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Up to date knowledge on different treatment strategies for phenylketonuria

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Abstract

Dietary management for phenylketonuria was established over half a century ago, and has rendered an immense success in the prevention of the severe mental retardation associated with the accumulation of phenylalanine. However, the strict low-phenylalanine diet has several shortcomings, not the least of which is the burden it imposes on the patients and their families consequently frequent dietary non-compliance. Imperfect neurological outcome of patients in comparison to non-PKU individuals and nutritional deficiencies associated to the PKU diet are other important reasons to seek alternative therapies. In the last decade there has been an impressive effort in the investigation of other ways to treat PKU that might improve the outcome and quality of life of these patients. These studies have lead to the commercialization of sapropterin dihydrochloride, but there are still many questions regarding which patients to challenge with sapropterin what is the best challenge protocol and what could be the implications of this treatment in the long-term. Current human trials of PEGylated phenylalanine ammonia lyase are underway, which might render an alternative to diet for those patients non-responsive to sapropterin dihydrochloride. Preclinical investigation of gene and cell therapies for PKU is

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ongoing. In this manuscript, we will review the current knowledge on novel pharmacologic approaches to the treatment of phenylketonuria.

Keywords

Phenylketonuria; Tetrahydrobiopterin; Sapropterin; Phenylalanine ammonia lyase; Gene therapy

1. Introduction

Phenylketonuria (PKU; OMIM 262600) is one of the most common inborn errors of metabolism. It is a recessively inherited disease caused by mutations in the gene encoding the enzyme phenylalanine hydroxylase (PAH; EC 1.14.16.1) [1]. It is also the first metabolic disorder in which a toxic agent, phenylalanine (Phe), was identified to cause mental retardation, and in which treatment was found to prevent clinical symptoms. The dietary management of PKU was established 60 years ago [2], with the first effects of treatment published in 1953 [3]. The PKU diet consists in a restriction of dietary natural protein in order to minimize Phe intake. It requires supplementation with special medical formulas that supply sufficient essential aminoacids, energy, vitamins and minerals. To avoid mental retardation, the diet should be started in the first weeks of age; therefore, neonatal screening programs are essential in the early identification of these patients. Treatment should be maintained for life, as hyperphenylalaninemia in adulthood has been associated with attention problems, mood instability and white matter degeneration leading to seizures and gait disturbances [4,5]. During pregnancy, moderately high Phe levels in the mother can cause microcephaly, mental retardation and congenital heart defects in the fetus [6].

Dietary treatment has been very effective in preventing severe mental retardation, but it has several shortcomings [5,7]. Even though medical formulas have improved in nutritional quality and palatability over the years, the severely restrictive diet necessary for the treatment of PKU still carries risk of associated nutritional deficiencies. There have been reports of growth retardation and specific deficits such as calcium, iron, selenium, zinc or vitamin D and B12 deficiencies [8–10]. Signs of osteoporosis may develop at an early age [11]. Besides nutritional considerations, the PKU diet imposes a heavy burden, both economical and social, upon the patient and their families [12]. It is therefore not surprising that non-compliance, particularly among adolescents and adults, is common [13,14]. Most disturbingly, it is evident that even early and continuously treated PKU patients may not attain their full neurodevelopmental potential. Treated PKU patients typically exhibit normal intellectual quotients (IQ), but there is, still an IQ gap when compared to their non-PKU siblings or classmates [5,15,16]. Treated PKU patients frequently show delays in certain neurological functions, which likely contribute to poor results in school and long-term employment [17]. Executive function seems to be the most consistently affected area in PKU patients [18–20]. Anxiety, depression and a low self esteem have also been reported [21].

For all these reasons, novel alternative therapies for PKU have been sought. Due to the strong link between neurological outcome and blood Phe levels, most new treatment

approaches will try aim to decrease blood Phe levels. However, clinical objectives are no longer centered exclusively on blood Phe levels, but also on the impact of blood Phe upon brain aminoacid concentrations, early neurodevelopment and long-term neurologic outcome. Attaining normal growth and body mass, and avoiding nutritional deficiencies are other important factors in the consideration of outcome. Finally, the patient's psychosocial well-being has become a key point in PKU treatment objectives, as other approaches lead to non-compliance and eventually important clinical manifestations.

