotypes previously have been well documented in the best established, most widely used PKU mouse model, the Pah^{enu2} model (e.g., Villiger et al., 2018).

PAH^{R408W}/PAH^{R408W} mice have the same three phenotypes that emerge within several weeks of birth (**Figures 12 and 13**), and they have lifespans of 1-2 years:

(1) Highly elevated blood Phe levels (typically in the 1000-1500 µmol/L range)

(2) Hypopigmentation of the fur

(3) Reduced weight

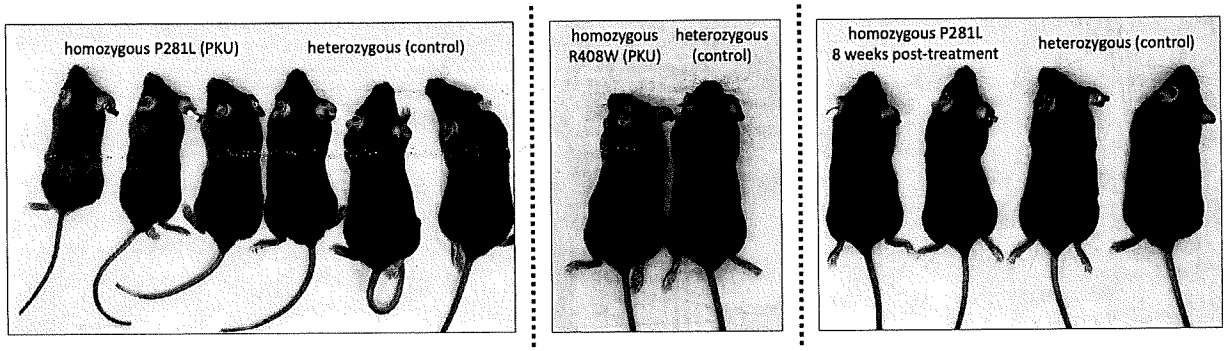
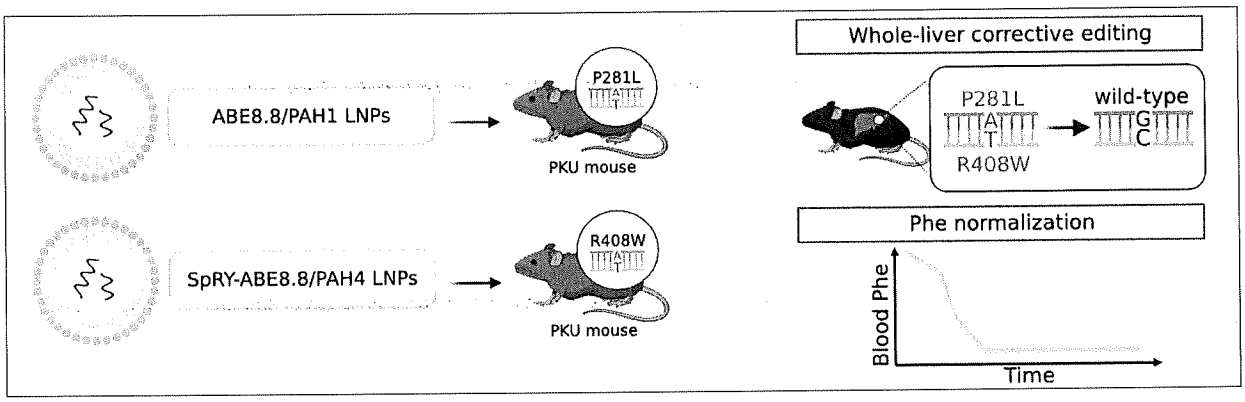
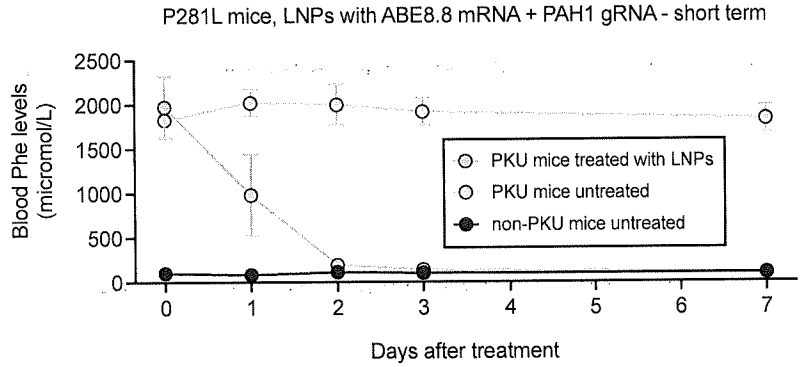


Figure 13. Hypopigmentation of fur in humanized PKU mice. Shown are mice with the *PAH* P281L variant (left) or *PAH* R408W variant (middle), and reversion of the phenotype after LNP base editing treatment (right).



P281L



R408W

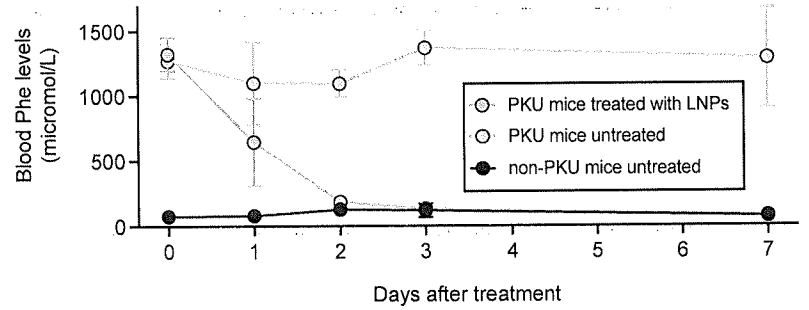


Figure 14. Rapid normalization of blood Phe levels in humanized PKU mice. Shown are data for mice with the *PAH* P281L variant or the *PAH* R408W variant following treatment (ABE8.8/PAH1 LNPs for P281L, SpRY-ABE8.8/PAH4 LNPs for R408W), after single 2.5 mg/kg LNP dose at ≈8 weeks of age.

Thus, the Sponsor has developed **disease-relevant animal models** for preclinical *in vivo* studies.

Proof-of-concept* in vivo *studies in humanized mouse models

The Sponsor next demonstrated clinically relevant *in vivo* activity by the lead ABE/gRNA sets, performing the following experiments (Brooks et al., 2023a; Brooks et al., 2024):

- The Sponsor formulated LNP test articles [redacted] with either ABE8.8 mRNA in combination with PAH1 gRNA, or SpRY-ABE8.8 mRNA in combination with PAH4 gRNA. [redacted]

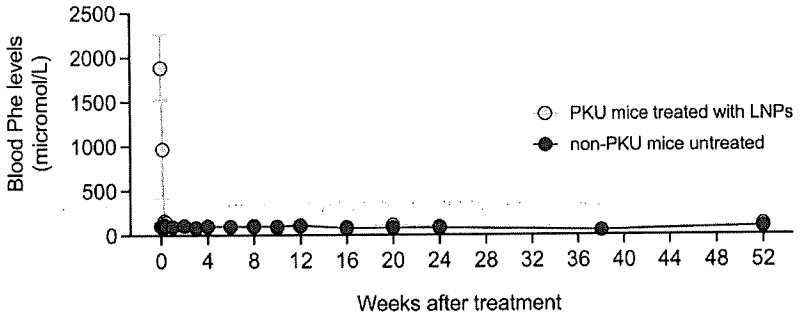


Figure 15. Long-term normalization of blood Phe levels in homozygous PKU mice. Shown are data out to 1 year for mice with the *PAH* P281L variant, after single 2.5 mg/kg ABE8.8/PAH1 LNP dose at ≈8 weeks of age.

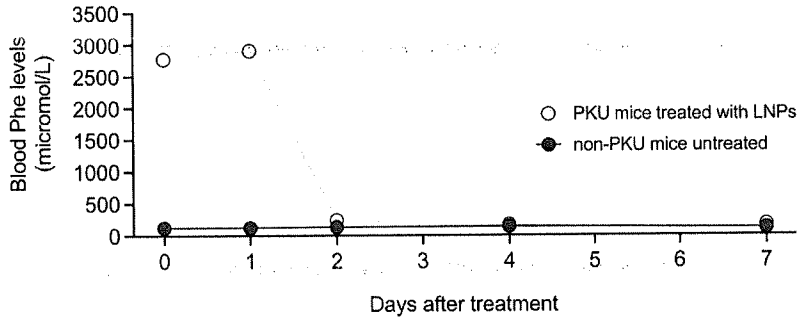


Figure 16. Rapid normalization of blood Phe levels in compound heterozygous PKU mice. Shown are data for mice with 1 copy of the *PAH* P281L variant, after single 2.5 mg/kg ABE8.8/PAH1 LNP dose at ≈4 weeks of age.

- Homozygous P281L mice were treated with ABE8.8/PAH1 LNP test articles, administered via IV injection at a 2.5 mg/kg dose. The elevated blood Phe levels were normalized within 48 hours after treatment, with levels indistinguishable from those of heterozygous littermates by 1 week after treatment (**Figure 14**). There was mean 40% whole-liver corrective editing of the P281L variant, with 0.8% bystander editing.
- Homozygous R408W mice were treated with SpRY-ABE8.8/PAH4 LNP test articles, administered via IV injection at a 2.5 mg/kg dose. The elevated blood Phe levels were normalized within 48 hours of treatment, with levels indistinguishable from those of heterozygous littermates by 1 week after treatment (**Figure 14**). There was mean 26% whole-liver corrective editing of the R408W variant, with 2.8% bystander editing.
- In a long-term study, homozygous P281L mice treated with ABE8.8/PAH1 LNPs at a 2.5 mg/kg dose had reversal of hypopigmentation by 8 weeks after treatment (**Figure 12**) and maintained normal blood Phe levels past 12 months (**Figure 15**). The longest survivor passed away at **≈2.7 years of age**, with 62% whole-liver corrective editing on necropsy.
- Compound heterozygous (one humanized P281L allele and one non-P281L loss-of-function allele) mice were treated with ABE8.8/PAH1 LNPs, administered via IV injection at a 2.5 mg/kg dose. The elevated blood Phe levels were normalized within 48 hours of treatment, with levels indistinguishable from those of heterozygous littermates by 1 week after treatment (**Figure 16**). There was mean 39% whole-liver corrective editing of the P281L variant (among the P281L alleles, representing half the total alleles.)
- To evaluate the *in vivo* performance of a hybrid gRNA identified as having reduced off-target editing *in vitro* (see **Figure 9**), homozygous P281L mice were treated with ABE8.8/PAH1_hyb24 LNP test articles, administered via IV injection at a 2.5 mg/kg dose. (LNP test articles with two additional hybrid gRNA configurations, "hyb22" and

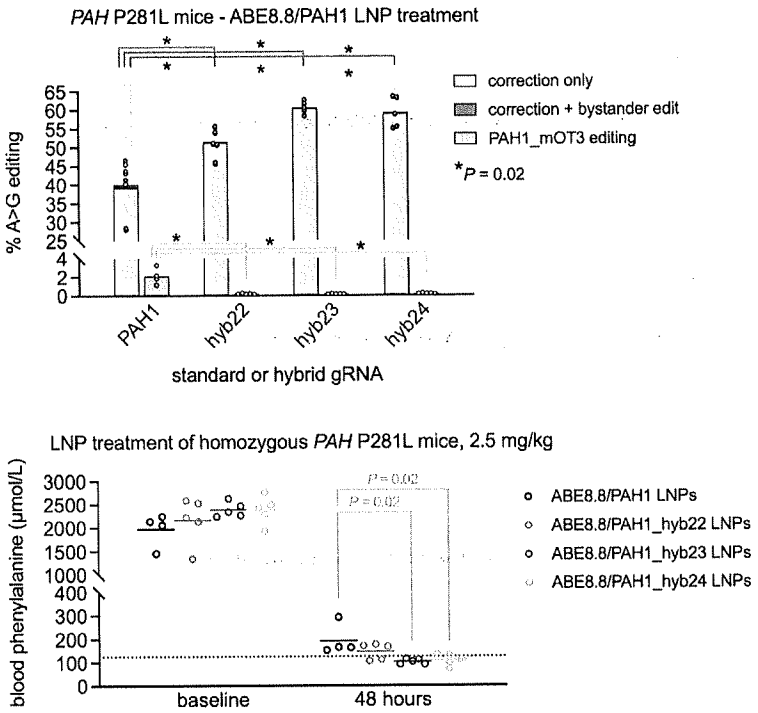


Figure 17. Comparison of treatment of homozygous PKU mice with standard versus hybrid gRNAs. Shown are data from mice with the *PAH* P281L variant, after single 2.5 mg/kg dose of standard ABE8.8/PAH1 LNPs versus ABE8.8/PAH1 LNPs with hybrid gRNAs, with effects on editing outcomes (by NGS) and blood Phe levels. A verified site of off-target editing in the mouse genome (PAH1_mOT3) was assessed along with on-target corrective editing of the P281L variant. Each point in the top graph represents the average editing % from 8 liver samples from a single mouse. The horizontal dotted line in the bottom graph indicates the upper limit of normal Phe levels for healthy individuals.

