



Review

The time has come: a new scene for PKU treatment

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ABSTRACT. Phenylketonuria (PKU) is one of the few genetic diseases in which mental retardation can be prevented. Hence, diagnosis and treatment must be established early. PKU treatment consists of a phenylalanine-restricted diet supplemented with a phenylalanine-free mixture of amino acids. However, it is difficult to adhere to this diet. In the last decade, a better comprehension of the biochemistry, genetics and molecular basis of the disease, as well as the need for easier treatment, led to the development of several new therapeutic strategies for PKU. In the present study, we evaluated these new therapeutic options in terms of theoretical basis, methodologies, efficacy, and costs.

Key words: Phenylketonuria, PKU treatment, Therapeutic strategies, Phenylalanine ammonia-lyase, Large neutral amino acids, Gene therapy, Tetrahydrobiopterin

INTRODUCTION

Some of the most important contributions from basic research to disease comprehension are those insights derived from variations in both clinical manifestation and therapeutic response. Phenylketonuria (PKU; OMIM 261600) has always been considered to be the classical example of a simple genetic disease for which an effective therapy is possible. However, a closer look at the different phenotypic levels of the disease has brought about evidence of an unexpected complexity. Besides, PKU diet is extremely restrictive and consequently adherence to treatment is poor. In this context, some questions can be put forward: How far are we from a less burdensome but truly

effective PKU treatment? What is the real impact of our basic knowledge of PKU on clinical management and therapy? How far are we from therapeutic strategies contemplating the underlying complexity of the illness? When are we going to be able to adapt therapeutics to patient individualities? Which perspectives of PKU treatment deserve our attention for the next few years?

Hyperphenylalaninemia (HPA) refers to all those clinical conditions leading to abnormally high phenylalanine (Phe) levels. Usually, HPAs are induced by mutations in the gene coding for phenylalanine hydroxylase (PAH; EC 1.14.16.1), the enzyme responsible for the conversion of Phe to tyrosine. PAH requires tetrahydrobiopterin (BH₄) as co-factor. Mutations in those genes responsible for BH₄ biosynthesis or regeneration lead to about 2% of the HPAs. On the other hand, PKU refers specifically to those HPAs caused by mutations in the PAH gene, which are severe enough to require therapeutic intervention.

PKU is an autosomal recessive disorder, with a mean frequency of 1:10.000 in Caucasian populations (Acosta et al., 2001; Scriver and Kaufman, 2001; Christ, 2003; Perez et al., 2004). Untreated patients are nowadays only seldom seen. The clinical picture includes delayed psychomotor development, mental retardation, a mousy odor in the urine, irritability, light cutaneous pigmentation, eczema, and epilepsy (OMIM - Online Mendelian Inheritance in Man, <http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=OMIM>; Surtees and Blau, 2000; Scriver and Kaufman, 2001).

Among PKU patients, a remarkably wide variation is observed in both clinical manifestation and therapeutic response (Dipple and McCabe, 2000; Gjetting et al., 2001; Scriver, 2002). The genotype at the PAH locus is the main determinant of plasma Phe levels and, consequently, of phenotypic variability. More than 490 mutations have already been described at this locus. Therefore, most of the PKU patients are compound heterozygotes (PAH Mutation Analysis Consortium, <http://www.pahdb.mcgill.ca/>; Scriver et al., 2003). Due to the large number of mutations, some genotypes are only seldom seen and phenotype-genotype correlations in PKU are not easy to establish. Besides, the active form of the enzyme is a tetramer and interactions between subunits bearing different mutations are possible but rarely predictable (Clark, 1998; Guldborg et al., 1998; Dipple and McCabe, 2000; Rivera et al., 2000; Scriver et al., 2003; Pey et al., 2003; Kasnauskiene et al., 2003).

Some other factors can still contribute to the phenotypic variation observed among PKU patients, such as individual differences in Phe handling with regard to enteric absorption, hepatic uptake, incorporation into proteins, and transport across the blood-brain barrier (Kayaalp et al., 1997; Scriver and Waters, 1999; Greeves et al., 2000; Jennings et al., 2000; Rivera et al., 2000; Weglage et al., 2002). Therefore, alterations in many other genes can intervene producing the final phenotype. A growing comprehension of the origins of its phenotypic variation have hinted to some new strategies for therapeutic intervention in PKU.

