Clinical therapeutics for phenylketonuria

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REVIEW ARTICLE

Clinical therapeutics for phenylketonuria

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Abstract Phenylketonuria was amongst the first of the metabolic disorders to be characterised, exhibiting an inborn error in phenylalanine metabolism due to a functional deficit of the enzyme phenylalanine hydroxylase. It affects around 700,000 people around the globe. Mutations in the gene coding for hepatic phenylalanine hydroxylase cause this deficiency resulting in elevated plasma phenylalanine concentrations, leading to cognitive impairment, neuromotor disorders and related behavioural symptoms. Inception of low phenylalanine diet in the 1950s marked a revolution in the management of phenylketonuria and has since been a vital element of all therapeutic regimens. However, compliance to dietary therapy has been found difficult and newer supplement approaches are being examined. The current development of gene therapy and enzyme replacement therapeutics may offer promising alternatives for the management of phenylketonuria. This review outlines the pathological basis of phenylketonuria, various treatment regimes, their associated challenges and the future prospects of each approach. Briefly, novel drug delivery systems which can potentially deliver therapeutic strategies in phenylketonuria have been discussed.

Keywords Phenylketonuria · Phenylalanine · Hyperphenylalaninemia · Phenylalanine hydroxylase · Phenylalanine ammonia lyase

Abbreviations

BH4	Tetrahydrobiopterin
DHPR	Dihydropteridine reductase
DNA	Deoxyribonucleic acid
EC	Enzyme Commission
EMEA	European Medicines Agency
ENU	Ethylnitrosourea
ERT	Enzyme Replacement Therapy
FDA	Food and Drug Administration
GMP	Glycomacropeptide
GTPCH	Guanosine triphosphate cyclohydrolase
HPA	Hyperphenylalaninemia
IQ	Intelligence quotient
LNAA	Large neutral amino acid
OMIM	Online Mendelian Inheritance in Man
PAH	Phenylalanine hydroxylase
PAL	Phenylalanine ammonia lyase
PCD	Pterin-4a-carbinolamine dehydratase
PEG	Polyethylene glycol
Phe	Phenylalanine
PKU	Phenylketonuria
qDHB	Quinonoid dihydrobiopterin
t-CA	*Trans*-cinnamic acid
Tyr	Tyrosine

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Introduction

Phenylketonuria (PKU; OMIM 261600) is a known metabolic disorder linked to high concentrations of phenylalanine (Phe) in body and tissue fluids. The disease was first characterised by Asbjorn Folling as 'imbecillitas phenylpyruvica' in two young siblings in 1934 in Oslo, Norway, where Folling's perseverance led to the establishment of the link between PKU and high concentrations of urinary Phe [1]. Subsequently generated interest and intensive research around the globe in the past 75 years has led to revelation of the clinical problem at the genetic level. Kwok et al. were the first to isolate phenylalanine hydroxylase (PAH) complementary DNA clone from human liver [2]. This paved the way for research, which gained momentum in the last two decades

resulting in global databases being established [3, 4]. The databases can be accessed at http://www.pahdb.mcgill.ca and www.biopku.org. PKU has been ascribed to mutations in the gene coding for hepatic PAH (EC 1.14.16.1) enzyme. About 560 such mutations have been identified in the *PAH* gene in accordance to the database.

In a normal subject, 25 % of the free pool of Phe is shunted to protein synthesis, whereas the rest of it is hydroxylated by hepatic PAH to tyrosine (Tyr) and a small fraction is transaminated to phenylpyruvic acid (PPA) [5, 6]. This hydroxylation of Phe to Tyr in the presence of PAH requires another enzyme dihydropterin reductase and two co-factors tetrahydrobiopterin (BH4) and nicotinamide adenine dinucleotide (NADH), in the presence of oxygen and iron [7, 8]. In this reaction, BH4 is converted to quinonoid dihydrobiopterin (qDHB), from which BH4 is regenerated by dihydropteridine reductase (DHPR) and pterin-4a-carbinolamine dehydratase (PCD) [8, 9].

Hyperphenylalaninemia (HPA) may be either due to PAH or BH4 deficiency (Fig. 1). In PAH's absence (classical PKU) or reduced activity as in case of non-PKU HPA, the free pool of Phe in a subject increases, which leads to the building-up of surplus Phe. PAH deficiencies are characterised by autosomal recessive inheritance of a mutant *PAH* gene, leading to changes in enzymes kinetics and altered affinity towards Phe and BH4 [10]. On the other hand, BH4 deficiencies are rare inherited neurological disorders, with nearly 193 mutant alleles or molecular lesions being identified in the various enzymes involved in its biosynthesis (GTP cyclohydrolase I, GTPCH and 6-pyruvoyl tetrahydropterin synthase (PTPS)) and regeneration (DHPR) [11]. Since PAH is a BH4-dependent aromatic amino acid hydroxylase, BH4 is an important cofactor for the conversion of excess Phe to Tyr. Hence, the subnormal activity of either PAH, BH4 (and its biosynthetic enzymes GTPCH and PTPS) or BH4 regenerative enzyme DHPR, may lead to elevated Phe concentrations in the blood (Fig. 1).