"hyb23", were used as well.) The elevated blood Phe levels were largely normalized within 48 hours after treatment, with the PAH1_hyb24 gRNA outperforming the standard PAH1 gRNA over that timeframe (**Figure 17**). There was **increased** on-target editing and **decreased** off-target editing with hybrid gRNAs compared to the standard gRNA.

- A dose-response study with ABE8.8/PAH1_hyb22 LNPs in homozygous P281L mice showed that doses as low as 0.25 mg/kg could achieve normalization or near-normalization of blood Phe levels in all treated mice, even when whole-liver corrective editing of the P281L variant was <10% (**Figure 18**).

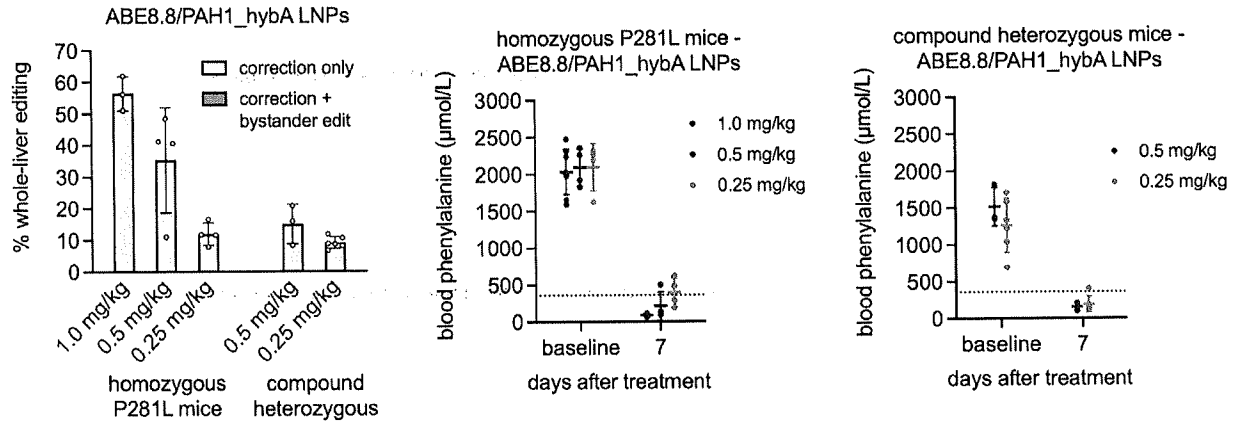
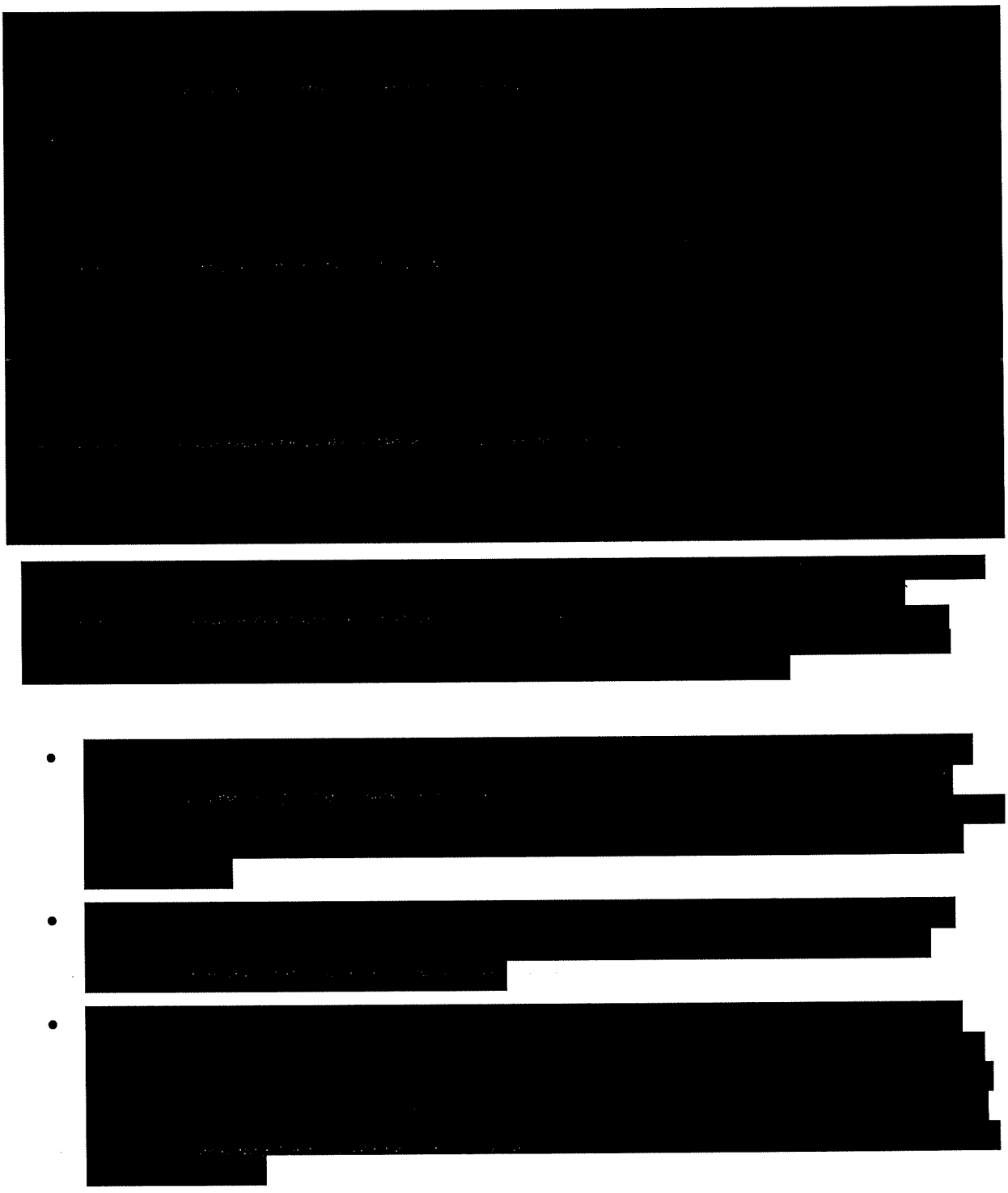


Figure 18. Dose-response editing studies in mice. Shown are data from homozygous PKU mice (P281L variant) or compound heterozygous PKU mice (P281L/R408W variants) and ABE8.8/PAH_hyb22 LNPs (hybA = hyb22). Each point in the left graph is the average editing % (by NGS) in 8 liver samples from 1 mouse. The dotted lines in the middle and right graphs indicate the guidelines-recommended goal Phe level for PKU patients.

[redacted]

- In these experiments, when assessing organs besides liver, the most substantial editing was observed in spleen (few percent), consistent with other studies of *in vivo* LNP-mediated gene editing (data not shown, available in Brooks et al., 2023a).
- In these experiments, post-treatment aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels had, at most, mild transient rises and remained within the normal ranges (data not shown, available in Brooks et al., 2023a and Brooks et al., 2024).
- [redacted]



Thus, the Sponsor's *in vivo* studies establish that ≥10% (and possibly less) corrective whole-liver editing (after subtracting out bystander editing) substantially reduces (by >>50%) and even normalizes blood Phe levels in homozygous PKU mice. Furthermore, ≥20% (and possibly less) corrective whole-liver editing of the targeted allele substantially reduces and even normalizes blood Phe levels in compound heterozygous PKU mice. The necessary degree of editing was achieved with LNP doses as low as 0.25 mg/kg. Normalization of blood Phe levels results in

[redacted]

correction of brain neurotransmitter and metabolite levels, along with some but not all neurobehavioral phenotypes, within several weeks of treatment.

Given that blood Phe reduction is the standard primary endpoint for trials of PKU therapeutics, these results demonstrate that nonclinical precursors of the investigational DP have clinically relevant *in vivo* activity in humanized PKU mice.

Pilot study of intended clinical LNP composition in nonhuman primates

The Sponsor undertook a pilot study of the intended clinical LNP composition for the LNP1.PAH.ABE1 DP in nonhuman primates (NHPs). [redacted]

For the pilot study of this LNP composition, the Sponsor used the ABE8.8 mRNA [redacted] and a gRNA targeting the *PCSK9* gene [redacted], using a published, validated gRNA sequence (Musunuru et al., 2021). The Sponsor formulated LNPs [redacted] and administered them at a 2.5 mg/kg dose to four ≈2-year-old cynomolgus monkeys [redacted]. Over the two-week period following LNP treatment, there were no clinical events, and the liver function tests (blood levels of ALT, AST, alkaline phosphatase, and total bilirubin) had minimal changes (**Figure 23A**). Compared to pre-treatment LDL cholesterol levels, there were reductions in LDL cholesterol ranging from 46% to 58% (**Figure 23B**). After necropsy, whole-liver editing was assessed, with *PCSK9* editing proportions ranging from 42% to 55%, quite concordant with the reductions in LDL cholesterol (Musunuru et al., 2021) (**Figure 24A**). A wide variety of other organs were also assessed to determine the biodistribution of editing (**Figure 24C**). There were low levels of editing in the spleen, adrenal

[redacted]

glands, and skin around the IV infusion site; in all the other organs, any signal was indistinguishable from background. These results are consistent with those of previous studies of LNP biodistribution (Gillmore et al., 2021; Musunuru et al., 2021; Lee et al., 2023).

[redacted]

7. MEETING PURPOSE

The purpose of the meeting is to seek input on the summarized nonclinical proof-of-concept and efficacy data; the proposed definitive animal studies; the proposed assessment of potential off-target editing; the proposed chemistry, manufacturing, and controls; and the proposed clinical study. The objectives of the meeting are to receive advice from the Agency as summarized in the enclosed questions.

8. PROPOSED AGENDA

The Sponsor's proposed agenda is presented below.