THE CONVENTIONAL TREATMENT: PHENYLALANINE-RESTRICTED DIET

Before considering new approaches to PKU management, it is fundamental to take a closer look at the low-Phe diet. PKU has always been targeted in newborn screening programs, because once low-Phe diet is started early, mental retardation can be prevented (Scriver, 1998; Clague and Thomas, 2002).

Variation in Phe tolerance depends not only on genetic background but also on metabolic status and general health conditions. Exercise, growth, pregnancy, and infections may change the needs for Phe. Therefore, the diet must be calibrated for each patient so that 1) Phe and/or secondary metabolites do not reach toxic levels and 2) intake of other amino acids must be sufficient to supply the patient's metabolic needs (Rose et al., 1955; Start, 1998; Arnold et al., 2002; Acosta et al., 2003).

The composition of the PKU diet has changed very little since its introduction in the 1950s. Basically, it is a low-protein diet supplemented with a Phe-free mixture of amino acids, plus added minerals, vitamins and other nutrients (MacDonald, 2000; Fisch, 2000; Przyrembel and Bremer, 2000; Scriver and Kaufman, 2001; Walter et al., 2002). Milk and dairy products, meat, eggs, wheat, beans, corn, peanuts, lentils, and other grains are prohibited. Breast milk can be encouraged once carefully controlled. Fruits and vegetables can be consumed, but in controlled quantities. Consequently, PKU diet is extremely restrictive and difficult to follow. Moreover, the Phe-free mixtures of amino acids have an unpleasant flavor and smell and must be consumed in relatively large amounts. The quality of life under the PKU diet is severely compromised and adherence to treatment diminishes as patients grow older (MacDonald, 2000; Fisch, 2000; Scriver and Kaufman, 2001; Walter et al., 2002; Koch et al., 2003).

Besides, in spite of all these sacrifices, even those patients who have been treated with low-Phe diet and who have adhered to it well show impaired frontal lobe functions and tend to score lower than their sibs in intelligence tests. Behavioral problems have also been reported. These results are frequently attributed to poor dietary control, and that the diet is too restrictive is a notion seldom expressed (Waisbren et al., 1987; Beasley et al., 1994; Smith and Knowles, 2000; Landolt et al., 2002; Arnold et al., 2002; Leuzzi et al., 2004). Despite being a huge advance and generally effective, low Phe-diet cannot be considered the dream management for PKU patients.

Therefore, alternatives to the PKU diet have been actively sought. In Table 1, a classification for the most recent therapeutic approaches for PKU is proposed. Some of them are already in use, while some others are still under development.

Table 1. Classification of new therapeutic strategies for phenylketonuria.

Theoretical principles	Therapeutic strategies
Substrate reduction	New phenylalanine-free formulae
Reduction of phenylalanine uptake	Purified phenylalanine ammonia-lyase
Alternative degradation pathways	Recombinant phenylalanine ammonia-lyase Pegylated phenylalanine ammonia-lyase
Increase of PAH enzymatic activity	Tetrahydrobiopterin (BH ₄) supplementation
Competition for blood-brain barrier carriers	Large neutral amino acids supplementation
Gene therapy	Low immunogenic vectors PAH expression in tissues other than liver

PAH = phenylalanine hydroxylase.

NEW PRODUCTS SUPPORTING LOW-PHENYLALANINE DIET: PROTEIN SUBSTITUTES WITH MORE PLEASANT FLAVOR

Determining factors for poor compliance to low-Phe diet during and after adolescence are the unpleasant flavor and smell of the Phe-free mixtures of amino acids. To solve this problem, some new products have been developed. New commercial presentations of the Phe-free mixture of amino acids include sachets, little bars with the flavor of fruits and tablets. Besides, vitamins and minerals are supplied separately. Some of these products have already been tested showing better acceptance than the conventional dietetic formulas (Rohr et al., 2001; MacDonald et al., 2003, 2004).

