High Phenylalanine Concentration Contributes to the Stability of the F263S-variant of Phenylalanine Hydroxylase

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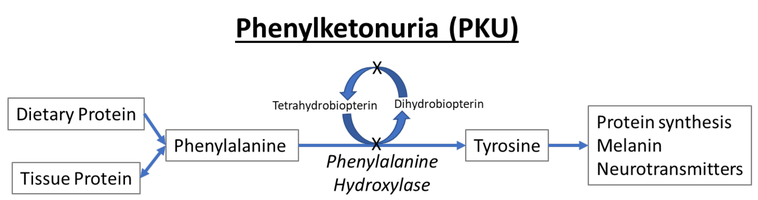
Introduction

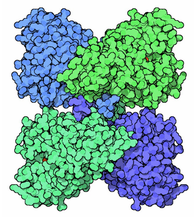
Phenylketonuria (PKU)

- PKU is an autosomal recessive disorder caused by deficiency in the activity of the enzyme phenylalanine hydroxylase (PAH) resulting in phenylalanine (Phe) accumulation and decreased levels of Tyrosine (Tyr), which are associated with phenotypic consequences, including deficient growth, light skin and hair coloration, cognitive deficits, sleep disturbance, psychiatric disorders and seizures.
- Recent reports have described a gene therapy based approach for normalization of blood Phe by expression of PAH in hepatocytes (ACMG 2019, Poster #111 Black, et. al). Such an approach has the potential to achieve sustained reduction of blood Phe levels by restoring PAH activity, allowing physiological conversion of Phe to Tyr and restoration of downstream metabolites (e.g., neurotransmitters).



Phenylalanine Hydroxylase

- PAH protein undergoes multiple stages of oligomerization and changes in tertiary structure under normal physiological conditions, as well as under elevated Phe levels. The active PAH enzyme exists as a tetramer, and can form heterotetramers with mutated PAH subunits due to interallelic complementation.



ENU2 mouse model (F263S mutation)

- The ENU2 mouse model of PKU, first described by Shedlovsky et. al., was created by chemical mutagenesis, using N-ethyl-N–nitrosourea (ENU).[2] ENU2 mice have phenotypes which mimic several of those seen in humans with classical PKU, including high plasma and tissue levels of Phe, low levels of Tyr, small size/body weight, and a light-brown coat color (while wild-type [WT] counterparts are black).
- ENU2 mice contain a mutation in exon 7 of the gene coding for PAH. Phe263 (F263) is replaced by Ser263 (S263), resulting in a mutant PAH protein with no detectable PAH catalytic activity.[2] This mutation is analogous to one found in a large subset of human PKU patients where Phe263 has been mutated to Leu263 (F263L).
- The negative effects of interallelic complementation due to ENU2-PAH were observed in a composite model of ENU1-ENU2 mice. (ENU1 mouse model, generated in a similar manner as ENU2, contains the V106A mutation in PAH protein resulting in mild phenylketonuria).[3]

Impact to gene therapy

- Herein, we attempted to study the effect of interallelic complementation between ENU2-PAH and gene-therapy-derived native PAH in hepatocytes. We describe a serendipitous observation that ENU2-PAH stability is positively correlated with high levels of plasma Phe. Normalization of plasma Phe reduces the risk of negative effects on gene-therapy-derived-PAH activity due to F263S ENU2-PAH, and we propose a similar mechanism may apply to the human F263L mutation.

Methods

AAV5-PAH vector design

- Adeno-associated virus serotype 5 (AAV5)-mediated delivery of the human PAH (hPAH) or murine PAH (mPAH) gene (under a liver-specific promoter) was utilized for these studies

In vivo studies

- All studies were conducted by BioMarin's Translational Biology Department in the vivarium located at the Buck Institute, Novato, CA. Animal breeding and experimental protocols were approved by the Institutional Animal Care and Use Committee
- Male ENU2 mice (C57BL/6-*Pah*^{enu2}) and WT control mice (C57Bl/6J) were received from Jackson Laboratory. Mice had unrestricted access to normal chow (normal protein content).
- Mice were dosed at 8 weeks of age via tail vein injection. Blood samples were taken every two weeks post-dosing by tail vein nick. At the conclusion of the study, blood was drawn via cardiac puncture and tissues (brain and liver) were perfused with phosphate buffered saline prior to collection