Topic	Estimated Duration
Introductions	5 minutes
Discussion of questions	50 minutes
Summary and review of action items	5 minutes

9. LIST OF QUESTIONS, GROUPED BY DISCIPLINE

Nonclinical

Question #1: Does the Agency agree that the completed proof-of-concept (POC) studies of a nonclinical precursor LNP test article, with an identical mRNA (ABE8.8) and gRNA (PAH-001) to those being used in one version of the clinical LNP1.PAH.ABE1 DP, in a mouse disease model with one *PAH* variant (*PAH*^{P281L}/*PAH*^{P281L})—documenting treatment-related effects on blood amino acid levels, PKU-related neurobehavioral changes, and biochemical changes in the brain—provide sufficient data to support an IND application for the administration of any of the six versions of the LNP1.PAH.ABE1 DP to PKU patients?

Question #2: Does the Agency agree that the proposed definitive pharmacology/POC mouse study of the LNP1.PAH.ABE1 DP, with both ABE8.8/PAH-001 and SpRY-ABE8.8/PAH-002, in a compound heterozygous mouse disease model (*PAH*^{P281L}/*PAH*^{R408W}) will provide sufficient data to support an IND application for all six versions of the LNP1.PAH.ABE1 DP?

Question #3: Does the Agency agree that the proposed definitive toxicology rodent study of the LNP1.PAH.ABE1 DP, with both ABE8.8/PAH-001 and SpRY-ABE8.8/PAH-002, in wild-type rats will provide sufficient data to support an IND application for all six versions of the LNP1.PAH.ABE1 DP?

Question #4: Does the Agency agree that the proposed definitive biodistribution/toxicology nonhuman primate (NHP) study of the LNP1.PAH.ABE1 DP, with ABE8.8/PAH-001 only, in wild-type NHPs will provide sufficient data to support an IND application for all six versions of the LNP1.PAH.ABE1 DP?

Question #5: Does the Agency agree that the proposed studies of the LNP1.PAH.ABE1 DP in rodents and NHPs will provide sufficient data to support re-dosing of patients with the LNP1.PAH.ABE1 DP?

Question #6 Does the Agency agree that the proposed off-target editing studies of all six versions of the LNP1.PAH.ABE1 DP will provide sufficient data to support an IND application for the administration of any of the six versions of the LNP1.PAH.ABE1 DP to PKU patients?

Question #7: Does the Agency agree that the overall nonclinical development plan is sufficient to support an IND application for all six versions of the LNP1.PAH.ABE1 DP?

Chemistry, Manufacturing and Controls (CMC)

[redacted]

Question #9: Does the Agency agree that the proposed potency assay for the LNP1.PAH.ABE1 DP is acceptable to support an IND application for all six versions of the LNP1.PAH.ABE1 DP?

Clinical

Question #10: Does the Agency agree that the general design, including the proposed safety and exploratory efficacy outcome measures, enrollment criteria, and long-term follow-up plan are appropriate for the Phase I/II umbrella trial protocol outlined in the protocol synopsis?

10. PROPOSED NONCLINICAL STUDIES

Table 4 summarizes the proposed nonclinical studies to be performed for each version of the LNP1.PAH.ABE DP. Some studies are to be performed only with **one** or **two** versions of the DP, with the intent of using those studies to support the use of **all other** versions of the DP (i.e., a platform approach).

Table 4. Proposed nonclinical studies to support platform use of the LNP1.PAH.ABE DP.

PAH variant	Editor (ABE)	gRNA sequence	Hybrid config.	*In vitro* POC (potency) in HuH-7 cell line	*In vitro* off-target analysis		*In vivo* POC in PKU mice	*In vivo* toxicology in wild-type rats	*In vivo* biodistrib./ toxicology in NHPs
					Nomination	Verification			
c.842C>T (P281L)	ABE8.8	PAH-001	[redacted]	D	D	D, E*	E	E	E
c.1222C>T (R408W)	SpRY-ABE8.8	PAH-002	[redacted]	D	D	D, E*	E	E	Leverage 1 study for platform approach
[redacted]	SpRY-ABE8.8	PAH-003	[redacted]	D	D	D, E*	Leverage 2 studies for platform approach	Leverage 2 studies for platform approach	
[redacted]	SpRY-ABE8.8	PAH-004	[redacted]	D	D	D, E*			
[redacted]	SpRY-ABE8.8	PAH-005	[redacted]	D	D	D, E*			
[redacted]	SpRY-ABE8.8	PAH-006	[redacted]	D	D	D, E*			

DP = drug product; config. = configuration; POC = proof of concept; PKU = phenylketonuria; biodistrib. = biodistribution; NHPs = nonhuman primates; D = development batch of DP; E = engineering batch of DP; * = on verified sites only.

Question #1: Does the Agency agree that the completed proof-of-concept (POC) studies of a nonclinical precursor LNP test article, with an identical mRNA (ABE8.8) and gRNA (PAH-001) to those being used in one version of the clinical LNP1.PAH.ABE1 DP, in a mouse disease model with one *PAH* variant (PAH^{P281L}/PAH^{P281L})—documenting treatment-related effects on blood amino acid levels, PKU-related neurobehavioral changes, and biochemical changes in the brain—provide sufficient data to support an IND application for the administration of any of the six versions of the LNP1.PAH.ABE1 DP to PKU patients?

Sponsor Position: The Sponsor notes the recommendations of the **FDA Guidance for Industry on Human Gene Therapy Products Incorporating Human Genome Editing (2024)**, particularly **Section IV: Considerations for Nonclinical Studies**: "*The animal species and/or models selected for in vivo studies should demonstrate a biological response to the human GE product.*" The Sponsor also notes the feedback from the Agency provided in the Annotated Preliminary Meeting Responses for the INTERACT meeting (PTS #PS008879, Meeting ID #15422, dated March 1, 2024): "Regarding your generated humanized PKU mouse model … that is used in pharmacology proof-of-concept (POC) studies, please provide":

- "A detailed description of how this mouse model was generated." [see **Figure 11**]
- "A summary discussion, along with data and/or supporting references, regarding the biological relevancy of the generated humanized PKU mouse model to the proposed patient population with PKU, including information describing": [see **Figures 12–22**]
    - progression of abnormal phenotypes
    - abnormal biochemistry (e.g., brain phenylalanine [Phe] and tyrosine [Tyr] levels, brain neurotransmitter concentrations [dopamine, norepinephrine, serotonin, and phenylethylamine], brain neurotransmitter metabolites [homovanillic acid [HVA], 3-methoxy-4-hydroxyphenylglycol [MOPEG], and 5-hydroxyindoleacetic acid [5-HIAA]) levels)
    - neurobehavior phenotypes
    - the disease onset and age at the time of product administration
    - life span of the animals
- "Regarding the definitive pharmacology/POC studies in the compound heterozygous mouse disease model":
    - longer-term timepoints to assess durability of the treatment effects
    - general safety (daily observations for mortality, morbidity, weekly body weights, etc.)
    - treatment related effects on PKU related neurobehavioral changes at multiple timepoints in addition to the originally proposed phenylalanine (Phe) and tyrosine (Tyr) levels in the blood
    - (terminal) on-target editing efficiency in the liver
    - (terminal) biochemical changes in the brain (Phe and Tyr levels, neurotransmitter and neurotransmitter metabolites, etc.)
    - (terminal) neuropathology in the brain
    - (terminal) histopathology of the target tissues (liver) and any gross lesions
    - for any unscheduled deaths, clinical pathology, gross pathology and histopathology on a comprehensive list of tissues and other analyses as appropriate to determine cause of death

The Sponsor has incorporated most of the requested readouts in the design of the proposed definitive pharmacology/POC study (#1 listed in **Figure 25** and **Table 5**) using *PAH*^{P281L}/*PAH*^{R408W} mice and engineering/GMP-like batches of the intended clinical LNP1.PAH.ABE1 DP with ABE8.8 mRNA and PAH-001 gRNA (matched to the P281L variant) and with SpRY-ABE8.8 mRNA and PAH-002 gRNA (matched to the R408W variant). However, the Sponsor holds that specific **neurological readouts**—namely, treatment-related effects on PKU-related neurobehavioral changes and biochemical changes in the brain—are entirely tied to **blood Phe levels**, and **not** to the nature of the LNP used for the corrective editing of *PAH* in the liver. [redacted]

Relatedly, the Sponsor holds that because the treatment-related neurological readouts are entirely tied to **reduction of blood Phe levels**, rather than the specific *PAH* variant that is responsible for elevated blood Phe levels, the POC data **already generated** with *PAH*^{P281L}/*PAH*^{P281L} mice are sufficient to support the clinical use of all six versions of the LNP1.PAH.ABE1 DP.

Question #2: Does the Agency agree that the proposed definitive pharmacology/POC mouse study of the LNP1.PAH.ABE1 DP, with both ABE8.8/PAH-001 or SpRY-ABE8.8/PAH-002, in a compound heterozygous mouse disease model (*PAH*^{P281L}/*PAH*^{R408W}) will provide sufficient data to support an IND application for all six versions of the LNP1.PAH.ABE1 DP?

Question #3: Does the Agency agree that the proposed definitive toxicology rodent study of the LNP1.PAH.ABE1 DP, with both ABE8.8/PAH-001 or SpRY-ABE8.8/PAH-002, in wild-type rats will provide sufficient data to support an IND application for all six versions of the LNP1.PAH.ABE1 DP?

Question #4: Does the Agency agree that the proposed definitive biodistribution/toxicology nonhuman primate (NHP) study of the LNP1.PAH.ABE1 DP, with ABE8.8/PAH-001 only, in wild-type NHPs will provide sufficient data to support an IND application for all six versions of the LNP1.PAH.ABE1 DP?

Question #5: Does the Agency agree that the proposed studies of the LNP1.PAH.ABE1 DP in rodents and NHPs will provide sufficient data to support re-dosing of patients with the LNP1.PAH.ABE1 DP?

Sponsor Position: The proposed definitive animal studies (**Figure 25**, **Table 5**, and **Appendix 1 – Definitive Animal Study Synopses**) have been designed specifically in accordance with the recommendations of the **FDA Guidance for Industry on Human Gene Therapy Products Incorporating Human Genome Editing (2024)**, particularly **Section IV: Considerations for Nonclinical Studies.** In light of the Agency's feedback during the INTERACT meeting, in response to one of the Sponsor's Questions, that it would be more appropriate to do a toxicology study in wild-type rodents than in a PKU mouse model, the Sponsor proposes (1) assessment of pharmacodynamics of the LNP1.PAH.ABE1 DP in a non-GLP definitive pharmacology/POC study in a PKU mouse model (#1 in **Figure 25** and **Table 5**) and (2) assessment of toxicology of the DP in a GLP-like definitive study in wild-type rats (#2 in **Figure 25** and **Table 5**).

