Phenylalanine ammonia lyase, enzyme substitution therapy for phenylketonuria, where are we now?

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Received 3 May 2005; received in revised form 20 June 2005; accepted 24 June 2005
Available online 13 September 2005

Abstract

Phenylketonuria (PKU) is an autosomal recessive genetic disorder in which mutations in the phenylalanine-4-hydroxylase (PAH) gene result in an inactive enzyme (PAH, EC 1.14.16.1). The effect is an inability to metabolize phenylalanine (Phe), translating into elevated levels of Phe in the bloodstream (hyperphenylalaninemia). If therapy is not implemented at birth, mental retardation can occur. PKU patients respond to treatment with a low-phenylalanine diet, but compliance with the diet is difficult, therefore the development of alternative treatments is desirable. Enzyme substitution therapy with a recombinant phenylalanine ammonia lyase (PAL) is currently being explored. This enzyme converts Phe to the harmless metabolites, trans-cinnamic acid and trace ammonia. Taken orally and when non-absorbable and protected, PAL lowers plasma Phe in mutant hyperphenylalaninemic mouse models. Subcutaneous administration of PAL results in more substantial lowering of plasma and significant reduction in brain Phe levels, however the metabolic effect is not sustained following repeated injections due to an immune response. We have chemically modified PAL by pegylation to produce a protected form of PAL that possesses better specificity, prolonged half-life, and reduced immunogenicity in vivo. Subcutaneous administration of pegylated molecules to PKU mice has the desired metabolic response (prolonged reduction in blood Phe levels) with greatly attenuated immunogenicity.

Keywords: Phenylketonuria; Metabolic disease; Enzyme replacement therapy; Phenylalanine ammonia lyase; Phenylalanine hydroxylase

The hyperphenylalaninemas (HPA) are multifactorial metabolic disorders (OMIM 261600) due primarily to mutations in the gene for phenylalanine hydroxylase (EC 1.14.16.1). Approximately 500 identified mutations in the phenylalanine hydroxylase gene (PAH) lead to a defective PAH enzyme that impairs the disposal of the surplus phenylalanine (Phe) from normal diet. In excess, Phe itself is the neurotoxic molecule [1–3], however it is also an essential nutrient and in addition to its requirement for protein synthesis, it serves as a precursor for tyrosine (Tyr) and its derivatives. In the absence of Phe, Tyr becomes an essential amino acid, therefore, the treatment of HPA requires the balanced reduction of systemic Phe concentration without excessive depletion (restoring euphenylalaninemia) and satisfactory Tyr provision [1,2].

An artificial diet to reduce Phe intake became available in the mid-1950s [4–6]. It was uniformly accepted by the late 1960s [1,2] and was a success due to a population-screening test [7] that identifies new-born patients and allowed treatment to begin prior to the onset of neurological damage. PKU therapy is one of the first effective treatments of a genetic disease.

The treatment regimen today is not much different. The diet products have been refined [8–11] but the mode remains the same. It involves the careful regulation of dietary L-phenylalanine intake to <500 mg/day, sufficient
to support protein synthesis while preventing excess accumulation of Phe in the free pool. These are the present standards, and the current guidelines [12,13] advise treatment to restore blood phenylalanine to levels as near normal as possible, as early as possible, for as long as possible, and for a lifetime in some patients.

A low-Phe diet can prevent cognitive impairment, but it is a difficult option for adolescents and adults. Multiple challenges exist: although much improved, the low-phenylalanine products continue to have unsatisfactory organoleptic properties, making long-term full compliance very laborious and requiring a great deal of social support [13–15]; pregnancies (maternal PKU) are another concern, because elevated Phe levels in the fetus are teratogenic [16]; furthermore, the diet has known deficiencies of several nutrients, all of which can be detrimental to brain development [17–19]; finally, a shift in homeostasis, causing protein catabolism, will result in increased blood Phe levels [20].

An alternative to dietary treatment is desirable because the current therapy incurs high personal costs to affected individuals and families, and because the normal culture of nutrition is greatly distorted. Although for the infant and young child, this aspect may be less important, for the teenage years and onward a common consequence is social isolation. With the recommendations [12,13] for dietary therapy, possibly for life, and for reintroduction of diet during conception and pregnancy for the affected woman, a change in the therapeutic mode is coveted.

