Trends in Enzyme Therapy for Phenylketonuria

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Phenylketonuria (PKU) is an inborn error of amino acid metabolism caused by phenylalanine hydroxylase (PAH) deficiency. Dietary treatment has been the cornerstone for controlling systemic phenylalanine (Phe) levels in PKU for the past 4 decades. Over the years, it has become clear that blood Phe concentration needs to be controlled for the life of the patient, a difficult task taking into consideration that the diet becomes very difficult to maintain. Therefore alternative models of therapy are being pursued. This review describes the progress made in enzyme replacement therapy for PKU. Two modalities are discussed, PAH and phenylalanine ammonia-lyase PAH. Developing stable and functional forms of both enzymes has proven difficult, but recent success in producing polyethylene glycol-modified forms of active and stable PAH shows promise.

Key Words: phenylketonuria, enzyme replacement therapy, phenylalanine ammonia-lyase, phenylalanine hydroxylase, PEGylation, microencapsulation

INTRODUCTION

Phenylketonuria (PKU; OMIM 261600) is an inborn error of phenylalanine (Phe) metabolism. Mutations in the phenylalanine hydroxylase (PAH; EC 1.14.16.1) gene resulting in enzyme deficiency lead to hyperphenylalaninemia (HPA). It has an occurrence of approximately 1 in 16,000 live births in the United States [1]. Untreated patients with PKU show mental retardation, microcephaly, and seizures, if not treated immediately after birth through a low-Phe diet. Inactive PAH enzyme causes accumulation of the essential amino acid Phe. There have been more than 400 mutations identified in the PAH gene: http://www.pahdb.mcgill.ca/ [2].

PAH is a nonheme, homotetrameric, iron-containing enzyme that needs (6R)-L-erythro-5,6,7,8-tetrahydrobiopterin (BH4), molecular oxygen, and the active site-bound Fe2+ for conversion of Phe to Tyrosine (Tyr) (Fig. 1A). PAH is responsible for the majority of the catabolism of dietary Phe and is located mainly in the liver. Thus Tyr becomes an essential amino acid in PKU patients, and if not added through supplements to the PKU diet, low levels of Tyr may affect the biosynthesis of the neurotransmitters dopamine, noradrenaline, and adrenaline. Tyrosine supplementation provided by oral administration of large neutral amino acids (LNAA; Phe, Tyr, tryptophan, threonine, isoleucine, leucine, valine, methionine, and histidine) has proven effective in reducing high Phe levels in the brain of humans and mice with...
PKU [3,4]. The LNAAs share a common transporter across the blood–brain barrier and will therefore compete with Phe, the levels which are high in patients with PKU [5,6].

**CURRENT PKU THERAPY**

The current therapy for PKU/HPA involves limiting Phe intake by a special semisynthetic diet [7]. A Phe-restriction diet can lower plasma Phe levels and may prevent the mental impairments of PKU patients. The first dietary therapy for PKU was administered in 1958 [8] and it has been used for the treatment of many cases from classic PKU to mild HPA [9]. However, compliance with dietary treatment erodes as patients get older. The diet therapy has proven difficult to adhere to, particularly in adolescents [8]. Pregnant PKU/HPA women have a particular need for keeping the Phe levels low, since high level of Phe affects the embryo and fetus (maternal PKU) [1]. The UK MRC Study Group on PKU has concluded that there is a need for an alternative to the low-Phe diet [10]. The NIH Consensus Panel also encouraged research on therapeutics for PKU, including enzyme therapy and gene therapy [11].

**GENE THERAPY FOR PKU**

Due to the difficulties in adhering to diet therapy in the treatment of PKU, efforts are directed toward alternatives to the unpalatable diet. The goal of gene therapy is to restore PAH expression permanently in liver and to eliminate the need for the special diet. Gene therapy has been performed using different methods of delivery and also using heterologous nonhepatic gene targeting attempts. A general and up-to-date review on PKU gene therapy was recently published [12]. With all the optimistic plans, all attempts to use gene therapy to treat PKU have failed [12,13].

**TETRAHYDROBIOPTERIN-RESPONSIVE PKU**

Recently, patients with mild PKU have been shown to respond to BH4 [14]. The patients display a lowering of their blood Phe levels upon an oral load of 10–20 mg/kg body weight of the BH4 cofactor to the PAH enzyme. Subsequent studies have found that ~60% of mild PKU patients are BH4-responsive [15]. A recent report showed for the first time that a relatively large number of patients with classical PKU responded positively to BH4 [16]. The genotypes of known PKU/HPA patients that are BH4-responsive have been gathered in the BH4 database (http://www.bh4.org/biopku.html). Even though most of the BH4-responsive patients have the milder form of PKU, the diet is still necessary in most cases, and thus an alternative to the PKU-diet would be the addition of BH4 to a normal diet [17]. The advantage of BH4 supplementation is that it can be taken orally; however, some major disadvantages at the moment are that BH4 is expensive and can be used mostly for the mild forms of PKU/HPA. Also, due to the relatively short elimination half-life of BH4 (3.3–5.1 h) [18], it needs to be given in doses at least two or three times a day. Sublingual injection may lower the required dosage of BH4 and subsequently the cost.