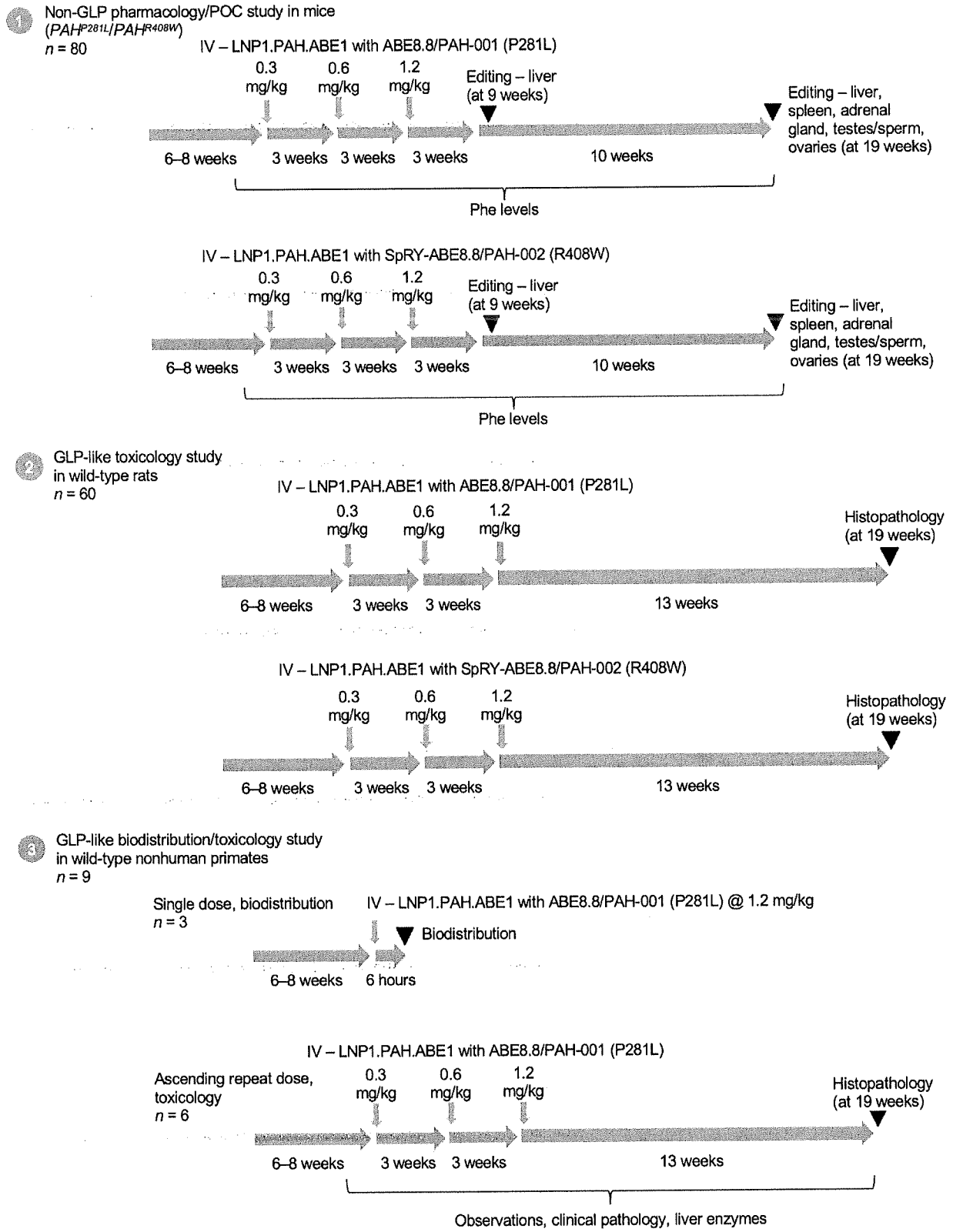


Figure 25. Schematics of proposed definitive animal studies. Green arrows indicate dosing; black arrowheads indicate necropsy.

A more comprehensive summary of the proposed definitive animal studies is available in **Appendix 1 – Definitive Animal Study Synopses**.

Table 5. Proposed definitive animal studies for the LNP1.PAH.ABE1 DP.

Study Design	Dose Groups	Primary Assessments
(1) Non-GLP pharmacology/POC study in compound heterozygous mouse disease model (PAH^{P281L}/PAH^{R408W}), with versions of DP matched to P281L and R408W	Repeat doses separated by 3 weeks—0.3 mg/kg, 0.6 mg/kg, 1.2 mg/kg—or vehicle control with two necropsy groups (9 weeks, 19 weeks) for each DP version; n = 10-20 mice (treated at 6-8 weeks of age) per necropsy group; equal numbers of females and males in each group, otherwise random, blinded assignment	General safety, blood Phe and Tyr levels at various timepoints up to necropsy; editing levels in liver (short-term group) and in organs including liver, spleen, adrenal glands, gonads, etc., as well as sperm isolated from males (long-term group) at necropsy; Phe, Tyr, and neurotransmitter levels in the brain at necropsy; anti-Cas9 antibodies
(2) GLP-like biodistribution/toxicology study in wild-type rats, with versions of DP matched to P281L and R408W	Repeat doses separated by 3 weeks—0.3 mg/kg, 0.6 mg/kg, 1.2 mg/kg—or vehicle control with one necropsy group (19 weeks) for each DP version; n = 20 rats per group (treated at 6-8 weeks of age); equal numbers of females and males in each group, otherwise random, blinded assignment	General safety, clinical observations, and clinical pathology including liver function tests at various timepoints up to necropsy; gross and histological pathology in liver and other organs at necropsy and for unscheduled deaths
(3) GLP-like biodistribution/toxicology study in wild-type NHPs, with version of DP matched to P281L	Single administration, 1.2 mg/kg dose, with one necropsy group (6 hours), or repeat doses separated by 3 weeks—0.3 mg/kg, 0.6 mg/kg, 1.2 mg/kg—or vehicle control with one necropsy group (19 weeks); n = 3 NHPs per group (treated at ≈3 years of age); at least 1 female and 1 male in each group, otherwise random, blinded assignment	General safety, clinical observations, and clinical pathology including liver function tests at various timepoints up to necropsy; lipid excipients and mRNA in blood at various timepoints up to necropsy (long-term group) and in a wide variety of organs at necropsy (short-term group); gross and histological pathology in liver and other organs at necropsy and for unscheduled deaths (long-term group)

Because there are no NHP models of PKU, particularly models with any of the targeted *PAH* variants, studies with wild-type NHPs would have limited utility for assessing on-target editing efficiency of the LNP1.PAH.ABE1 DP. Rather, the use of NHPs will be reserved for a GLP-like biodistribution/toxicology study. The Sponsor holds that biodistribution in NHPs is much more reflective of biodistribution in human patients, compared to biodistribution in rodents, and as such the Sponsor proposes to assess **biodistribution in NHPs only**, not in rodents. Moreover, the biodistribution of editing observed in the pilot NHP study of the intended clinical LNP composition (see **Figure 24C**) is highly concordant with the LNP biodistribution observed in other studies (Gillmore et al., 2021; Musunuru et al., 2021; Lee et al., 2023) and so is highly likely to match the biodistribution in the definitive toxicology NHP study of the LNP1.PAH.ABE1 DP (#3 in **Figure 25** and **Table 5**).

The Sponsor proposes to assess **toxicology in both rodents and NHPs**. The Sponsor holds that performing the proposed definitive **rodent** studies with **two versions** of the LNP1.PAH.ABE1 DP—with the ABE8.8 mRNA and PAH-001 gRNA, matched to the *PAH* P281L variant, and with the SpRY-ABE8.8 mRNA and PAH-002 gRNA, matched to the *PAH* R408W variant—are sufficient to support the IND application for **all six versions** of the LNP1.PAH.ABE1 DP.

The six versions of the LNP1.PAH.ABE1 DP will be nearly identical. All will be formulated in the same way using **identical lipid excipients, which drive the biodistribution and toxicology**. The only distinctions will be in the mRNA and gRNA components. The ABE8.8 and SpRY-ABE8.8 mRNA components, shown in **Table 2**, are extremely similar. The full-length mRNAs, spanning the coding sequence, the 5′ and 3′ untranslated regions, and the 3′ polyadenylate sequence, are identical in length at about 5.1 kilobases. The mRNAs differ within the Cas9 coding portion by 27 bases distributed throughout the Cas9 sequence; the remainder of the sequence, including the adenosine deaminase portion, is identical. Thus, just 27 out of ≈5100 positions in the mRNAs differ (99.5% identity). The difference in the mRNAs is not expected to affect the toxicology of the DP, which will be confirmed in the definitive **rodent** studies.

Across all six versions of the DP, the gRNA components will be identical in the tracrRNA portions (the final 80 nucleotides of the 100-nucleotide RNA molecule), with the differences being in the spacer portions (the first 20 nucleotides). The sequences of the gRNAs are shown in **Table 1**. Even accounting for DNA nucleotide substitutions for RNA nucleotides in order to reduce off-target editing, a maximum of 20 out of 100 positions differ among the gRNAs, which are not expected to affect the toxicology of the DP. This will be confirmed via comparison of the versions of the DP matched to the P281L and R408W variants in the definitive **rodent** studies.

The version of the DP matched to the *PAH* P281L variant, which will use ABE8.8 mRNA and PAH-001 gRNA, will be the only version tested in the definitive **NHP** study. Because minor differences in the mRNA and gRNA components are not expected to affect the toxicology of the DP—which will be confirmed by the definitive **rodent** studies—the Sponsor holds that it is unnecessary to confirm this same observation in the definitive biodistribution/toxicology NHP study by separately testing versions of the DP with the ABE8.8 mRNA and with the SpRY-ABE8.8 mRNA, or with different gRNAs. Furthermore, by testing only a single version of the DP, **the experimental design of the definitive NHP study respects the Replacement, Reduction, and Refinement framework to minimize animal use**.

All the definitive animal studies (#1, #2, and #3 in **Table 5** above) comprise groups that undergo **repeat dosing** with three consecutive treatments with the LNP1.PAH.ABE1 DP, with the doses

separated by 3 weeks, followed by a long-term necropsy endpoint matched with a control group (13 weeks following the third treatment). In each instance, the first dose is 0.3 mg/kg, the second dose is 0.6 mg/kg, and the third dose is 1.2 mg/kg. [redacted] Additional goals of this nonclinical study design are to (1) demonstrate >95% clearance of LNP components [redacted] from the blood within 3 weeks after each dosing, (2) establish that the expected sequelae of LNP treatment, e.g., ALT elevations, are either absent or rapidly self-resolve within the 3-week intervals following each of the three doses, and (3) document that long-term toxicological effects with repeat dosing are absent.

Because (1) the pilot study of the intended clinical LNP composition—effectively a development batch that differed from the ABE8.8/PAH-001 version of the LNP1.PAH.ABE1 DP only with respect to the gRNA, which targeted the *PCSK9* gene instead of the *PAH* P281L variant—has already shown no adverse clinical events and minimal changes in LFTs at a dose of 2.5 mg/kg (see **Figure 23**), [redacted] the Sponsor proposes not to test higher than 1.2 mg/kg in the repeat-dose protocols of the definitive animal studies. Of note, **the Sponsor has previously established the clinical safety of a single-patient ascending dose protocol in the subject of a recent single-patient expanded access IND** (Musunuru et al., 2025).

In regard to specific subsections of the FDA Guidance:

"The use of in vitro models … should be considered for evaluating the activity of a human GE product in the target cell type(s) for genomic modification."

The Sponsor will use lentivirus-transduced HuH-7 cells harboring all six *PAH* variants (P281L, R408W[redacted]), as outlined in **Figure 6**, to assess for on-target editing activity (specific single-nucleotide changes) by the six versions of the LNP1.PAH.ABE1 DP. Based on the results outlined in **Section 6, History of the Project/Background, Status of Product Development**, especially **Figures 5**, **7**, **8**, and **10**, it is expected that the version matched to the P281L variant will demonstrate substantially higher potency than the version matched to the R408W variant, with the versions for the other four variants all demonstrating intermediate potency. As explained above, the Sponsor holds that the *in vivo* data generated in the proposed definitive animal studies for the versions of the LNP1.PAH.ABE1 DP matched to the P281L and R408W variants, in combination with the *in vitro* data generated for the versions matched to the other four variants, are sufficient to support inclusion of the other four variants in the IND application, **without the need for *in vivo* studies of the other four variants**.