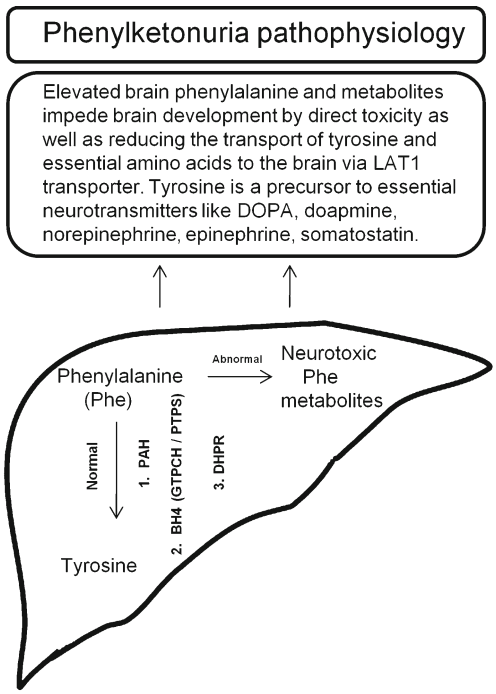

Fig. 1 Pathophysiology of phenylketonuria. A deficient (1) phenylalanine hydroxylase (*PAH*) or (2) its co-factor tetrahydrobiopterin (*BH4*) due to mutant enzymes involved in its biosynthesis (*GTP* cyclohydrolase I, *GTPCH* and *PTPS*) or its regeneration (3) dihydropteridine reductase (*DHPR*), lead to accumulation of phenylalanine (*Phe*) and its neurotoxic transaminated metabolites, which enter the brain and lead to cognitive impairment. During normal Phe metabolism, Tyr is formed, which acts as a precursor for vital neurotransmitters like DOPA, dopamine, norepinephrine, epinephrine and somatostatin

Although being an essential amino acid and a key molecule for protein synthesis, Phe has been shown to possess neurotoxic potential [12]. Elevated concentrations of Phe have been associated with impaired cognitive development in children and lead to mental retardation, microcephaly and seizures. PKU has also been associated with certain motor disturbances and skin abnormalities thus requiring an immediate reduction in systemic Phe concentrations [13]. Elevated serum Phe concentrations have been shown to be associated with oxidative stress as measured by low antioxidant enzyme activities and antioxidant concentrations in blood [14]. Also, in the absence of Phe, the onus of neurotransmitter biosynthesis is on Tyr which has to be supplemented in the diet. Tyr is a precursor to several important neurotransmitters synthesised in brain. Anomalous PAH affects catecholamine and serotonin biosynthesis [15]. Exogenous administration of amino acids such as Tyr, tryptophan, threonine, isoleucine, valine, methionine and histidine thus becomes essential [7]. It is imperative to maintain a balance between the Phe concentration, without causing excessive depletion and providing adequate concentrations of Tyr.

The severity of HPA can be used as a basis to classify PKU [16]. The normal circulating concentrations of Phe are 50–110 μmol/l. Individuals having Phe blood concentrations between 120 and 600 μmol/l before initiating any therapy are classified under mild (non-PKU) HPA group. Those with a concentration range of 600–1,200 μmol/l fall under the category of mild PKU and those exceeding 1,200 μmol/l are the cases of classical PKU. However, this classification is ambiguous and differences exist in the regulations amongst different countries. Whether or not a particular case requires therapeutic intervention, depends on these regulations [17–19]. A compilation of these regulations has been provided in a recent review by Feillet et al. [20]. Prevalence of PKU varies in different regions in the world from 1 in 10,000 in Europe to 1 in 15,000 in USA. PKU is less prevalent in Africa and Asia with only 1 in 70,000 to 200,000 cases reported [21].

A special low Phe diet has been characteristic of PKU management since it first became available in the middle of the twentieth century; some two decades after PKU was first discovered [13, 22]. Guidelines for instituting such a low protein diet have been based primarily on the neonatal screening of PKU [23]. Developed by Dr. Robert Guthrie, the test has been the mainstay of PKU diagnosis. It involves a simple bacterial inhibition test [23, 24], based on a semi quantitative measurement of bacterial growth in the presence of Phe. The test is usually carried out on a 3- to 5-day-old neonate, whereby blood samples are withdrawn by a small puncture in the heel of the infant onto a 'Guthrie filter paper'. Development of tandem mass spectrometry in the last two decades has increased the pace of screening, improving its credibility and making it appealing to wider category of healthcare professions [25].