PHENYLALANINE DEGRADATION IN THE INTESTINAL LUMEN: PHENYLALANINE AMMONIA-LYASE

Another therapeutic resource currently under development for PKU treatment is based on the oral administration of phenylalanine ammonia-lyase (PAL; EC 4.3.1.5). This enzyme acts by degrading Phe in the intestinal lumen preventing its absorption (Sarkissian et al., 1999; reviewed by Kim et al., 2004). Problems associated with this approach are PAL inactivation by digestive enzymes and the high costs of conventional protein purification. When intravenously injected, PAL exhibited low stability and high immunogenicity. To prevent PAL inactivation by digestive enzymes, PAL is immobilized in semi-permeable microcapsules. With this preparation, a reduction in plasma Phe levels has been reported in both mice and humans (Sarkissian et al., 1999; Levy, 1999).

In addition, a recombinant PAL has also been produced to reduce costs and increase PAL availability. The PAL gene was cloned under a high-expression *Escherichia coli* promoter. A 30 to 40% reduction of blood Phe concentration was observed in mice after oral administration of *E. coli* cells expressing PAL. This reduction reached ca. 50%, when the recombinant PAL was administered combined with a protease inhibitor (Sarkissian et al., 1999).

In another study, *Petroselinum crispum* PAL gene was subcloned in *Lactococcus lactis*. This modified probiotic was administered to HPA rats leading to significant reduction of plasma Phe levels (Liu et al., 2002). *L. lactis* do not permanently adhere to the intestinal mucosa, conferring to this therapeutic strategy the necessary plasticity with minor risks of excessively reducing Phe absorption.

Recently, a pegylated phenylalanine ammonia-lyase (PEG-PAL) was tested. When intravenously injected, PEG-PAL was less immunogenic and more stable than the native non-pegylated PAL. Besides, PEG-PAL retained full catabolic activity (Ikeda et al., 2005; Gamez et al., 2005).

LARGE NEUTRAL AMINO ACID SUPPLEMENTATION

Phe, as well as other large neutral amino acids (LNAAs: asparagine, cysteine, glutamine, histidine, isoleucine, leucine, methionine, serine, threonine, tyrosine, tryptophan, and valine), are transported across the blood-brain barrier by means of L-type amino acid carrier. High Phe levels, such as those usually seen in PKU patients, reduce the brain uptake of other LNAAs (Figure 1; Pietz et al., 1999; Koch et al., 2003; Kanai and Endou, 2003). Some LNAAs such as tyrosine and tryptophan are precursors of neurotransmitters, and it has been suggested that impaired neurotransmitter synthesis would be an additional factor contributing to the cognitive dysfunction observed in PKU (Pietz et

al., 1999; Surtees and Blau, 2000; Weglage et al., 2002; Koch et al., 2003; Matalon et al., 2003).