The Sponsor wished to assess the potency of the LNP test articles [redacted] used in the initial POC mouse studies described above (**Section 6, History of the Project/Background, Status of Product Development, Figures 12–22**). The Sponsor made use of the P281L homozygous HuH-7 cell line and the R408W homozygous HuH-7 cell line used in the initial base editing screening experiments (see **Figure 5**). In a dose-response study with a

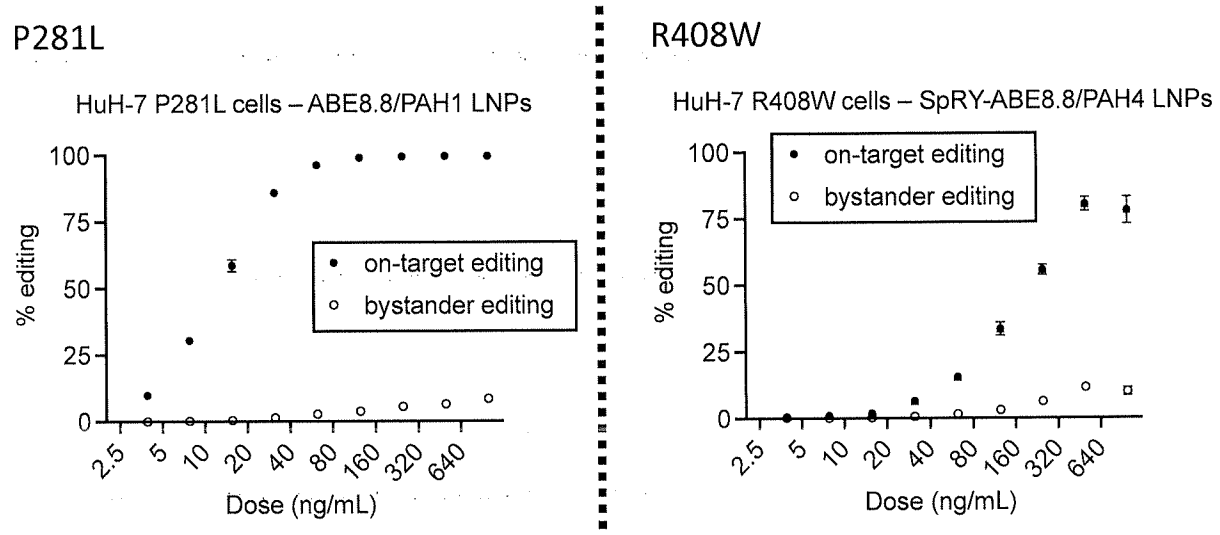


Figure 26. Cellular dose-response experiments with LNPs. On-target editing and bystander editing (by NGS) by lead ABE/gRNA candidates—ABE8.8/PAH1 for the *PAH* P281L variant, SpRY-ABE8.8/PAH4 for the *PAH* R408W variant—via transfection of LNP test articles in homozygous variant HuH-7 cell lines.

ABE8.8/PAH1 LNP test article using the P281L homozygous HuH-7 cell line, there was almost 100% total on-target editing at higher doses, with low levels of bystander editing at the higher doses (**Figure 26**). In a dose-response study with a SpRY-ABE8.8/PAH4 LNP test article using the R408W homozygous HuH-7 cell line, there was ≈80% total on-target editing at higher doses, with modest levels of bystander editing at the higher doses (**Figure 26**).

In principle, these experiments could serve as the basis for a **potency assay** for the LNP1.PAH.ABE1 DP, with the EC_{50} for corrective editing of the target variant [as measured by next-generation sequencing (NGS)] serving as a quantitative measure of potency. However, the use of homozygous variant HuH-7 cell lines for a potency assay has significant limitations. First, the potency assay needs to be able to assess all six versions of the LNP1.PAH.ABE1 DP (i.e., targeting each of the six *PAH* variants). Six distinct homozygous variant HuH-7 cell lines would need to be generated to allow for measurement of corrective editing of each of the targeted *PAH* variants, but it might not be possible to generate all six cell lines via prime editing or another technique (notwithstanding the prior success in generating P281L and R408W homozygous HuH-7 cell lines). Second, being derived from a transformed cell line (HuH-7) with a heterogeneous mix of cells, individual clonal cell lines might be quite different with respect to key characteristics, e.g., transfectability by LNPs. This would make it challenging to compare the potency of a DP batch for correction of one variant, e.g., P281L, to the potency of another DP batch for correction of another variant, e.g., R408W. Third, while it is theoretically feasible to introduce all six variants into the endogenous *PAH* locus to make a pan-homozygous clonal HuH-7 cell line, the technical obstacles to generating such a cell line are formidable.

Accordingly, the Sponsor proposes to instead use the lentivirus-transduced HuH-7 cells harboring all six *PAH* variants (see **Figure 6**) as the basis of the potency assay. (Refer to **Section 11, Chemistry, Manufacturing, and Controls, LNP1.PAH.ABE1 Drug Product, Potency Assay** for a comprehensive description of the proposed potency assay.) Once created, the cell line will be used to establish a Master Cell Bank that would provide a continual supply of cells

for a potency assay that can measure editing of any of the six variants—and, perforce, allow not only for comparisons of potency of multiple batches of the same version of the LNP1.PAH.ABE1 DP, but also for comparisons of potency between different versions of the DP. The latter would allow for determination of whether a particular clinical batch of the DP meets a minimum potency threshold that would make it appropriate for use in the patient dosing scheme laid out in the clinical protocol (**Section 12, Clinical Program Overview**).

"The animal species and/or models selected for in vivo studies should demonstrate a biological response to the human GE product … Given the differences in the genomic sequences between humans and animals, analysis of the biological activity may be done in a species-specific context (e.g., using a surrogate product), as appropriate."

To assess for activity of the LNP1.PAH.ABE1 DP *in vivo*, the Sponsor will use **humanized** PKU mice, which have phenotypes consistent with human PKU disease (**Section 6, History of the Project/Background, Status of Product Development, Figures 12–22**). The Sponsor has demonstrated the humanized mice to have the appropriate biological responses to nonclinical precursors of the LNP1.PAH.ABE1 DP (**Section 6, History of the Project/Background, Status of Product Development, Figures 12–22**). Because the humanized mice have perfect matches to the gRNA spacer sequence in each of the versions of the LNP1.PAH.ABE1 DP matched to the *PAH* P281L variant or *PAH* R408W variant, **no surrogate products are needed**.

The Sponsor will use compound heterozygous mice with one humanized P281L allele and one humanized R408W allele (PAH^{P281L}/PAH^{R408W}) as the primary *in vivo* model for the definitive pharmacology/POC study (#1 in **Figure 25** and **Table 5**). PAH^{P281L}/PAH^{R408W} mice were chosen because (1) most PKU patients are compound heterozygous rather than homozygous for pathogenic *PAH* variants (e.g., see **Figure 2**), and (2) the same model can be used for two versions of the LNP1.PAH.ABE1 DP, matched to the P281L and R408W variants.

"In vivo nonclinical safety studies for a human GE product (or surrogate product) should incorporate elements of the planned clinical trial (e.g., dose level range, ROA, delivery device, dosing schedule, study endpoints, concomitant therapies, etc.), to the extent feasible."

The definitive animal studies (#1, #2, and #3 in **Figure 25** and **Table 5**) have all been designed with the planned clinical trial in mind. [redacted]

"Assessment of biodistribution should be conducted to characterize the distribution, persistence, and clearance of the GE product, any expressed GE components in vivo, editing activity in target and non-target tissues, and the potential for inadvertent germline modification. These evaluations may be conducted independently or in conjunction with POC and/or safety studies."

In prior studies with LNPs, it has been observed that LNPs predominantly distribute to the liver, with minor distribution to the spleen and adrenal glands (Gillmore et al., 2021; Musunuru et al., 2021; Lee et al., 2023; see **Figure 24C**). The Sponsor will assess distribution, persistence, and clearance of GE components in NHPs (study #3 in **Figure 25** and **Table 5**). The Sponsor proposes a tiered approach, wherein lipid excipients [redacted] will be assessed in a broad selection of tissues first, and the expressed GE component, the ABE8.8 mRNA, will then

be assessed only in tissues that are positive for lipid excipients. The Sponsor will also assess distribution of on-target corrective editing (of either the P281L variant or the R408W variant) in selected tissues in compound heterozygous *PAH*^{P281L}/*PAH*^{R408W} mice (study #1 in **Figure 25** and **Table 5**), including liver, spleen, adrenal glands, testes/sperm, and ovaries—the latter to assess for the potential for inadvertent germline modification.

"The intended clinical GE product should be evaluated in the definitive POC and safety studies, as feasible."

Engineering/GMP-like batches of the LNP1.PAH.ABE1 DP, made [redacted] with the intended clinical manufacturing process (as described in **Section 11, Chemistry, Manufacturing, and Controls**) will be used for the definitive animal studies.

"Specificity and efficiency of editing in target and non-target cells."

The definitive pharmacology/POC mouse study (#1 in **Figure 25** and **Table 5**) will assess editing in target cells (liver) and selected non-target cells.

"Functionality of the corrected or expressed gene product (e.g., protein, RNA), if applicable."

The definitive pharmacology/POC mouse study (#1 in **Figure 25** and **Table 5**) will directly assess for rescue of PAH activity via measurement of blood Phe levels, with a reduction of blood Phe levels expected with the correction of either *PAH* variant in *PAH*^{P281L}/*PAH*^{R408W} mice.

"Editing efficiency required to achieve the desired biological activity or therapeutic effect."

The necessary editing threshold (5-10% whole-liver corrective editing) has been established by prior studies (summarized in **Section 6, History of the Project/Background, Status of Product Development**, especially **Figure 18** and **Figure 22**).

"Durability of the genomic modification and resulting biological response."

The long-term durability of corrective editing and normalization of blood Phe levels has been established by prior studies (summarized in **Section 6, History of the Project/Background, Status of Product Development**, especially **Figure 15**) and will be confirmed by the definitive pharmacology/POC mouse study (#1 in **Figure 25** and **Table 5**).

"Assessment of immunogenicity of the GE components and expressed transgene(s)."

The definitive pharmacology/POC mouse study (#1 in **Figure 25** and **Table 5**) will monitor the development of anti-drug antibodies, specifically against the Cas9 component of the ABE.

"Evaluation of the potential for inadvertent germline modification."

The definitive pharmacology/POC mouse study (#1 in **Figure 25** and **Table 5**) will assess editing in the gonads (testes in males, ovaries in females) and will additionally assess editing in sperm isolated from the testes on necropsy. The definitive biodistribution/toxicology NHP study (#3 in **Figure 25** and **Table 5**) will assess lipid excipients [redacted] and the expressed GE component, ABE8.8 mRNA, in the gonads. The Sponsor holds that a germline transmission mouse study with the LNP1.PAH.ABE1 DP is unnecessary if the proposed definitive pharmacology/POC mouse study and biodistribution/toxicology NHP study document a lack of detectable on-target editing and mRNA delivery in gametes, within the limit of detection of available assays.

If the Agency deems it to be necessary for the Sponsor to perform a germline transmission mouse study with the LNP1.PAH.ABE1 DP, the Sponsor proposes that if the study with one version of the LNP1.PAH.ABE1 DP shows no transmission, it would be unnecessary to perform a germline transmission study for any of the other versions of the LNP1.PAH.ABE1 DP. As explained above, all six versions of the LNP1.PAH.ABE1 DP will be nearly identical. All will be formulated in the same way using the same lipid excipients. The only distinctions will be in the extremely similar gRNA and mRNA components. Given the near identity of the gRNA and mRNA components, which are entirely enclosed within the LNPs and are not released until internalization of the LNPs into cells, the distribution of the DP and its components *in vivo* into gametes is not expected to differ, **due to the identical lipid excipients, which drive biodistribution and toxicology**. Moreover, all versions of the DP target the same *PAH* locus, and so there will be no difference in accessibility of the locus to editing in the gametes.