New dietary therapies include more palatable medical formulas, large neutral aminoacids supplementation [22] and the development of medical foods based upon glycomacropeptide, a naturally low-Phe protein [23]. The increasing knowledge of the genetic basis of disease and enzymology has allowed for the investigation of novel pharmacologic therapies to directly ameliorate the effects of a mutant enzyme. For example, pharmacologic chaperones, including the naturally occurring cofactor, 5,6,7,8-tetrahydrobiopterin (BH4), help stabilize misfolded mutant enzymes and prevent proteolysis. BH4 the cofactor of PAH, has been used to treat a subset of PKU patients for almost a decade under experimental conditions. In the past 2–3 years, BH4 has begun to be commercialized in the form of sapropterin dihydrochloride, a synthetic form of BH4, and is beginning to be widely available. Orthotopic liver transplantation successfully cured PKU in a single patient who was transplanted because of cryptogenetic cirrhosis [1]. Therapeutic liver repopulation following hepatocyte transplant continues to be investigated in preclinical models [24]. Clinical gene therapy trials are underway in several inborn errors of metabolism, and preclinical gene transfer experiments continue in PKU animal models. Finally, a novel enzyme substitution approach that utilizes subcutaneous injection of phenylalanine ammonia lyase to metabolize circulating blood Phe, is currently in clinical trial. This paper aims to summarize and review the current knowledge on the pharmacological approach to the treatment of PKU.

2. Tetrahydrobiopterin (BH4)

Tetrahydrobiopterin is the natural cofactor of the PAH enzyme. The lack of BH4 gives rise not only to disfunction of PAH (and therefore hyperphenylalaninemia), but also to disfunction of brain tyrosine and tryptophan hydroxylases, leading to the very severe neurologic manifestations neurotransmitter deficiencies in the different BH4 deficient syndromes [25]. In the 1980s, colleagues began to employ oral BH4 challenges to detect neonates with inherited BH4 deficiencies [26]. Unfortunately, these short-term challenge protocols did not typically detect individuals with of BH4-responsive PAH deficiency, which requires test protocols of longer duration and higher BH4 doses [25]. It was not until 1999 that Kure et al. first showed that in some patients with mutations in the *PAH* gene pharmacological doses of BH4 (20 mg/kg) could reduce blood Phe levels [27]. Since then, 10 to 60% of patients with a deficiency of the PAH enzyme have been found to be BH4-responsive, depending on the mutations prevalent in the different populations [28–36]. Initially, only some centers had access to a non-commercial form of BH4 (Shircks Laboratories, Jona, Switzerland), but since then, sapropterin dihydrochloride was developed and commercially launched after regulatory approval in the US in 2007 and in a growing number of countries in Europe and worldwide since 2008.

Not all PAH-deficient patients respond to BH4 and among those who do, there is a wide range in the extent of the response. In order to assess whether a PAH-deficient patient is responsive to BH4, an oral challenge is employed. Different protocols have been developed. In general, European centers favor shorter tests, similar to that proposed by Blau et al. [37]. This test consists of blood Phe monitoring at 0, 8, 16 and 24 h during 2 consecutive days, while receiving a 20 mg/kg/day. It is recommended that basal Phe levels be $>400 \mu\text{mol/L}$ and that blood Phe levels are closely monitored during the previous days in order to avoid false negative or positive results. A decrease of the blood Phe levels with respect to the basal value of at least 30% is considered a positive response. A negative result would be a reduction of less than 20%, while if the reduction is between 20% and 30% a longer response protocol (1 to 3 weeks), with daily blood Phe concentration measurements, is recommended to establish BH4 responsiveness. In the USA, longer-term challenges are the common. In the protocol proposed by Levy et al., 20 mg/kg of BH4 is given daily for 2 to 4 weeks, with blood Phe samples taken weekly [38]. Cutoff Phe levels are similar to European standards, considering positive a reduction of more than 30%. These are general recommendations that unite the most common trends followed in the different centers, but different goals can be set for individual patients. In general, 48-hour tests detect most BH4-responders if multiple blood Phe measurements are taken. Short-term challenge protocols allow treatment decisions to be made sooner, at a lower cost and with less interference of possible dietary interventions or infectious episodes. On the other hand, longer tests are less likely to miss possible late-responders [39] and can be used to assess other benefits of the treatment such as effects upon neurocognitive function.