Plasma Phenylalanine

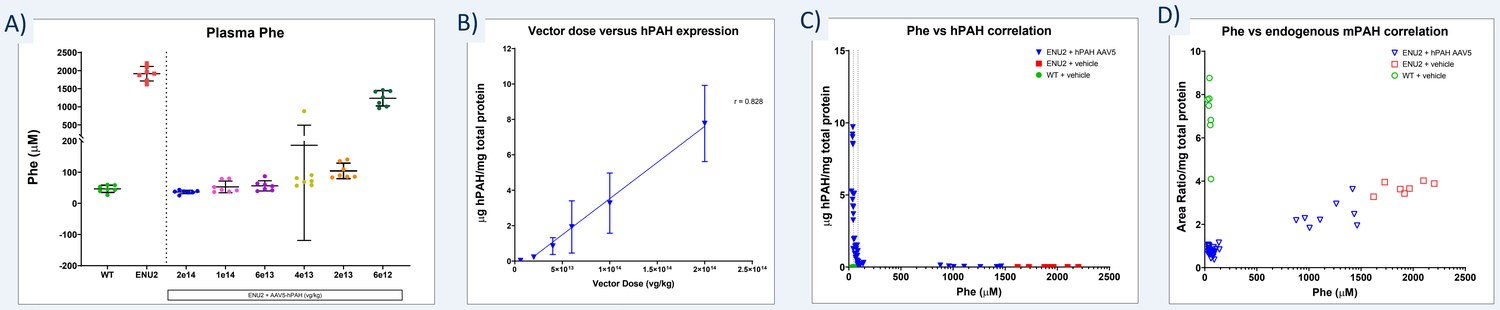
- Plasma Phe was measured by LC-MS/MS following derivatization with benzoyl chloride (Sigma)

Liver PAH protein expression

- Frozen liver sections were homogenized and resulting lysates were reduced, alkylated and digested with Trypsin (Promega)
- PAH level was measured by LC-MS/MS using tryptic peptides specific to human or mouse sequences
- PAH was quantified against a recombinant human PAH standard and normalized to total protein concentration as measured by Bradford Assay (Thermo)

Results

Fig 1: AAV5 mediated delivery of hPAH in ENU2 mouse model: Dose response curve



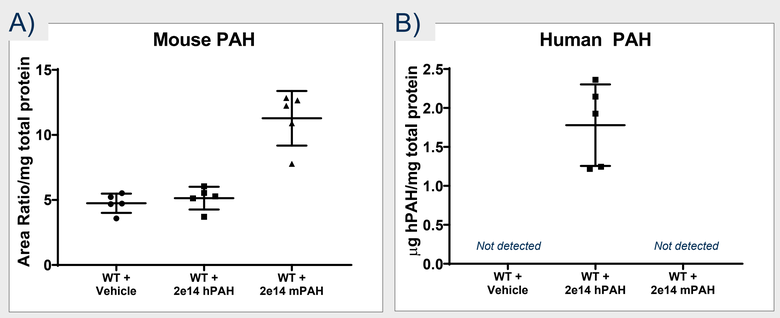
Study Design:

- Wild type C57Bl/6J (WT) mice (1 group, n=7)
- ENU2 mice (7 groups, n=7)
- Dosing:
  - WT and ENU2 + Vehicle
  - ENU2 + hPAH AAV5 (6e12, 2e13, 4e13, 6e13, 1e14, and 2e14 vg/kg)

Observations:

- Phe levels were reduced to a level statistically similar to WT mice in doses 2e14, 1e14, 6e13, 4e13 and 2e13, whereas a dose of 6e12 did not normalize Phe levels (**Fig 1A**)
- Linear correlation between dose and liver hPAH expression (**Fig 2B**)
- Negative correlation between Phe levels and liver hPAH expression at high Phe (>500 μM), which does not exist at low Phe levels (<100 μM), where additional increases in hPAH protein do not lower Phe beyond WT levels due to allosteric regulation of PAH (**Fig 2C**)
- Decrease in endogenous F263S-mouse PAH level with Phe level (**Fig 2D**)