Table 6 summarizes the germline transmission mouse study that would be performed if biodistribution studies document on-target editing and/or mRNA delivery in gametes. The version of the LNP1.PAH.ABE1 DP with ABE8.8/PAH-001, matched to the *PAH* P281L variant, has been chosen for this study, should the study be required. ABE8.8/PAH-001 has high potency compared to the other ABE/gRNA sets being used in other versions of the DP, maximizing the chance of observing germline transmission. Moreover, the homozygous PAH^{P281L}/PAH^{P281L} mouse model will be used, because the presence of two editable P281L alleles (rather than just the one editable P281L allele present in compound heterozygous mice) maximizes the chance of observing germline transmission since all gametes (rather than only half of gametes) will have the P281L allele.

Table 6. Provisional germline transmission study for the LNP1.PAH.ABE1 DP.

Study Design	Dose Groups	Primary Assessments
(4) Non-GLP germline transmission study in homozygous mouse disease model (PAH^{P281L}/PAH^{P281L})	Single administration, 1.2 mg/kg LNP dose, with dosing timed to allow for a full cycle of gametogenesis before mating; numbers of females and males chosen to generate ≈250 viable offspring of LNP-treated female PAH^{P281L}/PAH^{P281L} mice and LNP-treated male PAH^{P281L}/PAH^{P281L} mice	*PAH* genotypes at the site of the P281L variant by NGS of genomic DNA samples of offspring

Of note, both humans and mice experience **maternal PKU syndrome**, in which the health of offspring is adversely affected by elevated maternal blood Phe levels (>360 μM) during pregnancy (Zeile et al., 2018). In mice, maternal PKU syndrome results in the death of offspring by 24 hours after birth. The Sponsor has found that normalization of blood Phe levels in female humanized P281L and R408W PKU mice with LNP-mediated corrective base editing restores the viability and health of their offspring. Accordingly, all female mice used in the germline transmission mouse study will receive treatment with the LNP1.PAH.ABE1 DP with ABE8.8/PAH-001 as part of the study design.

In light of the deleterious consequences of maternal PKU syndrome, the Sponsor proposes to defer any developmental and reproductive toxicology (DART) studies until after the initiation of the Phase I/II clinical study. The risk of harm to the fetus from elevated blood Phe levels (in the case of mice, prenatal or early postnatal death) far outweighs the risk of harm to the fetus from maternal treatment with the DP and reduction/normalization of maternal blood Phe levels.

Question #6 Does the Agency agree that the proposed off-target editing studies of all six versions of the LNP1.PAH.ABE1 DP will provide sufficient data to support an IND application for the administration of any of the six versions of the LNP1.PAH.ABE1 DP to PKU patients?

Sponsor Position: The Sponsor notes the recommendations of the **FDA Guidance for Industry on Human Gene Therapy Products Incorporating Human Genome Editing (2024)** related to off-target editing, contained in **Section IV: Considerations for Nonclinical Studies**.

In regard to specific subsections of the FDA Guidance:

"Identification of on- and off-target editing events, including the type, frequency, and location ... Multiple methods (e.g., in silico, biochemical, cellular-based assays) that include a genome-wide analysis are recommended to reduce bias in identification of potential off-target sites."

The Sponsor is planning to use **three orthogonal methods** to **nominate** candidate sites of **gRNA-dependent off-target editing** for all six versions of the LNP1.PAH.ABE1 DP. Of note, these same methods were used to support a recent single-patient expanded access IND (Musunuru et al., 2025).

The **first nomination method** is Circularization for High-throughput Analysis of Nuclease Genome-wide Effects by sequencing adapted for adenine base editing (CHANGE-seq-BE) (Lazzarotto et al., 2024), a **homology-independent** biochemical assay that provides an **unbiased genome-wide analysis** (**Figure 27**). CHANGE-seq uses Tn5 tagmentation to fragment genomic DNA obtained from cells (e.g., human hepatocytes), followed by circularization of the DNA fragments via intramolecular ligation. After enzymatic degradation of any remaining linear DNA, the circular DNA will be mixed *in vitro* with a ribonucleoprotein (RNP) comprising recombinant ABE protein (ABE8.8 or SpRY-ABE8.8, as appropriate) complexed with the synthetic gRNA (PAH-001 through PAH-006). The RNP will nick certain oligonucleotide sequences on one strand and deaminate an adenine base on the other strand. EndoV will be used to cleave the other strand adjacent to the deaminated base, resulting in the equivalent of a double-strand break that linearizes the circular DNA molecule. After end-repair and adaptor ligation to the ends of linearized DNA molecules, next-generation sequencing (NGS) will identify the sequences that were edited *in vitro* and the frequency of editing, generating a rank-ordered list of candidate (i.e., potential) off-target sites.

[An alternative for the first nomination method is Digenome-seq adapted for adenine base editing (Liang et al., 2019; Kim et al., 2019; Musunuru et al., 2021). Like CHANGE-seq-BE, Digenome-seq is a homology-independent biochemical assay that provides an unbiased genome-wide analysis by *in vitro* mixing of genomic DNA isolated from cells (e.g., human hepatocytes) with an RNP and then EndoV. Deep whole-genome sequencing (WGS) identifies sites with disproportionately high frequencies of double-strand breaks, generating a rank-ordered list of candidate off-target sites.]

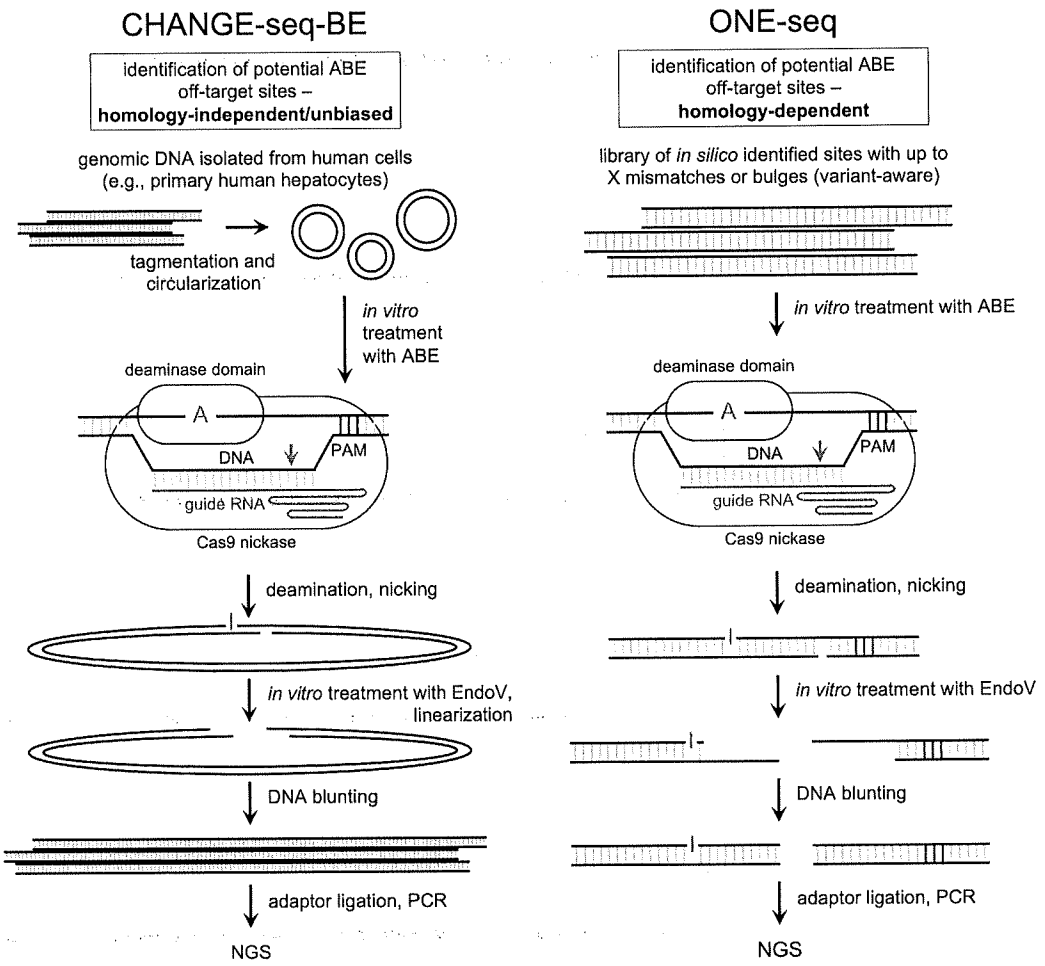


Figure 27. Off-target nomination methods. These methods nominate candidate off-target sites, which are subsequently evaluated to verify whether or not off-target editing genuinely occurs at the sites in target cells.

The **second nomination method** is bioinformatic prediction, which relies on sequence similarity between genomic sites and the protospacer/PAM sequence specified by the ABE/gRNA set (e.g., ABE8.8/PAH-001 or SpRY-ABE8.8/PAH-002). This method uses bioinformatic tools such as Cas-OFFinder and GuideScan (Bae et al., 2014; Perez et al., 2017) to identify sites in the reference human genome with up to three mismatches, or up to two mismatches plus up to one RNA or DNA bulge. These sites will automatically be regarded as candidate (potential) off-target sites.

The **third nomination method** is OligoNucleotide Enrichment and sequencing (ONE-seq), a **homology-dependent** biochemical assay that uses a synthetic human genomic library selected by sequence similarity to the protospacer/PAM sequence specified by the ABE/gRNA (Petri et al., 2021; Musunuru et al., 2021) (**Figure 27**). Thus, ONE-seq is an extension of the second method (bioinformatic prediction). The Sponsor will design a library with sites in the reference genome with up to five mismatches, or up to three mismatches plus up to one RNA or DNA bulge, to the on-target protospacer/PAM sequence. After synthesis by a commercial vendor, the library will be mixed *in vitro* with an RNP comprising recombinant ABE protein complexed with

the synthetic gRNA. The RNP will nick certain oligonucleotide sequences on one strand and deaminate an adenine base on the other strand. EndoV will be used to cleave the other strand adjacent to the deaminated base, resulting in the equivalent of a double-strand break. NGS will then quantify the frequency with which each unique oligonucleotide sequence was edited *in vitro*, generating a rank-ordered list of candidate (potential) off-target sites (typically, the on-target site is at or near the top of the list).

Standard off-target assessment techniques share a critical limitation: each is tied to the specific individual genome represented by the cells or the genomic DNA sample used for analysis. For this reason, most off-target analyses have overlooked the potential for naturally occurring human genetic variation to create novel off-target editing sites in some patients. Furthermore, even if one were to predict that a common or rare genetic variant might create an off-target editing site, it can be challenging to evaluate whether editing actually occurs at that site in the therapeutically relevant cells (e.g., hepatocytes) if there is no way to obtain such cells from a patient with that variant. The Sponsor proposes to use the ONE-seq methodology to empirically identify candidate off-target sites created by genetic variation. **Variant-aware** ONE-seq uses oligonucleotide libraries designed not just using the reference human genome but also incorporating data from the 1000 Genomes Project, the Human Genome Diversity Project, etc., with bioinformatic tools like CRISPRme (Cancellieri et al., 2023).