Diagnosis based on BH4 loading test has also been recommended in determining the specific pathology and distinguish between BH4 responsive PKU and DHPR deficiency [26–28], which has been presented in reviews by Blau et al. [21, 29]. Recently, cerebrospinal fluid analysis has been used to analyse the concentrations of 5-hydroxyindoleacetic acid and homovanillic acid as well as ratios of various neurotransmitters as a marker of BH4 deficiency [30]. In addition, DNA analysis has been adopted for some pilot studies in the newborn screening for severe combined immunodeficiency diseases and is expected to be used for PKU screening in the future [31].

The neonatal screening programs which have been made mandatory in many countries to recognise specific inborn errors of metabolism in infants [32] have helped a great deal in instituting a low Phe diet [20]. Specific algorithms have been devised by various healthcare organisations to help physicians to decide the course of action and to start a low protein diet. An example of such an algorithm is presented in Fig. 2 [33].

Although PKU was among the first metabolic disorders to be recognised, its cure still remains elusive. Till recently, dietary management was the only measure used to control Phe concentrations, with therapy started during the postnatal period and continued until normal cognitive development is observed. In recent years, several other therapies have been suggested by researchers. These include newer dietary supplements such as tetrahydrobiopterin, glycomacropeptide and large neutral amino acids. These supplements aim to increase the palatability of the low protein diet and achieve better therapeutic control. The dietary approach aims to maintain the plasma Phe concentrations within an acceptable range. Other strategies such as gene therapy offer a potentially permanent solution for the genetic anomaly by correcting the genetic sequence coding for the PAH enzyme. A novel approach of either replacing the endogenous PAH or substituting it with phenylalanine ammonia lyase (PAL), has been explored and offers an exciting avenue in PKU management. Currently, clinical trials are going on to determine the human dose and safety of administering subcutaneous formulations of PAL [34].

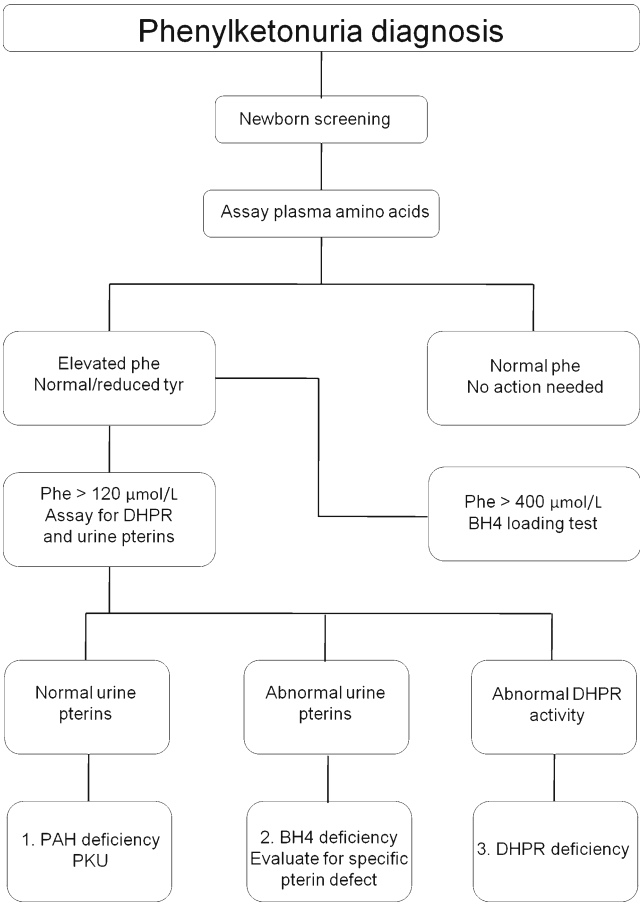

Fig. 2 Diagnostic algorithm for determining the course of therapy following newborn screening in PKU. The lack of enzyme (1) PAH leads to classic PKU, whereas lack of (2) BH4 and (3) DHPR also lead to elevated Phe concentrations, necessitating therapeutic intervention

Although there have been several reviews published previously [7, 16, 35–37], by various research groups, they have either focused on the treatment modalities developed and researched by them or have tried to present all possible treatment modalities, with a rather brief description of each. Sarkissian et al. developed oral and parenteral formulations for delivering enzyme PAL and their reviews reflect more on the benefits of enzyme substitution with PAL [36, 37]. On the other hand, Eavri and Lorberboum-Galski focussed on enzyme replacement with the endogenous enzyme, namely, PAH [13]. Their review is thus inclined towards the therapeutic effects of PAH. Other reviews [16, 35] pursue a general outlook with brief details of all aspects.

In this review, we discuss the various therapeutic approaches including the classical dietary management, which have been used in the management of PKU (Fig. 3).