Alternative forms of therapy are now being explored. Somatic gene therapy by therapeutic liver repopulation has some promises, but continues at the experimental stage [21,22]. Liver transplantation will correct the metabolic phenotype but it is not a clinical option for all but the rarest of patients [23]. Large neutral amino acid supplementation to compete with Phe for transport across the blood–brain barrier will help reduce brain Phe levels in adults who have PKU, but this treatment is still controversial, and recommended only for non-compliant adults who have difficulty following their diet [24,25].

The current promising potential therapy for some patients harbouring missense PAH mutations is tetrahydrobiopterin (BH4) PAH-cofactor administration. The combination of milder HPA-inducing alleles found in the patient’s genotype influences the degree of a patient’s BH4 responsiveness [26–29]. BH4 supplementation therapy will potentially address the treatment needs of part of the hyperphenylalanimemic population; however patients with the more severe forms of classical PKU and some with non-PKU HPA do not respond to BH4 treatment. These non-responders could benefit most from enzyme therapy, which is currently under pre-clinical development. Unlike BH4 treatment, it is not dependent on the PAH genotype.

**Enzyme therapy** for PKU can be done either by replacement with phenylalanine hydroxylase or by substitution with phenylalanine ammonia lyase enzyme (PAL, EC 4.3.1.5), a “foreign” protein involved in Phe degradation. Both approaches can reduce the elevated Phe by acting as proxy to the deficient PAH enzyme of the PKU patient.

Replacement of the native PAH enzyme (the primary choice) presents a number of challenges: PAH is inherently unstable, making large-scale isolation and purification of the enzyme an ambitious task; its complex activity and cofactor (BH4) requirement in addition to the inherent protease sensitivity and potential immunogenicity in a person lacking the functional enzyme further complicate the venture.

Enzyme replacement therapy with PAH requires the intact multi-enzyme complex for catalytic hydroxylating activity [1,2]. To make it therapeutically viable, modification of the PAH molecule is required. Truncated forms of PAH have been constructed in an attempt to stabilize and increase catalytic activity [30]. In addition, protection of the enzyme against immune response has been achieved by polyethylene glycol (PEG) chemical conjugation (pegylation) of the protein [31]. Human and various bacterial PAH have been successfully pegylated with retention of full or near-full and in some cases augmented catalytic activity; the modified molecules are also more stable than their non-derivatized PAH counterparts [32]. The growing number of pegylated enzyme therapeutics, displaying improved pharmacokinetic and pharmacodynamic profiles that are currently approved by the Food and Drug Administration (FDA) [31,33] tells a promising story. Exploring pegylated-PAH as a long-term injectable molecule for PKU is ongoing, but given the drawbacks of the enzyme, its viability as a therapeutic remains debatable [32].

PAL is a potential substitute for the native PAH protein. In fungal cells, PAL has a strictly catabolic role [34], while in plants, it is a key biosynthetic enzyme catalyzing the first reaction in the synthesis of a variety of polyphenyl compounds [35–37]. PAL is an autocatalytic protein responsible for the non-oxidative deamination of L-phenylalanine to form trans-cinnamic acid and ammonia [38–40]. trans-Cinnamate has no embryotoxic effects in laboratory animals [41] and is excreted as hippurate in urine [42] along with small amounts of cinnamic and benzoic acids [39]. The ~3 g trans-cinnamic acid, that would be generated daily with complete dietary Phe conversion by PAL, is predicted to be harmless to both PKU and normal individuals; the ammonia formed would be metabolically insignificant [39,43].

The optimal pH and temperature for PAL activity is 8.5 at 30 °C, a feature compatible with an enteral route for PAL therapy [44]; but the enzyme will normally experience proteolytic degradation in the intestinal lumen [45] unless it is protected [46]. If it were injected
parenterally, as another option, it would be immunogenic [47] (a reaction already reported when animals have been injected with PAL [43,48]), unless the protein is modified to suppress its immunogenicity [31].

Initial attempts to avoid eliciting an immune response used an extracorporeal multitubular enzyme reactor with immobilized PAL to reduce plasma Phe levels significantly without the enzyme entering the circulation [49]. However this is not a viable long-term option, and for treatment, the original proposed route for protected therapeutic PAL administration was oral. This approach capitalized on PAL depleting the Phe pool as it passed through the intestine, while evading problems with immune response associated with accumulation of synthetic injectable drugs [50–54].