An example of the use of CHANGE-seq-BE and standard vs. variant-aware ONE-seq with ABE8.8/PAH1, nominating candidate sites for the standard gRNA and for hybrid configurations of the gRNA, is shown in **Figure 28**. The use of hybrid gRNAs substantially reduced the number of candidate sites to be verified, reflecting their reduced off-target propensity (see **Figure 9**).

"Verification of off-target sites should be conducted using methods with adequate sensitivity to detect low frequency events … For in vivo GE products, the analysis should also include the major cell types in which editing events are detected. Appropriate controls should be included to confirm the quality of the assay and to assure interpretability of the results and its suitability for the intended use."

The Sponsor is planning to use **a primary approach** and, if needed, **a secondary approach** to **verify** candidate sites as *bona fide* off-target sites, i.e., sites where off-target editing genuinely occurs in hepatocytes.

The **primary verification approach** is the rhAmpSeq system (Integrated DNA Technologies), which uses highly multiplexed, targeted amplicon sequencing. rhAmpSeq can readily accommodate hundreds or even thousands of candidate sites. Any sites that either (1) are flagged by rhAmpSeq as having off-target editing, or (2) are dropouts with rhAmpSeq, can be reassessed with individual targeted amplicon sequencing (PCR followed by NGS). Regarded as the gold standard, this approach typically has a lower limit of detection of ≈0.1% editing.

[An alternative for the primary verification approach is the hybrid capture methodology. Hybrid capture employs custom-designed probes (synthesized by a commercial vendor) to capture a set of target sequences—in this case, sequences spanning the candidate off-target sites—from genomic DNA samples and to subject them to deep NGS. Like rhAmpSeq, hybrid capture can interrogate hundreds or even thousands of genomic sites in a single assay. As with rhAmpSeq, any sites that either (1) are flagged by hybrid capture as having off-target editing, or (2) are dropouts with hybrid capture, can be reassessed with individual targeted amplicon sequencing.]

The Sponsor proposes to assess off-target editing by the LNP1.PAH.ABE1 DP in three groups of cell types:

- Lentivirus-transduced HuH-7 cell line bearing the targeted *PAH* variant (as shown in **Figure 6**), untreated vs. treated with a supersaturating dose of the LNP1.PAH.ABE1 DP (20× the EC_{90} value calculated from a dose-response study of the DP in the HuH-7 cell line)
- Primary human hepatocytes (PHHs) from at least three donors (obtained via a commercial vendor), untreated vs. treated with a supersaturating dose of the LNP1.PAH.ABE1 DP, with ABE expression confirmed via comparison of treated PHHs to treated lentivirus-transduced HuH-7 cells by quantitative reverse transcription PCR (RT-PCR) of the ABE mRNA
- Additional cultured or primary cell types nominated by the proposed definitive pharmacology/POC mouse study and biodistribution/toxicology NHP study (#1 and #3 in **Figure 25** and **Table 5**), due to substantial on-target editing and/or mRNA delivery, and untreated vs. treated with a supersaturating dose of the LNP1.PAH.ABE1 DP, with ABE expression confirmed with quantitative RT-PCR of the ABE mRNA

The lentivirus-transduced HuH-7 cells have the advantage that they can be used to directly assess for on-target editing efficiency **simultaneously** with off-target editing, confirming that the LNP1.PAH.ABE1 DP has successfully transfected the cells and has exposed them to supersaturating amounts of the mRNA and gRNA components. HuH-7 cells are highly proliferative, reflecting a distinct cellular state from the quiescent PHHs. In all cases, genomic analysis of DP-treated versus untreated cells will be performed three days after DP treatment.

The **secondary verification approach**, termed Lenti-seq, would involve only high-priority candidate off-target sites that are created by human genetic variants and are not present in readily available hepatocytes. HuH-7 cells will be transduced with a lentivirus bearing a concatenated sequence bearing 100-bp fragments spanning (1) the on-target *PAH* variant sequence and (2) each of the high-priority candidate variant off-target sites, like the scheme showed in **Figure 6**. Treatment of these off-target lentivirus-transduced HuH-7 cells with a supersaturating dose of the LNP1.PAH.ABE1 DP will be followed three days later by genomic analysis for on-target corrective editing of the *PAH* variant and for off-target editing in each of the candidate variant off-target sites.

"Assessment of genomic integrity, including chromosomal abnormalities, insertions or deletions, and potential oncogenicity or insertional mutagenesis."

Besides gRNA-dependent off-target editing, base editors have the potential for **gRNA-independent off-target editing** incurred by activity of the TadA deaminase domain independent of the Cas9 component of the ABE. Although ABEs have proven to be relatively inert compared to cytosine base editors, the Sponsor will use two methods to rule out gRNA-independent **DNA editing** and gRNA-independent **RNA editing** by the ABE8.8 TadA deaminase domain. **For the former**, WGS will be performed in PHHs treated with supersaturating amounts of the LNP1.PAH.ABE1 DP, to assess for evidence of genome-wide DNA editing above background levels observed in untreated PHHs. **For the latter**, RNA sequencing (RNA-seq) will be used to assess for evidence of RNA editing (above background levels) in PHHs treated with supersaturating amounts of the LNP1.PAH.ABE1 DP. The Sponsor proposes that this analysis be

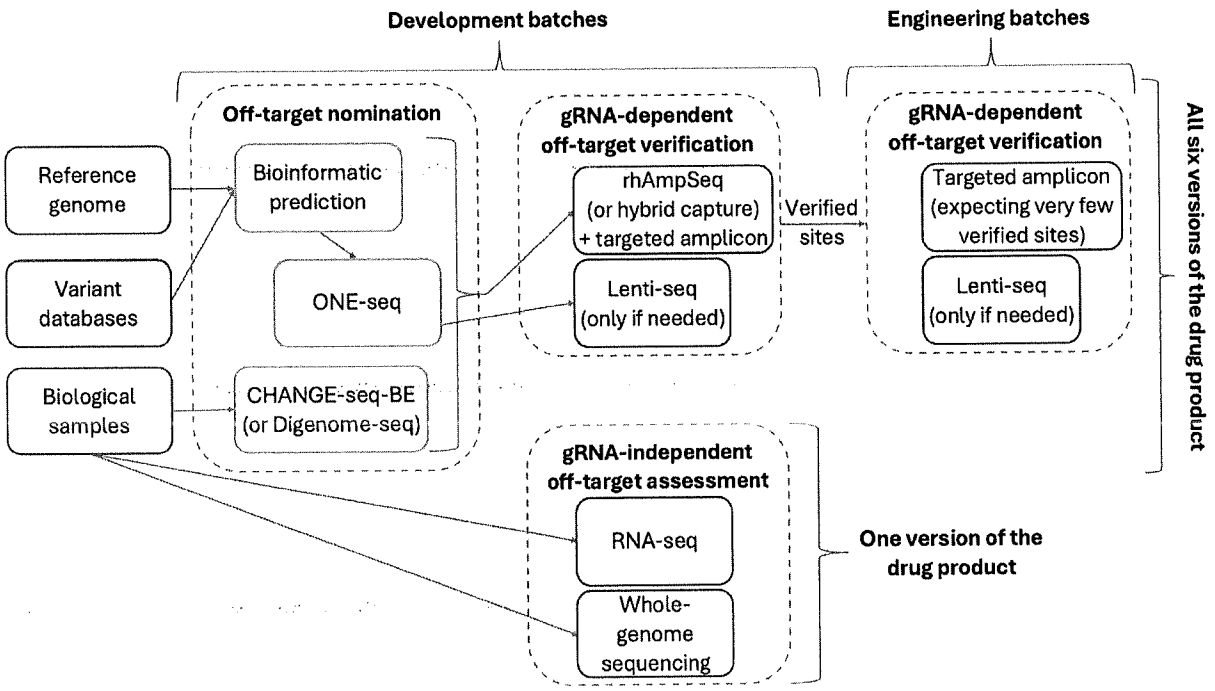


Figure 29. Overview of testing plan for off-target analyses.

performed **with only one version** of the LNP1.PAH.ABE1 DP (e.g., with ABE8.8/PAH-001), since gRNA-dependent off-target editing is determined by the TadA deaminase domain alone (shared by all versions of the DP) and not by PAM preference or gRNA. Based on the negative findings of previous studies of LNP-delivered ABE8.8 mRNA expression in PHHs (Musunuru et al., 2021), the Sponsor does not expect to detect gRNA-independent off-target editing.

Aligned WGS reads from pre- and post-treated PHH genomic DNA will be analyzed with the bioinformatics tool Manta (Chen et al., 2016) to detect rare **structural variants**. Briefly, Manta identifies split reads (i.e., single read that spans a structural variation breakpoint such as in inversions or translocation) to precisely locate the breakpoints of SVs, and then performs local *de novo* assembly of the regions surrounding the breakpoints. Based on the negative findings of previous studies of LNP-delivered ABE8.8 mRNA expression in PHHs, the Sponsor does not expect to detect treatment-related structural variants.

Because there are no DNA elements in the LNP1.PAH.ABE1 DP, there is no concern for insertional mutagenesis.

For the **overall testing plan** (**Figure 29**) the Sponsor proposes to do the CHANGE-seq-BE (or Digenome-seq) and ONE-seq **nomination methods** with **development batches** of the various gRNAs (PAH-001 through PAH-006). **Verification approaches** will initially be undertaken with **development batches** of each of the six versions of the LNP1.PAH.ABE1 DP. Any **verified** sites of off-target editing (which are expected to be very few in number) in any of the tested cell types with a development batch will then be **re-tested** with the **engineering batch** of each of the six versions of the LNP1.PAH.ABE1 DP.

"Evaluation of the biological consequences associated with on- and off-target editing, including, but not limited to, viability and function of the edited cells (e.g., differentiation capacity of progenitor cells)."

Because on-target editing entails the correction of a disease-causing variant to wild-type, it is expected to have only favorable biological effects or neutral effects on the edited cells, even non-target cells.

For any site for which off-target editing by an LNP DP has been **verified** by rhAmpSeq (or hybrid capture) and/or targeted amplicon sequencing, the Sponsor will apply a **risk assessment framework** to assess the biological risk of the edit(s) at the site:

(1) Is the edit in or near a cancer gene, e.g., in the Catalogue of Somatic Mutations in Cancer (COSMIC) database (Tate et al., 2019)?

(2) Does the edit affect a genomic site that is likely to have functional impact: e.g., coding versus non-coding, Ensembl Variant Effect Predictor analysis (McLaren et al., 2016), and Combined Annotation-Dependent Depletion (CADD) score (Rentzsch et al., 2019)?

(3) Is the edit likely to affect gene expression in the target tissue (hepatocytes) or other tissues in which on-target editing is evident?

(4) Do structural variants involving the site of the edit occur?

(5) Is the edit likely to occur at pharmacological doses of an LNP DP administered to patients (rather than the supersaturating doses of an LNP DP used in off-target assays)?