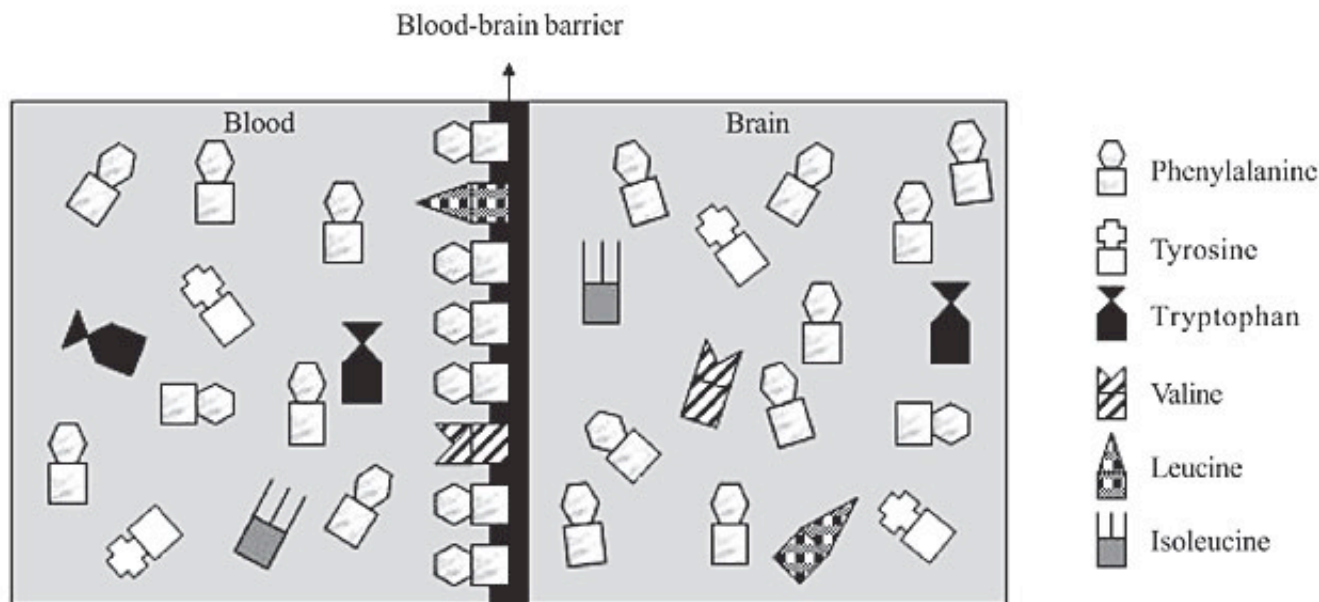


Figure 1. Phenylalanine competes with other LNAAs for the same carrier to pass the blood-brain barrier. High phenylalanine levels, as seen in PKU patients, reduce the brain uptake of other LNAAs and their availability in the brain.

Consequently, a new therapeutic strategy based on moderate protein restriction combined with the supplementation of LNAAs except for Phe was introduced (Koch et al., 2003; Matalon et al., 2003). This strategy has been offered since 1985 at the John F. Kennedy Institute, DK, to those adolescent and adult PKU patients with difficulties in adhering to the Phe-restricted diet (National PKU News <http://www.pkunews.org/>). Despite the large number of tablets to be consumed, about ten before each meal, the LNAA supplement has had a good acceptance. In a fifteen year-follow-up of those patients, there were no differences in cognitive performance or other adverse effects, when adolescents under conventional low-Phe diet were compared to those under a less restrictive low-protein diet plus a Phe-free LNAA supplement (Koch et al., 2003). Similar results have been published elsewhere by an independent study (Moats et al., 2003).

BH₄ SUPPLEMENTATION FOR PATIENTS WITH PHENYLALANINE HYDROXYLASE MUTATIONS

Another relevant alternative to the low-Phe diet was pointed out by the observation that some PAH-deficient PKU patients show reduced plasma Phe levels with the BH₄ loading test. Recently, it has been shown that a large group of patients with PAH mutations do respond to treatment with BH₄ (Lindner et al., 2001; Bernegger and Blau, 2002; Cerone et al., 2004; Steinfeld et al., 2004). The most important factor determining BH₄ responsiveness seems to be the allelic combination at the PAH locus, and the intensity of the response to BH₄ therapy in these patients correlated with the residual enzymatic activity of the mutations identified in the patient (Blau and Erlandsen, 2004). BH₄-responsive PAH mutations are listed elsewhere (<http://www.bh4.org/biopku.html>). However, there is some controversy in the literature regarding the effectiveness of BH₄ supplementation in the presence of some specific mutations or genotypes (Lindner et al., 2001). Recently, it has been shown that long-term BH₄ treatment may also increase Phe tolerance in many patients with severe

PKU phenotype (Hennermann et al., 2005).

Approximately 60% of the patients with plasma Phe concentrations between 400 and 800 $\mu\text{mol/L}$ respond to the BH_4 . However, some patients with higher Phe levels also respond to BH_4 therapy. Best results are obtained with a dose of 20 mg/kg. A few hours after BH_4 administration, blood Phe concentrations decrease considerably (Bardelli et al., 2002; Bernegger and Blau, 2002; Spaapen and Rubio-Gozalbo, 2003; Desviat et al., 2004; Perez-Ducñas et al., 2004). It has also been suggested that BH_4 supplementation combined with the diet would suffice for the prevention of the maternal PKU syndrome (Trefz and Blau, 2003).

