

## Advances and challenges in phenylketonuria

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Phenylketonuria (PKU; OMIM 262600), one of the most common inborn errors of metabolism, is caused by recessively inherited deficiency of the enzyme phenylalanine hydroxylase (PAH; EC 1.14.16.1) (Blau et al. 2010). PAH catalyses the irreversible hydroxylation of phenylalanine (Phe) to tyrosine. PAH is expressed primarily in the liver but also in the kidney and pancreas, and its activity requires the unconjugated pterin co-factor, tetrahydrobiopterin (BH<sub>4</sub>). PAH deficiency causes hyperphenylalaninaemia, but hyperphenylalaninaemia can also be caused by inherited deficiency of enzymes involved in BH<sub>4</sub> synthesis or recycling (Blau et al. 2010). Chronic, untreated, severe hyperphenylalaninaemia in infants and children leads to seizures and mental retardation. Newborn screening and early initiation of PKU therapy has eliminated the major manifestations of the disease, but shortcomings in our current therapeutic approach remain.

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Contemporary therapy for PKU is centered upon tight restriction of dietary Phe intake and requires supplementation with special medical foods that supply sufficient essential amino acids and energy from fat and carbohydrate. Institution and maintenance of the PKU diet are difficult, and the required medical foods are often unpalatable. Dietary therapy is recommended for life (Anon 2001), but non-compliance with the dietary prescription is commonplace, particularly during adolescence and adulthood. Hyperphenylalaninaemia in adults is often associated with attention problems, mood instability and poor job performance. Chronically elevated Phe may cause a progressive neurodegenerative disorder affecting white matter that leads to seizures and gait disturbance. Finally, untreated maternal hyperphenylalaninaemia during pregnancy is the only teratogen guaranteed to cause birth defects, which include microcephaly, mental retardation and congenital heart disease (Levy et al. 2003), the so-called maternal PKU syndrome. For all these reasons, novel alternative therapies for PKU are being sought. Phe itself, accumulating to extremely high levels, is primarily responsible for the phenotype associated with untreated PKU. Accumulation of Phe in blood (normally less than 150 µM but often over 2,000 µM in untreated patients) severely interferes with growth and maturation of the developing brain, although the exact molecular mechanism by which this occurs has yet to be elucidated. Over 50 years of experience with PKU treatment based upon the restriction of dietary Phe intake have firmly demonstrated the link between clinical outcome and blood Phe levels (Azen et al. 1996). So, any treatment approach that results in decreased blood Phe levels will ameliorate the PKU phenotype.

BH<sub>4</sub> supplementation is a novel therapeutic approach that is effective in a subset of individuals with PKU (Blau 2010; Harding 2010). In addition to PAH, BH<sub>4</sub> is a cofactor

for numerous metabolic enzymes, including tyrosine hydroxylase, tryptophan hydroxylase, nitric oxide synthase and glyceryl-ether monooxygenase (Thöny et al. 2000). BH<sub>4</sub> is synthesized from guanosine triphosphate (GTP) in a multi-step reaction catalyzed by GTP cyclohydrolase I (GTPCH), 6-pyruvoyl-tetrahydropterin synthase (PTPS) and sepiapterin reductase (SR). During the catalytic activity of BH<sub>4</sub>-requiring enzymes, BH<sub>4</sub> is oxidized to quinonoid dihydrobiopterin, but then fully reduced BH<sub>4</sub> is regenerated through the sequential activities of pterin-4a-carbinolamine dehydratase (PCD) and dihydropteridine reductase (DHPR). Mutations in any of the genes encoding BH<sub>4</sub> synthetic or recycling enzymes can, therefore, lead to BH<sub>4</sub> deficiency (Thöny et al. 2006), and individuals with these deficiencies require treatment with exogenous BH<sub>4</sub>.

PKU due to PAH deficiency is not associated with BH<sub>4</sub> deficiency, but in a subset of individuals with PKU, oral supplementation with further BH<sub>4</sub> can lead to reduction in blood Phe concentration (Kure et al. 1999). Although two-thirds of patients with mild PKU are BH<sub>4</sub>-responsive, little is yet known concerning the mechanisms underpinning responsiveness (Blau et al. 2004). Three mechanisms involved in BH<sub>4</sub>-responsiveness have been postulated, including reduced binding affinities for BH<sub>4</sub> in PAH Km mutants, the stabilization of mutant PAH by BH<sub>4</sub> through protection of the active tetramers or dimers from either cleavage or degradation by the ubiquitin-dependent proteasome pathway, and finally effects of exogenous BH<sub>4</sub> supplementation upon the regulation of BH<sub>4</sub> biosynthesis. It is quite likely that the mechanism (Pey et al. 2004; Gersting et al. 2008) underlying BH<sub>4</sub>-responsiveness will be multi-factorial and that further in vitro studies involving mutant PAH enzymes, and BH<sub>4</sub> knock-out mice will be necessary to aid characterization of the BH<sub>4</sub>-responsive genotype (Pey et al. 2007).