**ENZYME REPLACEMENT THERAPY**

There is an increasing interest in enzyme replacement therapy (ERT) for metabolic diseases. For example, ERT is gaining popularity in the treatment of lysosomal storage disease, thus circumventing the difficulties with gene therapy. Two enzyme systems are being developed for treatment of PKU: the PAH enzyme and the Phe-degrading enzyme from plants; phenylalanine ammonia-lyase (PAL).

**ENZYME REPLACEMENT THERAPY USING PAL**

In comparison to PAH, PAL therapy for PKU has some advantages. PAL requires no cofactors for degrading Phe, and *trans*-cinnamate has a very low toxicity and no embryotoxic effects in experimental animals. The PAL product *trans*-cinnamic acid is converted in the liver to benzoic acid, which is then excreted via the urine mainly as hippurate [19]. PAL is very stable under a wide temperature range. Purified PAL from *Rhodotorula glutinis* at a concen-
tration of 20–40 mg/ml showed no loss of activity at −60°C for at least 6 months [20].

A nonmammalian enzyme, PAL is widely distributed in plants [21,22] and some fungi [23] and yeasts [20] (Table 1) and also produced from E. coli. PAL (Fig. 1B) was investigated to treat PKU as early as 1980 and ERT studies in human PKU patients began with the oral administration of PAL in enteric-coated gelatin capsules [24]. The purified PAL from the yeast R. glutinis was packed into hard gelatin and enteric-coated capsules (50 U each, SA 1.2 U/mg). PAL enteric-coated capsules reduced the blood Phe levels in PKU patients by 22%. The pH optima of PAL from R. glutinis and Rhodotorula rubra were 8.75 and 8.0, respectively [20,25]. These pH ranges, which are close to the average pH of the small intestine, may have potential advantages in oral enzyme therapy of PKU. For investigating oral administration of PAL therapy, both enzymatic activity and its stability should be evaluated in gastrointestinal fluid. PAL from Rhodospiridium toruloides was reported to have no activity at pH 2.2 and a half-life in duodenal juice of 3.5 min [25]. PAL from R. glutinis was also inactivated rapidly by duodenal juice. This inactivation of PAL in duodenal juice was due to the enzyme being more susceptible to chymotrypsin than to trypsin [26]. To preserve the activity of PAL in intestinal fluids, PAL has to be protected from intestinal proteolysis and also from pH levels found in the upper gastrointestinal tract. Therefore, pretreatment was necessary to protect the PAL enzyme against gastric acidity and pancreatic proteases. Chang et al. immobilized PAL (from R. glutinis) within artificial cells and the result was an enzymatic PAL system that acted effectively on permanent external substrates, such as Phe [27].

Immobilized PAL within artificial cells was more effective than a phenylalanine-free diet in PKU rats and lowered Phe in the plasma and intestinal and cerebrospinal fluids more than a low-Phe diet [28,29]. Consequently, the depletion of intestinal phenylalanine by ENC PAL could significantly lower the plasma phenylalanine levels [30]. However, oral administration of ENC PAL would be limited to mild PKU patients and diet control should also be recommended for better results [29,30]. Another restriction was that the ENC PAL displayed an activity only 20% of the native enzyme activity. The \( V_{\text{max}} \) values for ENC PAL and native PAL were 9 and 55 \( \mu \text{mol/min} \), respectively [27]. Accordingly, additional modifications for enhancing enzyme activity are needed for immobilization of PAL in artificial cells to work in reducing Phe levels.

An alternative approach has been investigated to overcome the reduction of enzyme activity by microencapsulation. PAL was entrapped in silk fibroin to maintain its activity in the intestinal fluids. Entrapped (ENT) PAL was resistant against chymotrypsin and trypsin in vitro [31]. The ENT PAL was injected directly into rat duodenum. The activity of ENT PAL was retained since it circumvents the intestinal proteases. This approach also actively degraded Phe in the intestinal tract. Although the ENT enzyme showed similar \( K_m \) for Phe compared to the native enzyme, there was no discussion of the protective effect that the silk fibroin produced toward gastric acidity.