Although not generally available, different methods of detecting a response to BH4 exist. The ^{13}C -Phe oxidation test may be a useful and safe diagnostic tool to assess the effect of BH4 on *in vivo* enzyme activity in the individual patient. In this test, the percentage of expired $^{13}\text{CO}_2$ recovered after an oral ingestion of BH4 rises significantly in responsive patients indicating that BH4 improves *in vivo* PAH enzyme activity [29,40].

In general, patients with milder phenotypes have a higher response rate to BH4 than those with low Phe tolerance [29–36]. A mutation database (BIOPKU database), which includes all mutations reported to respond to BH4, is available (www.biopku.org). In Europe, southern countries have the highest predicted response rates based on their most prevalent mutations [34]. It has been shown that missense mutations in the *PAH* gene induce PAH loss of function by protein misfolding, destabilization and early degradation [41,42]. BH4 was shown to act as a molecular chaperone by increasing the stability of partially misfolded PAH proteins and by this the effective intracellular concentration of functional PAH enzyme [43–46]. It is clear, therefore, that for a patient to respond to BH4, sufficient residual liver PAH protein must be present to interact with BH4. This explains why patients with milder forms and milder mutations are more likely to respond than those with null mutations. It also clarifies why as the phenotype worsens, the response to BH4 is often slow and incomplete. Using mutations to predict residual activity has been successful in predicting BH4-responsiveness [36]. However, responses have been described across the phenotypic spectrum of PAH deficiency, and there are also inconsistencies in the response of patients

that share the same genotype, particularly among compound heterozygous (which are the majority of patients) [47,48].

Why are there genotype inconsistencies in the response to BH4? One reason is that different BH4 challenge protocols have been employed by different centers, but these inconsistencies have been described even in the same center. Recent work on PAH enzyme kinetics using a technology that allows for the analysis of a broad range of substrate and cofactor concentrations on PAH activity showed that in addition to the chaperone effect of BH4, kinetic aspects also have to be taken into account [49]. These investigations demonstrate the importance of the relationship between baseline Phe concentrations and the BH4 dose used in the response to a BH4 challenge. In vitro, a given mutation may respond differently when these factors are modified. Since most patients are compound heterozygous, this effect would be magnified. This study suggests that patients with the same genotype can have different responses to BH4 depending on baseline Phe levels or the amount of BH4 administered [49]. In the future, optimal baseline Phe concentrations or the BH4 dose recommendations might have to be individualized. At present, recommendations are standard for all individuals but if a patient with a very suggestive genotype is found to be non-responsive, it is recommended that testing be repeated while taking careful notice of basal Phe levels and/or using longer duration response tests.

BH4 challenge to detect BH4-responsive PAH deficiency is effective at all ages, but there is concern that sensitivity of this testing could be decreased in newborns. After the first 3 months of age, when hepatocytes are sufficiently mature, testing is thought to be reliable. However, testing during the neonatal period has several advantages. It helps in the differential diagnosis of PAH and BH4 deficiencies, but regarding exclusively PKU, it allows the diagnosis of BH4-responsive patients early enough to avoid introducing restrictive diets, favoring breastfeeding and natural protein intake (with better protein, fat, vitamins, microelements and immune composition). During the neonatal period, only short tests (24 h-long) can be used in order to avoid delaying treatment in non-responders. Late-responders can be missed with this protocol; longer term challenges should then be considered at a later age. Although the few publications regarding this subject have not mentioned any side-effects of BH4 in very young patients [50–52], there have been no standardized tests to measure safety in children under 4 years of age. For this reason, the European authorities do not recommend its use in this age group, and the centers that do so must do it under compassionate use.