Some hypotheses have been proposed for explaining BH_4 responsiveness: 1) some PAH mutant proteins have a reduced binding affinity for BH_4 , which can be overcome at higher BH_4 concentrations; 2) BH_4 helps stabilize PAH by protecting the protein against proteolytic cleavage or degradation, as a chaperon; 3) BH_4 helps stabilize PAH mRNA, and 4) BH_4 regulates PAH gene expression (Erlandsen and Stevens, 2001; Steinfeld et al., 2003; Spaapen and Rubio-Gozalbo, 2003; Blau and Erlandsen, 2004). However, it has been suggested that BH_4 affects the conformational stability of PAH and consequently its activity (Thony et al., 2004).

GENE THERAPY

The ideal treatment of genetic diseases would consist of taking a normal copy of the defective gene and transferring it into the patient's cells, which should express it. PAH gene is expressed mainly in the liver. Vectors derived from a recombinant retrovirus can efficiently transduce the PAH cDNA into PAH-deficient hepatocytes *in vitro*, but the transduction efficiency is low *in vivo* (Eisensmith and Woo, 1996). Beyond this barrier, studies have been initiated in which vectors derived from a recombinant adenovirus expressing PAH cDNA have been placed in the portal circulation of PAH-deficient mice. This approach allowed the restoration of 10 to 80% of hepatic PAH activity, normalizing the plasma phenylalanine levels. Antibodies against recombinant adenoviral vector are a great obstacle to this strategy (Eisensmith and Woo, 1994, 1996; Fang et al., 1994). However, it has been shown that the concurrent administration of an immunosuppressant blocking host immune response prolongs PAH gene expression, and promotes the reversal of hypopigmentation (Nagasaki et al., 1999).

Recombinant adenovirus-associated vectors seem to be safer and more effective. They lead to minimal immune response and produce longer lasting therapeutic effects. This treatment was very satisfactory in male PKU mice, inducing a reduction of plasma Phe level from 1800 to 360 μM in two weeks. Unexpectedly, the treatment was less effective in females and further studies are needed to explain this difference (Kay and Nakai, 2003; Oh et al., 2004).

In addition to conventional gene therapy, some studies with heterologous therapy (PAH expression in tissues other than liver) have been developed for PKU. According to this strategy, epidermal keratinocytes and dermal fibroblasts were engineered by transducing retroviral vectors expressing genes coding for PAH and GTP-cyclohydrolase (one of the enzymes involved in BH_4 synthesis), and high Phe clearance was obtained (Christensen et al., 2000, 2002, 2005a). Moreover, it has been shown that concomitant overexpression of enzymes responsible for additional steps in Phe uptake and metabolism, such as LAT1 and 4F2hc subunits of the large neutral amino acid transporter and tyrosinase, also increases Phe transport into human keratinocytes improving its clearance

(Christensen et al., 2005b). PAH expression in erythrocytic bone marrow, T-lymphocytes and skeletal muscle has also been explored (Lin et al., 1997; Harding et al., 1998, 2003). Skeletal muscle therapy seems promising, but co-expression of BH₄ biosynthesis genes is necessary (Harding et al., 1998, 2004, reviewed by Ding et al., 2004).

In addition to technical difficulties typically associated with gene therapy, some other problems emerge from specific aspects of the illness. Although most PKU mutations lead to loss of function, the protein is frequently present. Allele interactions in PKU are still poorly understood. It is predictable, that in some cases it would also be necessary to inactivate the abnormal alleles.

FINAL COMMENTS

PKU is a hazardous condition and the low-Phe diet undoubtedly changes its course. In spite of that seemingly successful outcome, some patients still have subtle cognitive and emotional abnormalities. However, the worse consequence of a low-Phe diet is the loss of quality of life imposed on the patients and their families. Compliance to a low-Phe diet gradually decreases after infancy. By adolescence, 60 to 80% of the patients have partially or completely abandoned the treatment (Smith and Knowles, 2000; Walter et al., 2002; Merrick et al., 2003). Consequently, PKU therapy still needs to be improved. The pressure created by these difficulties, combined with the great deal of information generated in more than 50 years of PKU research, have produced an interesting situation, in which the perception of the complexity underlying the illness has led to the development of new therapeutic strategies. Some of them are not truly ready to use, such as PAH-based gene therapy. Some others have been submitted to clinical trials for some years now, such as the new Phe-free products and PAL, BH₄, and LNAA supplements.