In a recent study on yeast PAL ERT by Sarkissian et al., recombinant PAL was produced expressed at high levels [32]. PAL was encased in its original E. coli expression cells, and to evaluate the effect of recombinant PAL, PAH\(^{E7U2}\) mice were given either enteral or intraperitoneally injected PAL. Orally administered recombinant PAL (25 units) lowered plasma Phe in PKU mice by 31% in 1 h \((P < 0.04)\) and 44% in 2 h \((P < 0.0004)\). This formulation also reduced the Phe content significantly in in vitro solution, which contains mouse intestinal fluid. Although this treatment has promising effects, it has been stated that low specific activity compared to native PAL and relative inefficiency at pH 7.0 may be significant challenges to overcome.

**IN VIVO STUDIES OF PARENTERAL PAL THERAPY**

Although oral administration of PAL will be more comfortable for the patient, a parenteral modality for PAL therapy needs to be considered. The highly immunogenic property of PAL is a serious problem for parenteral PAL therapy, since it may lead to a short half-life of the enzyme in the blood and unwanted immunologic responses [33]. To overcome these problems, multitudes of enzyme-reactors with immobilized PAL (from R. glutinis) were investigated and resulted in a rapid, 77% removal of Phe in blood samples of PKU patients [34,35]. A sustained reduction of Phe was exhibited in less than 1 h, *in vitro* [36]. A series of experiments was

![FIG. 2. PEGylation mechanism and conditions for PEGylation using mPEG-succinimidyl propionate (Nektar Therapeutics).](image-url)
conducted with a large animal model to evaluate its safety for clinical use. Repeated use of PAL (from *R. glutinis*) reactors to induced HPA artificially in animals did not produce unwanted immunological reactions [37,38]. The PAL reactor was also applied to a PKU patient, and as a result the Phe concentration was decreased from 1.82 to 1.24 mmol/L after 5.5 h of treatment, without side effects [38]. However, extracorporeal hollow fibers containing PAL cannot be easily administered to young children, although it may be recommended for PKU management in pregnant women.

**ENZYME MODIFICATION BY PEGYLATION**

To reduce the degree of immunoreactions [39,40], the PEGylation method (Fig. 2) was applied to PAL from *R. glutinis* by Wieder *et al.* [41]. The half-lives of native PAL and linear PEGylated PAL after the 1st injection were 6 and 20 h, respectively. PEG-PAL had a much longer blood-circulating time in mice than native PAL. However, intravenously injected PEGylated PAL was cleared rapidly from circulating blood after the 13th injection.

The PEGylated enzymes may also require additional treatment before oral administration as outlined in Table 2. This review infers that complex microcapsules could be used as additional measures to protect the therapeutic enzymes from inactivation in both the stomach and the intestine. The semipermeable microcapsules can be further encapsulated by enteric-soluble materials to protect them from gastric juice. When the preparation passes into the intestine, the small molecule Phe will rapidly diffuse and equilibrate across the semipermeable membrane and can be converted to nontoxic products by the enveloped enzymes [42].

**ENZYME REPLACEMENT THERAPY USING PAH**

A recent report by Gamez *et al.* [43] described the first attempts at producing a stable and nonimmunogenic form of the PAH enzyme that can be used for ERT. PEGylation (Fig. 2) increased the *in vitro* activity of three forms of PAH (full-length, double-truncated, and bacterial PAH from *Chromobacterium violaceum*). The results were promising, but it has not been tested in PAH*enu2* mice, so it is not known whether it will be effective *in vivo*. Effectiveness may prove to depend upon method of delivery (i.e., oral route versus intraperitoneal). Additionally, for this to work, there will be a need to administer the PAH cofactor BH₄, either orally or by addition of the (BH₂ to BH₄) recycling enzyme dihydropteridine reductase.

Although the cofactor requirement is a disadvantage in the use of PAH for ERT, there are several advantages. These include that the protein is well expressed in bacteria, particularly the doubly truncated form; the expressed protein in the human form of the disease, the protein is easily PEGylated, unlike other enzymes that have been attempted; and the PEGylated protein is very stable after PEGylation. Another advantage of PAH is that additional Tyr supplementation may be unnecessary in PKU therapy.

**CONCLUSIONS**

There have been considerable advances in ERT to treat PKU. These include the use of various forms of bacterial and human PAH, PEGylated PAH as well as the Phe degrading enzyme PAL, and various encapsulation techniques. Although a great deal of work has been conducted to date, there are still hurdles to overcome, including the stabilization of the enzymes, consistency of response, and how to avoid an immune response.

**REFERENCES**