As neonates with increased Phe levels detected through newborn screening may have either BH<sub>4</sub> deficiency or PKU, it is essential that accurate diagnosis be carried out. The abnormal pattern of pterins in urine or reduction in DHPR activity are clear signs of BH<sub>4</sub> deficiency (Blau et al. 2001). In addition, a BH<sub>4</sub>-loading test should be performed to further evaluate the possibility of BH<sub>4</sub> deficiency as well as to categorize patients with PKU as either BH<sub>4</sub>-responsive or -non-responsive (Fiege et al. 2007; Blau et al. 2009). An orally active formulation of BH<sub>4</sub> (sapropterin dihydrochloride) is now available, and an optimized BH<sub>4</sub>-loading test has been proposed (Blau et al. 2010). Blood Phe levels should be monitored for 24 h before and after giving a loading dose of sapropterin, 20 mg/kg. In patients who do not experience a 30% reduction in Phe at 8, 16 and 24 h after dosing, a second 20 mg/kg sapropterin dose should be given. If a 30% reduction in Phe is still not achieved within 24 h, the test is stopped. In patients who experience a 30%

reduction in Phe after either loading dose, sapropterin treatment is continued, with the dose adjusted to 5–20 mg/kg to keep blood Phe within the therapeutic range. Only at this time can a patient's dietary restrictions be reduced or even lifted. The introduction of BH<sub>4</sub> and sapropterin treatment has proved to be an important step forward for patients with BH<sub>4</sub>-responsive PKU. Importantly, we are now starting to understand how to optimize both BH<sub>4</sub> and dietary restrictions so that patients achieve the best treatment outcomes and quality of life in the long term (Blau et al. 2010).

Substantial progress has been made over the past several years in the preclinical development of gene therapy, cell therapy and enzyme substitution for PKU (Harding 2008). As the liver is the primary mediator of Phe homeostasis, it is an obvious target for cell- or gene-directed therapies, but because the liver is not adversely affected by PAH deficiency, successful treatment of PKU does not necessarily require targeting of the liver. The best evidence for this is the success of contemporary dietary therapy that largely prevents the major manifestations of PKU without directly addressing liver PAH deficiency. Enzyme substitution, cell transplantation or gene therapy approaches directed at tissues other than the liver might also be successful if the normalization of blood Phe levels can be achieved.

Preclinical investigation of these novel therapies has been greatly facilitated by the availability of a mouse model that adequately recapitulates the human PKU phenotype. One of three PAH-deficient mice lines to result from a random mutagenesis project (McDonald et al. 2002), the *Pah*<sup>enu2</sup> mouse carries a T to C transition at nucleotide position 788 of the mouse *Pah* cDNA (McDonald et al. 1997). This missense mutation replaces a conserved Phe in the catalytic site with a serine and completely inactivates the PAH protein. Homozygous animals are hyperphenylalaninaemic, hypo-pigmented, mildly growth retarded, and cognitively impaired relative to wild-type or heterozygous mice. Affected females are fertile, but in a phenomenon that is quite similar to human maternal PKU syndrome, many of the offspring have structural defects including congenital heart lesions (McDonald et al. 1997). The *Pah*<sup>enu2</sup> mouse is an outstanding model for human PKU.

Therapeutic liver repopulation with wild-type hepatocytes or stem cells has been explored as a potential therapy for inborn errors of metabolism in both animal models and in humans (Harding et al. 2010). Transplantation of wild-type hepatocytes into PAH-deficient mice under conditions that provide a selective growth advantage for the donor cells has demonstrated complete correction of blood Phe concentrations in animals that had achieved at least 10% liver repopulation (Hamman et al. 2005). Blood Phe levels were only partly corrected in mice that had achieved only 5–10% repopulation. The therapeutic threshold of approx-

imately 10% PAH-expressing hepatocytes needed to fully correct Phe clearance is also important for the efficacy of liver-directed gene therapy for PKU. The major current limitation to the use of hepatocyte transplantation as a treatment for PKU is that PAH-expressing cells do not exhibit any natural selective growth advantage over PAH-deficient hepatocytes. Even under conditions that stimulate hepatocyte regeneration, such as following partial hepatectomy, a selective growth advantage for the donor cells is necessary to achieve a therapeutically relevant degree of liver repopulation in PKU (Harding et al. 2010).