Once a patient is labeled responsive, considerable effort to fine-tune the therapy is yet required. Continued responsiveness over the long-term must be documented, as fluctuation in blood Phe concentration and unintended alteration of dietary Phe intake during a short-term test may yield false positive results. Improved blood Phe control or a clear increase in dietary Phe tolerance confirm that an individual is truly BH4-responsive, but there is no consensus as to what degree of control or tolerance should be considered as sufficient evidence. BH4 treatment must be closely supervised both by the clinician and the dietician during the first year of treatment.

First, the appropriate individualized BH4 dose must be decided. Some studies suggest that the higher the dose, the greater the reduction on Phe levels [53]. For this reason in the US, the trend is to use sapropterin at 20 mg/kg/day. However, other studies suggest that each patient has a dose over which he does not experience any further benefit [54]. European publications tend to suggest the latter, as the dose is titrated to that at which no further tolerance is reached, and patients receive from 5 to 20 mg/kg/day of BH4 [55–60]. The dose per kilo is not age related, but rather it seems affected by the phenotype/genotype of the patient: the milder the phenotype, the smaller the BH4 dose needed for long-term treatment. As with other drugs, there might be a total daily dose, unrelated to weight, over which there is no additional effect of the medication, but no study has been performed to establish such a measure. The maximum dose for obese and adult patients is being decided following each center's protocol, maintaining a dose of 20 mg/kg/day in some centers, while others use maximum daily doses ranging from 1 g to 1.4 g/day. Few publications indicate that doses higher than 20 mg/kg/day have been tried [60]. No adverse-effects have been reported at these higher doses, but also little additional clinical benefit. It must be noted that animal studies revealed that compound heterozygous mice carrying both a mild and a severe mutation in the *PAH* gene, *Pah^{enu1/2}*, show an inhibitory effect at doses above 20 mg/kg/day, whereas this was not true in mice carrying a mild mutation in the homozygous state, *Pah^{enu1/1}*[61].

Pharmacokinetic studies suggest a peak blood concentration at 2–4 h after ingestion, and a half-life of 6 h[54]. Most patients are treated with single daily dosing, but dividing the total amount in 2–3 daily doses has been suggested to be beneficial for some patients in order to avoid daily Phe level fluctuations [57]. Again, this idea is supported by animal studies, where the *in vivo* effect of BH4 was significantly shorter in the compound heterozygous mouse *Pah^{enu1/2}* than in the homozygous mild phenotype *Pah^{enu1/1}*[61]. Taking the tablets with food, rather than fasting, has also been proven beneficial [62].

After identification of BH4 responsiveness, the diet must be progressively modified until the highest Phe tolerance possible is determined. The first months of treatment require a very close nutritional follow up, in order to explain the necessary dietary changes to the family, make sure they implement them and detect any possible food-phobias or misunderstandings that would lead to nutritional deficiencies in the long-term. The usual approach to determine the new Phe tolerance is to give a small amount of extra phenylalanine per week and closely monitor blood Phe levels. Phenylalanine can be administered as natural food, but some centers prefer initially to give only egg or milk powder, as calculation of the increase Phe is more straightforward and it avoids the introduction of foods the patient might like but not be able to tolerate in the long-term. Special Phe-free formulas should be reduced accordingly to the new protein requirements [63]. There are some suggested guidelines as to how to reach the new diet [64], but frequently the patients' expectations and each center's experience greatly influence the timing and approach to introducing natural foods. In published papers, patients with very mild phenotypes and an important reduction in their Phe levels during the testing period can attain a normal unrestricted diet. However, for patients with more severe phenotypes, a smaller reduction in their Phe levels or a late treatment response, BH4 administration may allow an increase in dietary Phe intake but not fully alleviate the need

for a restrictive diet in the long-term [57]. The successfully pharmacologically treated patient will show an increase in dietary Phe tolerance. If this does not occur it is probably due to a false positive result of the treatment test and long-term therapy should be reconsidered. However, there is no clear recommendation for using tolerance as a marker for responsiveness and suggestions vary from a rise in 2 g of daily natural protein, doubling the baseline Phe tolerance or reaching a completely liberalized diet.