Low-Phe diet has two main problems. One is the flavor and the smell of many of the Phe-free products. The other is the strong restriction in protein intake with which the patients must comply. The first problem has been successfully addressed by the introduction of more palatable Phe-free products, such as new formulae, tablets or bars. Therefore, a better compliance to the low-Phe diet is expected in the very near future. However, the second problem, that is, the no-almost-everything diet remains.

In this context, PAL, BH₄, and LNAA supplements represent very attractive alternatives where their use allows for a less restrictive diet. However, all of the three are only partially effective alternatives.

PAL-mediated Phe degradation in the intestinal lumen would be a good therapeutic option. In humans, it has only been tested as a purified enzyme, which has the disadvantage of high production costs. PAL-secreting genetically modified organisms offer better economical viability but, have been tested only in animal models. Despite being successful in pre-clinical trials, restrictions in genetically modified organisms commercialization are expected in some countries. Moreover, PKU treatment requires a 70-80% reduction in blood Phe concentrations, which has not been achieved by this method. Therefore, when and if this therapeutic strategy becomes practice, some restrictions in Phe intake will still be needed. Investigations of PEG-PAL have just started, but preliminary results appear promising. The discovery of BH₄-responsive PAH deficiency opened a new perspective in PKU treatment. However, BH₄-responsiveness depends on the presence of some residual enzymatic activity, and therefore, BH₄ supplementation in PAH deficiency is not

indicated for all the patients. BH₄-responsiveness correlates with the mutation present in the patients, but variation among patients bearing the same allele has also been described. This finding suggests that other factors must take part in determining BH₄ responsiveness in PAH-deficient individuals. However, the main difficulty associated with BH₄ supplementation is by far its high, almost prohibitive price. Only at lower costs will the expectation of a widespread BH₄ use in PKU be fulfilled.

A particularly attractive therapeutic option for those patients who do not cope with adhering to low-Phe diet is LNAA supplementation. The results published so far seem to justify an optimistic expectation. Nevertheless, some aspects should be stressed. The results reported up to now refer to a very small sample. Phe levels in the brain decrease slowly and a period of approximately six months is necessary for normal values to be achieved. There is only one article in the literature relating the results of long-term therapeutic tests of LNAA supplementation, and therefore, doses and side effects are still poorly characterized. Moreover, this treatment is not indicated for all patients. It has not been tested in children, and its use by women of reproductive age is not warranted, since its efficacy in preventing maternal PKU embryopathy has not been shown (Lee et al., 2005). However, it is a very important option for older patients, because the quality of life standards achieved are much better than those obtained with a low-Phe diet.

Research based on conventional gene therapy has seen some success, but the lack of truly appropriate vectors for gene transfer is still a limitation to overcome. Heterologous therapy remains far from clinical application due to the need of combining PAH and BH₄ gene expression in tissues. Methods such as ribozymes, triplex DNA, anti-sense RNA, which could help some patients, are not at our disposal. Options allowing some degree of individualization of the treatment are very welcomed.

Perhaps, the most interesting aspect of current PKU therapy research is the diversity of new strategies in development. All of them have advantages and disadvantages, and presently, only BH₄ supplementation allows the patient to completely depart from the diet. It is possible that in the next few years we will treat PKU patients with combinations of a less restrictive diet combined with flavorful Phe-free amino acid supplements, plus LNAAs, and in some cases, plus BH₄. Besides, changing therapy as patients grow older is a very attractive possibility. In the first years of life, the better option is still the low-Phe diet, but alternative treatments should be introduced as patients start to reveal difficulties in keeping to the diet. At this moment, individualization relies on identifying the specific therapeutic combination that better suits a specific patient.

Prospective, controlled studies, with large enough samples, should be developed before generalizing the use of these new therapeutic alternatives. Although the perfect PKU therapy, involving few sacrifices and low costs still does not exist, news on this front promises a significant improvement in the quality of life for patients and their families.

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