Various formulations and mechanisms, to prevent protease inactivation, have been tested. Measures to immobilize PAL [51] have shown significant plasma Phe reduction [55]. Preliminary studies in human PKU patients showed analogous responses after the administration of PAL in enteric-coated gelatin capsules [43]. Entrapment of enzyme in silk fibroin [56] and in adenocarcinoma cell line Caco-2 [57] were other proposals. These studies were not continued because at the time PAL was not available in sufficient amounts at an acceptable cost.

Treatment with PAL, purified from a natural source, has a predicted daily cost comparable to the approximated annual expense per patient of the dietary therapy. PAL enzyme substitution therapy became a viable option only when cloning technology allowed the production of virtually limitless quantities of recombinant PAL (the gene was acquired from yeast *Rhodospiridium toruloides*). Access to the orthologous mouse models of human PKU and HPA [58–60] made pre-clinical in vivo studies possible.

Our earlier work [61] used mutant ENU mouse orthologues of non-PKU HPA and PKU to demonstrate proofs of pharmacological and physiological principles in the efficacy of the recombinant PAL depleting systemic Phe levels. The first was achieved by intra-peritoneal injection of recombinant PAL, which acted in vivo to lower ambient blood Phe levels. The effect persisted over 24 h following treatment, with plasma Phe levels markedly lower than those measured at zero time prior to the administration of the enzyme. The second was demonstrated when recombinant PAL administered orally and protected from degradation by intestinal digestion, actively depleting phenylalanine. Two different systems were applied. First recombinant PAL expressed in a non-pathogenic *Escherichia coli* organism provided a protective environment for the enzyme from the action of digestive proteases, which were unable to penetrate the cells. The free Phe, in the intestinal lumen, was transported through the bacterial membrane and converted by the recombinant PAL to *trans*-cinnamic acid, reducing Phe levels by 44% within 2 h post-administration. The second system capitalized on recombinant PAL delivery in solution with aprotinin protease inhibitor. This mixture, placed in the intestinal lumen, again acted in vivo effectively depleting the systemic phenylalanine levels, this time by 54% within 2 h post-administration. Recombinant PAL has the desired effect on elevated plasma phenylalanine in the PKU phenotype. Moreover brain Phe levels are improved by parenteral (injectable) recombinant PAL administration [62]. However, parenteral use of native recombinant PAL produces an immune response and while an enteral route for PAL therapy is still the most desirable form of enzyme substitution therapy, the current protease-protected variants require a long contact time between the enzyme formulations and substrate, during passage through small and large intestine. In addition, low specific activity means that a large amount of the recombinant PAL formulations is required to lower plasma phenylalanine in PKU. All these factors make oral therapy a continued challenge.

Enzyme replacement therapy has become a significant approach to treatment of inborn errors of metabolism [33]; thus to treat PKU with an injectable enzyme would not be an unexplored therapeutic paradigm in genetic medicine. Current knowledge about the crystal structures of PAH [30,63–66] and of PAL [67] will allow the application of structure-based molecular engineering to optimize the recombinant PAL protein architecture. The desired product here would be an injectable recombinant PAL therapeutic, with satisfactory immune tolerance in vivo.

Our work now focuses on chemical modifications of the recombinant PAL molecule, to develop a protected form of the enzyme that retains satisfactory specific activity against phenylalanine substrate and yet with suppressed immunogenicity. A series of linear and branched polyethylene glycols chemically conjugated to recombinant PAL have set these wheels in motion [68]. These pegylated recombinant PAL conjugates reduce plasma Phe levels effectively and they mask immunogenicity in vivo after repeated challenge [68]. The new procedures for synthesis of modified recombinant PAL exclude unmodified (native) recombinant PAL from the final product [68]. Our findings so far constitute a new set of proofs-of-principle for the use of parenteral recombinant PAL enzyme substitution in the treatment of PKU under the conditions of the new guidelines [12,13].

**Acknowledgments**

The authors acknowledge their mentors Drs. Charles R. Scriver and Raymond C. Stevens for their vision, endless enthusiasm, and their invaluable advice in the development of PAL therapy for PKU treat-
We are also grateful to our colleagues in this project who made the work possible, Dr. Lin Wang, Mélanie Hutubise, and Mary Straub. This work was supported in part by the Medical Research Council (Canada), the Canadian Genetic Diseases Network (Networks of Centers of Excellence), IBEX Technologies, BioMarin Pharmaceutical Inc., the National PKU Society (UK), the Garrod Association (Canada), the Michaux Family, and the Piziali Family Foundation (USA).

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