Fig 2: AAV5 mediated delivery of hPAH and mPAH in WT mouse model



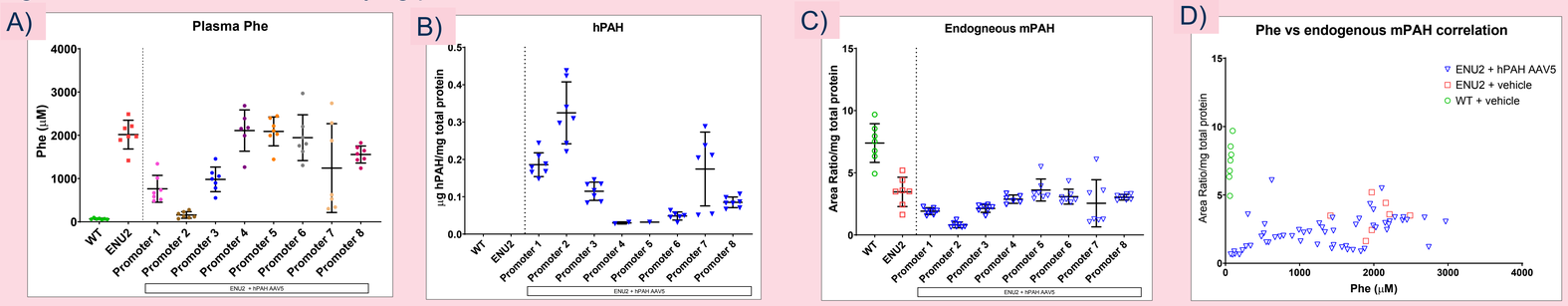
Study Design:

- WT mice, 3 groups, n=5/group
- Dosing:
  - Vehicle
  - 2e14 hPAH AAV5
  - 2e14 mPAH AAV5

Outcome:

- Mouse PAH relative quantitation (**Fig 2A**) shows similar levels of mouse PAH in vehicle (circles) and hPAH AAV (squares) treated groups, indicating that treatment of WT mice with hPAH AAV5 does not decrease levels of endogenous WT mouse PAH
- Treatment with 2e14 mPAH AAV (**Fig 2A**, triangles) showed increased mouse PAH levels
- hPAH (**Fig 2B**) was only detected in the hPAH AAV treated group (squares)

Fig 3: Fixed AAV5 dose with varying promoters in the hPAH construct



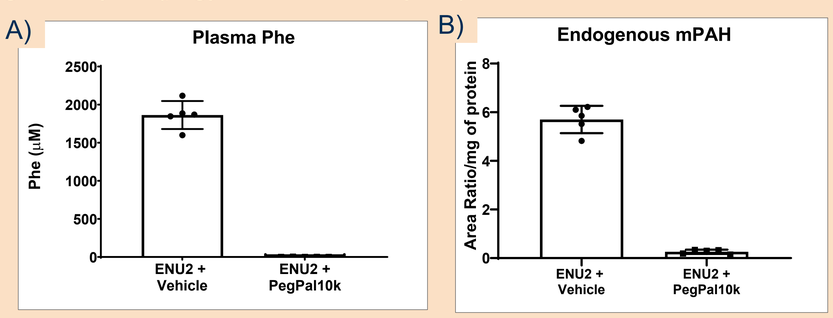
Study Design:

- WT mice (1 group, n=7)
- ENU2 mice (6 groups, n=7)
- Dosing:
  - WT and ENU2 + Vehicle
  - ENU2 + hPAH AAV5 2e13 vg/kg with variable promoters

Outcome:

- Groups receiving the same vector dose (2e13vg/kg) but with variable promoter constructs had different response in Phe level reduction (**Fig 3A**)
- Plasma Phe levels are inversely correlated with human PAH levels, and, positively correlated with ENU2 mouse PAH levels (**Fig 2B and 2C**)
- ENU2 mouse PAH levels are again correlated with Phe level as in previous study (**Fig 2D**)

Fig 4: Enzyme (pegylated ammonia lyase) mediated Phe reduction in F263S-mouse model



Study Design:

- ENU2 mice, 2 groups, n=5/group
- Dosing:
  - Vehicle
  - PegPal10K at 160mg/kg

Outcome:

- Plasma Phe (**Fig 4A**) and endogenous mPAH (**Fig 4B**) levels in ENU2 mice following treatment with PegPal 10K are significantly reduced
- Demonstration that ENU2 mouse protein level can be reduced by reducing Phe through enzymatic treatment

Conclusions

- We hypothesize that F263S (ENU2) PAH protein is stabilized by high Phe. Reduction of Phe, by gene therapy or enzymatically, specifically led to reduction of ENU2 PAH protein.
- Reduction of ENU2-PAH protein may minimize the risk of heterotetramer formation and negative complementation with gene-therapy-derived native PAH.
- Future studies will be aimed towards elucidating the cellular pathways, which contribute to stability of the F263S variant of PAH in ENU2 mouse model of classical PKU.

References

1. http://www.biopku.org/home/pah.asp
2. Shedlovsky et al. *Genetics* 1993;134:1205–1210.
3. Sarkissian et. al. Mol Genet Metab 2000 69(3):188-194.

Disclosures

All authors are full-time employees and shareholders of BioMarin Pharmaceutical, Inc.