After the initial months, patients can be followed with a similar routine as that of any other PKU patient. Few centers have a longer experience with the drug than for a few years [55–60,65,66], but benefits of BH4 other than just the increase in tolerance are starting to be noted, such as a reduction in Phe level fluctuation [58,67] improved growth [58] and improved nutritional status [68,69]. Changes in neuropsychological or quality of life results, although expected to be good, remain to be documented [70,71]. Large scale studies to evaluate long-term outcomes have been initiated, but results will not be known for several years.

Possible negative side-effects are the greatest concern of any pharmacological therapy. During the use of the previously available non-commercial form of BH4, the only published adverse event was mucositis following sublingual administration [72]. Commercialization studies for sapropterin were mainly designed to determine treatment efficacy but no severe adverse events attributable to the drug were reported. [73–75]. In an extension safety study following patients treated with 20 mg/kg/day for several weeks, it was observed that patients complained more often from headache, nasal discharge and gastrointestinal discomfort, mainly nausea and diarrhea. All these symptoms were reported mild and never required the discontinuation of the therapy [53]. Long-term studies need to be conducted to confirm these results and try to establish if side-effects are dose dependant and if other factors such as the diet, age or concomitant medication are involved.

The use of BH4 during pregnancy is an attractive option. In women determined to be BH4-responsive, sapropterin can help reach the strict blood Phe target levels recommended during pregnancy, something all clinicians know is usually not an easy task. In women with the benign form of PKU, who have never followed a protein-restrictive diet and are not accustomed to the taste of the special formulas, sapropterin could offer the perfect solution to their dilemma. Furthermore, avoidance of dietary protein restriction should allow improved nutritional status of the mother, and hence of the fetus. If BH4 is not widely prescribed during pregnancy, it is due to the scanty existing knowledge of the possible teratogenic effect of the medication. A few cases have been published in which no side-effects for the mother or the baby were noted [76]. However, in this report a very low dose of the medication was prescribed (100 to 300 mg/day when the usual dose for an adult PKU patient is 800–1400 mg/day) and there is no long-term safety evidence. The actual recommendation is that diet should be the first option during pregnancy, but when it cannot be followed and/or blood Phe levels are not sufficiently controlled, sapropterin dihydrochloride treatment can be used.

Many questions remain to be answered regarding the optimum method for evaluating BH4 responsiveness and regarding the long-term benefits of the treatment upon neurological

function, nutritional status and the psychosocial well-being of patients. The possibility of adverse events after prolonged treatment and the safety and/or benefits of this therapy in very young children or women during pregnancy and breastfeeding are still to be evaluated [77]. Now that sapropterin is becoming available worldwide, participation in the different ongoing registries is essential in order to gather sufficient data to reach solid conclusions.