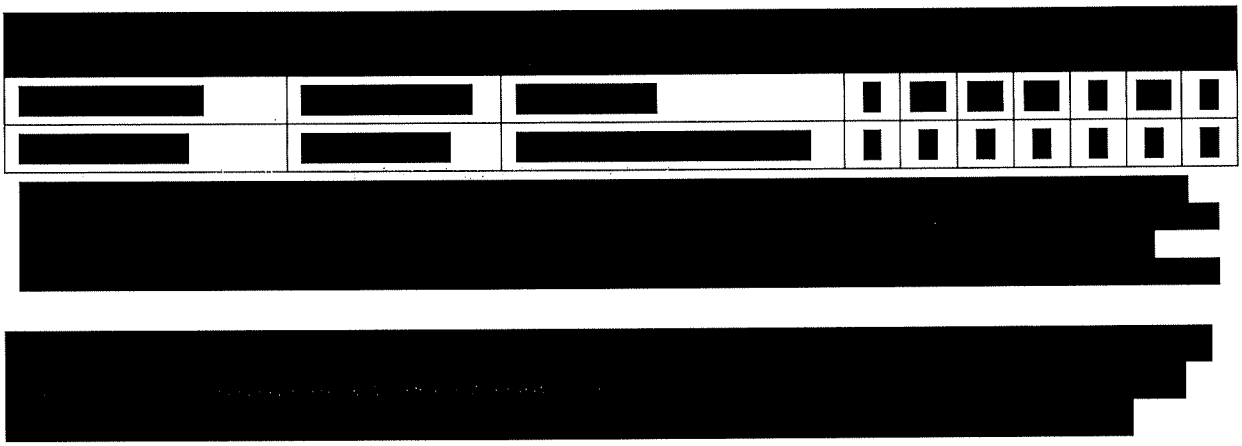
Should any off-target sites be identified in the studies described above with any of the versions of the LNP1.PAH.ABE1 DP, and if a particular off-target edit is deemed to be of high risk by the framework above, the Sponsor will seek guidance from the Agency on the proposed experimental path to address this risk directly.

Additional genotoxicity assessment.

[redacted]

Question #7: Does the Agency agree that the overall nonclinical development plan is sufficient to support an IND application for all six versions of the LNP1.PAH.ABE1 DP?

Sponsor Position: The overall nonclinical development plan is outlined in this section, above, and addresses each relevant recommendation of the **FDA Guidance for Industry on Human Gene Therapy Products Incorporating Human Genome Editing (2024)**.



Potency assay

Question #9: Does the Agency agree that the proposed potency assay for the LNP1.PAH.ABE1 DP is acceptable to support an IND application for all six versions of the LNP1.PAH.ABE1 DP?

Sponsor Position: The Sponsor notes the recommendations of the **FDA Guidance for Potency Tests for Cellular and Gene Therapy Products (2011)** and the recommendations of the **FDA Guidance for Industry on Human Gene Therapy Products Incorporating Human Genome Editing (2024)** related to potency assays, contained in **Section III.B.3.i: In vivo-administered Human Genome Editing Drug Products**, specifically the following points:

- *"For early phase studies, potency assays evaluating the ability of the GE components to perform the desired genetic sequence modification may be adequate."*
- *"We recommend that, whenever possible, the potency assays be performed in the target cells or tissues (or a representative surrogate)."*
- *"We also recommend inclusion of such a potency assay in the DP stability studies."*

The Sponsor proposes an early-phase-appropriate potency assay for the LNP1.PAH.ABE1 DP, in which a lentivirus-transduced HuH-7 cell line harboring all six *PAH* variants (see **Figure 6** for a schematic of this cell line) will be used to assess for the desired genetic sequence modification—namely, the on-target corrective base editing activity (specific single-nucleotide changes) appropriate to each of the six versions of the LNP1.PAH.ABE1 DP, as determined by amplicon-based sequencing, i.e., NGS. The justification for the use of this cell line is provided in **Section 10, Proposed Nonclinical Studies**, especially **Figure 26**.

The mechanism of action of LNP1.PAH.ABE1 is illustrated in **Figure 33**. The Sponsor proposes to assess the potency of the LNP1.PAH.ABE1 DP using the lentivirus-transduced HuH-7 cell line to evaluate two critical stages of the mechanism of action, i.e.:

- initially, the level of expression of the Cas9 component of the ABE, assessed by a quantitative enzyme-linked immunosorbent assay (ELISA); and
- ultimately, the efficiency of corrective base editing at the site of the variant targeted by the LNP1.PAH.ABE1 DP, assessed by amplicon-based sequencing.

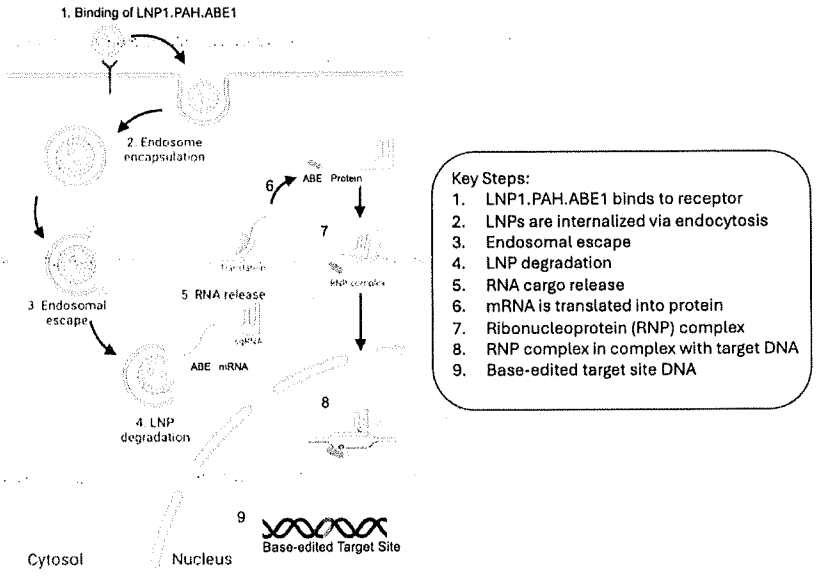


Figure 33. Mechanism of action of the LNP1.PAH.ABE1 LNP. Figure adapted from Lee and Han, 2024.

In the potency assay, lentivirus-transduced HuH-7 cells will be transfected with LNP1.PAH.ABE1 DP samples and, ultimately, the level of corrective base editing measured by amplicon-based sequencing with an eight-point dose range to establish an EC_{50} (see **Figure 26** for examples of this kind of assay). In parallel, the HuH-7 cells will also be transfected with a reference standard, i.e., a previously characterized lot of the LNP1.PAH.ABE1 DP. The editing level will be reported in relation to the reference standard.

For the reference standard, the Sponsor proposes to use a previously characterized lot of the version of the LNP1.PAH.ABE1 DP with SpRY-ABE8.8/PAH-002 (targeting the *PAH* R408W variant) for two reasons. First, this ABE/gRNA set has been validated to have clinical efficacy in a humanized mouse model (see **Figure 14**). Second, this ABE/gRNA set is matched or exceeded in corrective editing efficiency (for their respective targeted *PAH* variants) by the lead ABE/gRNA sets for the other five versions of the LNP1.PAH.ABE1 DP (see **Figure 10**), and so as a reference standard SpRY-ABE8.8/PAH-002 establishes a "baseline" potency that offers assurance that a qualified lot of the LNP1.PAH.ABE1 DP will have acceptable editing activity *in vivo* when administered to a patient in the proposed clinical trial.

The HuH-7 cell-based assay is now being optimized, and for the first proposed clinical trial under this IND, for DP release and stability testing [redacted], the Sponsor proposes to assess potency using a quantitative ELISA to determine Cas9 protein expression. An acceptance criterion for this attribute will be set based on accrued development data and submitted as an attribute in the specification for DP release in the IND.

As soon as the actual corrective base editing efficiency can be reproducibly quantified, and no later than for a trial intended to show proof of clinical efficacy, this attribute will replace the Cas9 ELISA and be implemented for the DP release specification and for DP stability testing with an amendment to the IND.

12. CLINICAL PROGRAM OVERVIEW

Question #10: Does the Agency agree that the general design, including the proposed safety and exploratory efficacy outcome measures, enrollment criteria, and long-term follow-up plan are appropriate for the Phase I/II umbrella trial protocol outlined in the protocol synopsis?

Sponsor Position: The Sponsor notes the recommendations of the **FDA Guidance for Industry on Human Gene Therapy Products Incorporating Human Genome Editing (2024)**, particularly **Section V: Considerations for Clinical Studies**: "*Clinical trial design should include an appropriately-defined patient population, an efficient and safe approach to product administration (including data-based dosing, dose schedule, and treatment plan), adequate safety monitoring, and appropriate safety and efficacy endpoints.*"

In accordance with the Guidance, the Sponsor proposes a phase I/II open-label umbrella clinical trial designed to evaluate the safety and efficacy of LNP1.PAH.ABE1 (**Table 20**). The full clinical protocol and informed consent form will be included in the IND submission.

Table 20. Clinical synopsis.

Study title:	A Phase I/II open-label safety and efficacy study of LNP1.PAH.ABE1, a lipid nanoparticle-delivered base editing therapy, in patients with phenylketonuria (PKU) due to variants amenable to corrective editing by LNP1.PAH.ABE1
Clinical phase:	Phase I/II
Number of subjects:	[redacted]
Study rationale:	To date, there are no one-time, disease-modifying therapies that durably correct neurotoxic blood phenylalanine elevations in adults with classic PKU. The goal of this study is to restore expression of functional PAH enzyme and reduce phenylalanine levels through corrective adenine base editing of any one of 6 pathogenic *PAH* variants including: • c.842C>T (P281L) • c.1222C>T (R408W) • [redacted]
Study objectives:	The primary outcome is safety. The secondary and exploratory outcomes explore efficacy.
Study design:	Open-label clinical trial of LNP1.PAH.ABE1 injected via intravenous (IV) infusion

Study dose:	[redacted]
Inclusion criteria:	[redacted]
Exclusion criteria:	[redacted]
Primary outcome:	[redacted]
Secondary outcome:	[redacted]

Exploratory outcomes:	[redacted]
Immunosuppression medications:	[redacted]
Study duration:	[redacted]
Long-term follow-up:	[redacted]

Genotype arm assignment

LNP1.PAH.ABE1 is designed to treat patients with classic PKU who are homozygous or compound heterozygous for a pathogenic variant targeted by a version of the DP (**Table 21**).

Table 21. Genotype arms.

Genotype arm name	Targeted *PAH* variant
PAH-001	c.842C>T (p.Pro281Leu)
PAH-002	c.1222C>T(p.Arg408Trp)
PAH-003	[redacted]
PAH-004	[redacted]
PAH-005	[redacted]
PAH-006	[redacted]

[redacted]

At screening, all subjects will have confirmatory *PAH* sequencing completed. Once a subject's genotype is confirmed, they will be assigned to the appropriate genotype arm for the target *PAH* variant that they harbor (**Figure 34**). [redacted]

Data safety monitoring board and subject enrollment timeline

A data and safety monitoring board (DSMB) will be created consisting of at least 3 people who are experts in PKU, gene editing therapy, and safety/pharmacovigilance. The DSMB will review safety data from all participants at predetermined intervals and as any concerns arise. [redacted] DSMB meetings will then occur at least quarterly.

[redacted]

Rationale for dose selection and dosing plan

To prioritize the safety of subjects, the dosing plan will begin with a low dose that is still predicted to provide benefit. This dose will be finalized after completion of the proposed definitive biodistribution/toxicology NHP study.

Pending the outcomes of the NHP study, the Sponsor anticipates proposing an initial dose of the LNP1.PAH.ABE1 DP. [redacted]

Rationale for immunosuppression plan

A single dose of IV methylprednisolone will be given at the time of the infusion of the DP to help minimize the risk of infusion reaction. [redacted]

[redacted] This regimen may be adapted as clinically indicated.

Plan for liberalization of dietary protein prescription

[redacted]

13. A LIST OF FDA STAFF ASKED TO PARTICIPATE IN THE REQUESTED MEETING

The participation of members of the Center for Biologics Evaluation and Research is requested.

14. TELECONFERENCE DATE/TIME

[redacted]

15. FORMAT OF THE MEETING

A webinar has been requested and scheduled.

16. REFERENCES

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