The laboratory of Dr Savio Woo was responsible for cloning the human (Woo et al. 1983) and mouse PAH cDNAs, and for early investigations into PKU gene therapy using the *Pah<sup>emu2</sup>* mouse. Experiments with a recombinant adenovirus vector, expressing PAH from the human PAH cDNA, demonstrated the potential for gene therapy (Fang et al. 1994). Blood Phe levels of adenovirus-treated *Pah<sup>emu2</sup>* mice were completely corrected to normal in animals that achieved more than 10% liver PAH activity. Unfortunately, this effect only lasted about 2 weeks, when immune-mediated destruction of the transduced hepatocytes led to loss of PAH activity and a return of hyperphenylalaninaemia. Re-administration of the vector was ineffective because of neutralizing antibodies against the adenovirus vector that had appeared in the blood of the treated mice (Fang et al. 1994). This experiment, however, was the first to demonstrate the potential efficacy of liver-directed gene therapy for PKU.

Liver-directed gene therapy using recombinant adeno-associated virus serotype 8 vectors (rAAV8) has achieved long-term correction (up to 1 year) of blood Phe concentration in *Pah<sup>emu2</sup>* mice without inducing the immune-mediated rejection seen following adenoviral therapy (Ding et al. 2006; Harding et al. 2006). Even simple intramuscular injection of rAAV8 vector can lead to successful liver transduction and correction of blood Phe (Rebuffat et al. 2010). However, rAAV8-mediated therapy does not lead to permanent correction of liver PAH deficiency; it is thought that gradual but continuous hepatocyte regeneration eventually leads to elimination of episomal rAAV vector genomes and loss of PAH expression. Reinjection of the same serotype vector is ineffective because of antibody-mediated destruction of the vector. Skeletal muscle is therefore an attractive target organ for gene therapy because adult muscle lacks ongoing cell division. Expression of a complete phenylalanine hydroxylating system including the BH<sub>4</sub> synthetic steps necessary to fully support PAH activity has been achieved in *Pah<sup>emu2</sup>* mice following intramuscular injection of a multicistronic rAAV serotype 5 vector (Ding et al. 2008); this treatment approach led to sustained reduction of blood Phe in the mice. Optimization of this muscle-directed approach and continued development of liver-directed gene therapy to improve sustainability are ongoing.

Enzyme replacement strategies have been successfully used to treat many inborn errors of metabolism, namely lysosomal storage disorders. Enzyme replacement in PKU would require introduction into liver of active PAH protein, a complex tetramer with multiple cofactor requirements for maintaining stability. Enzyme substitution with the non-mammalian enzyme phenylalanine ammonia lyase (PAL) provides an alternative approach that overcomes the technical challenge of targeting the protein to liver; PAL is active as a monomer and does not require any exogenous cofactors. Subcutaneous injection of PAL, modified with polyethylene glycol (PEG) to protect PAL from immune-mediated destruction, leads to sustained concentrations of PAL enzyme in blood where it converts circulating Phe to ammonia and trans-cinnamic acid, a harmless organic acid that is rapidly excreted in urine (Sarkissian et al. 2005). Further investigation of PAL proteins from different species led to preclinical development of PAL from the bacterium *Anabaena variabilis* as the kinetically most suitable version for the treatment of PKU (Sarkissian et al. 2008). Weekly subcutaneous injection of this PEG-modified recombinant PAL (rAvPAL-PEG) to *Pah<sup>emu2</sup>* mice led to complete and sustained correction of blood Phe levels. These highly promising results have subsequently led to the initiation and completion of a successful Phase I clinical trial, sponsored by BioMarin, of injectable rAvPAL-PEG in 25 patients with PKU. A multi-site phase II trial examining the safety and efficacy of repeated rAvPAL-PEG injection is underway in the US.

Progress in the development of alternative approaches to the treatment of PKU has been truly impressive over the past decade. Although much difficult work remains before the application of any of these technologies in the clinic becomes commonplace, the future of PKU research remains bright.

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