3. Phenylalanine ammonia lyase (PAL)

Enzyme substitution therapy with phenylalanine ammonia lyase (PAL; E.C.4.3.1.5) is currently under intensive clinical investigation as a possible alternative treatment for PKU. PAL catalyzes the deamination of phenylalanine to free ammonia and trans-cinnamic acid (Fig. 1) [78]. In humans, trans-cinnamic acid is safely and rapidly converted to hippuric acid which is then excreted in the urine [79]. In comparison to human phenylalanine hydroxylase (PAH), PAL is structurally and catalytically less complex than PAH, physically more stable, and does not require a cofactor. Prior attempts using oral presentations or subcutaneous implantation of PAL-containing bioreactors were unsuccessful [80–82]. However, simple subcutaneous injection of the PAL enzyme into *Pah^{enu2}* mice, a model of human PKU, yielded complete correction of blood phenylalanine concentrations [83] and this effect was sustained for up to a year with weekly enzyme injections. PAL from the algae *Anabaena variabilis* proved physically more resistant to proteolytic degradation, less prone to aggregation, and catalytically more favorable than PAL from other species [84]. Attachment of polyethylene glycol polymers to lysine side chains exposed on the surface of the enzyme (PEGylation) assist to elude immune-mediated detection and elimination of the injected enzyme [85]. Based upon these results, BioMarin Corp., Novato, CA has chosen to move enzyme substitution therapy with PEGylated recombinant *Anabaena variabilis* PAL (rAvPAL-PEG) into clinical trial. In a Phase 1 trial designed to evaluate safety of subcutaneous rAvPAL-PEG injection and carried out at seven centers in the US, twenty-five adults with PKU and blood phenylalanine >600 µM received a single rAvPAL-PEG injection in escalating dose cohorts. No severe adverse events were recorded and in the highest dose cohort, blood phenylalanine levels decreased significantly after a single rAvPAL-PEG injection (unpublished data). A multi-center Phase 2 clinical trial to assess the safety and efficacy of multiple, repetitive rAvPAL-PEG injections is currently underway. Meanwhile, efforts to develop an effective orally administered rAvPAL-PEG have also been initiated [86].

4. Gene therapy

The cloning of the human and mouse PAH cDNAs was reported in the 1980s [87]. Early investigations showed that only reaching about 10% enzyme activity is necessary to attain normal Phe metabolism in PAH-deficient mice [88] and recent gene transfer studies using murine models are promising. A recombinant adeno-associated virus (rAAV) vector is frequently employed which produces a lesser immune response than vectors used in early experiments, giving rise to fewer side effects and allowing for longer-term successful treatment. In the mouse model, liver directed gene therapy has allowed for up to one year correction of hyperphenylalaninemia [89,90]. However, liver-directed gene therapy does not lead to a permanent correction of PAH activity. The vector's genome is not integrated in the

hepatocyte's DNA and gradual but continuous hepatocyte regeneration eventually leads to elimination of episomal rAAV vector genomes. Reinjection of the same serotype vector is not effective, as it is destroyed by an antibody-mediated immune reaction.

Skeletal muscle is an attractive target organ for gene therapy because it does not suffer on-going cell division and it is more easily accessible. On the other hand, PAH expression alone in myocytes will not lead to functional Phe hydroxylation; expression of enzymes involved in the BH4 synthetic pathway and local production of BH4 is also required. This has been tried with promising results in mice [91,92].

The improvement in viral vector design has led to human gene therapy trials for other inborn errors of metabolism, including α 1-antitrypsin deficiency, Canavan disease, late-infantile neuronal ceroidlipofuscinosis and lipoprotein lipase deficiency [93]. Ongoing research, optimizing the muscle-directed approach and improving sustainability in liver-directed gene therapy might lead to human trials in PKU in the next few years.

5. Conclusions

Neonatal diagnosis of PKU, early institution of a Phe-restrictive diet and the possibility of avoiding devastating brain damage associated to hyperphenylalaninemia was a great success. Nevertheless, the difficulties of adhering to a strict diet for life and the presence of neurological deficits despite treatment make other therapeutic approaches indispensable. The past decade has been a very exciting time for investigators, clinicians and PKU patients, as the knowledge on the pathogenesis of the disease has been broadened and this has allowed for the investigation of truly novel therapeutic approaches. In some cases, such as dietary products or tetrahydrobiopterin, these novel treatments are already available in the clinical setting. Human trials are underway with rAvPAL-PEG enzyme substitution. Pre-clinical studies are being done in gene and chaperone therapy. Many questions regarding existing therapies remain to be answered and much difficult work has to be done before many of these novel technologies reach the patient, but the future of PKU treatment has never seemed brighter. In the future, different therapies will be available allowing individual treatment depending upon the particular genotype and other personal circumstances such as age or pregnancy.

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Fig. 1. Differences between phenylalanine hydroxylase and phenylalanine ammonia lyase. PAH requires the presence of its cofactor, BH₄, as well as a Fe⁺ molecule and oxygen. PAL does not need a cofactor. Adapted from [78].