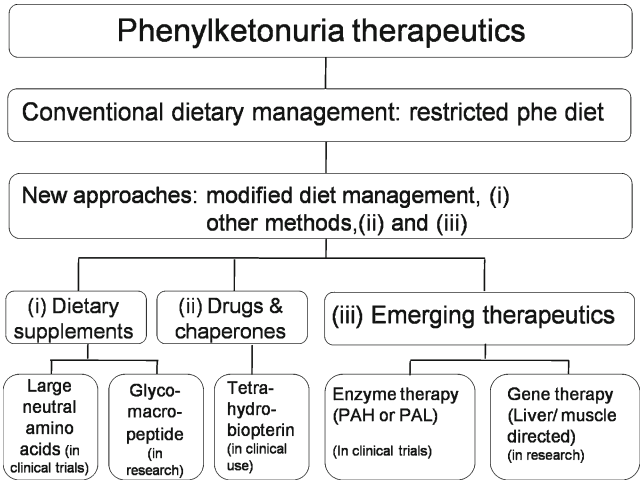

Fig. 3 Therapeutic regimens in PKU. Classical dietary management has been supplemented with new supplements like tetrahydrobiopterin (in clinical use, KUVAN™), LNAAs (in clinical trials) and glycomacropeptide (in research). Gene therapy (both liver and muscle directed) and enzyme therapy with phenylalanine hydroxylase (*PAH*) and phenylalanine ammonia lyase (*PAL*) have made rapid strides in research and aim to offer a lasting solution for management of PKU

It is sectioned into two broad categories: the classical dietary management with its recent developments and the emerging trends in PKU management, namely, gene and enzyme therapy. We review PKU management from therapeutic strategies used by clinicians over the years to the promising newer approaches from research labs, making this review suitable for scientists and clinicians. We look at potential drug delivery approaches that can be adapted in the management of PKU. As such, we attempt to provide an unrestrained view of all approaches, discussing the *pros* and *cons* of each.

Classical dietary management

Stage of development: clinical application

Since Folling established the connection between elevated serum Phe and cognitive derangement in his patients, the primary therapeutic approach has been to lower the elevated Phe concentrations to be within the therapeutic range as higher Phe concentrations are considered toxic. Still regarded as the first line therapy in PKU, the diet was first introduced by Bickel et al. in 1953, in a girl who was unable to stand, walk or talk. She showed classical symptoms of PKU with elevated Phe concentrations. Low Phe diet with normal concentrations of other amino acids showed significant therapeutic effect [22]. This was a breakthrough in the management of PKU and several modifications to the diet soon followed [38, 39], in pursuit of better therapeutic control. The diet has primarily consisted of restricted amounts of proteins and amino acids (especially protein-rich foods such as meat, dairy products and grains). The treatment can be made more efficient by starting the therapy as early as possible after neonatal screening. This resulted in improvements in cognitive behaviour and intelligence scores [40].

Over the years there has been a considerable improvement in the low Phe diet; still concerns have been raised over the compliance to such a restricted diet, notably in adults. A low Phe diet has been associated with unsatisfactory organoleptic properties, due to the uncharacteristic odour and bitter taste of amino acids [41, 42]. Compliance to such a strict regimen is laborious and demanding. It has been reported in a recent study that over 80 % of the patients between 15–19 years of age had elevated serum Phe due to dietary non-compliance [43]. Another recently concluded study in patients aged 3–18 reported only 56 % of the patients to be adherent to the dietary regimen, measured by reviewing their 3-day food records. An associated reduction in quality of life was also reported in these patients [44]. Although some studies have reported normal quality of life and capacity of performance in patients receiving special diet, behavioural patterns, such as reduction of positive emotions, lower autonomy and a lower propensity to form relationships remained [45, 46]. In addition, mood swings and lower sustained attention have been shown to be associated with individuals who consume a high Phe diet. The authors thus recommended a low Phe 'diet for life' in such patients [47]. Another recent study revealed patients with PKU had a lower propensity to have children as compared with normal population [48].

Although the diet has been effective in maintaining acceptable plasma Phe concentrations, some patients showed lower IQ levels and a certain degree of neurological derangement [49]. In addition, specialised diet is devoid of certain pivotal nutrients such as vitamin B_{12}, vitamin D, iron, calcium and certain fatty acids [50–52], all of which may impact on normal neurological and physical development [53, 54]. Particularly, conformance to the diet in pregnant women is a critical concern as high concentrations of Phe in the fetus have been branded as teratogenic and expecting females have to return to a restricted diet (low Phe diet that may be relaxed in individuals with PKU once they attain normal cognitive development) to ensure healthy foetal development [55].

The current goal in the dietary management of PKU is to make the diet more palatable and to find alternatives to classical low amino acid diets, so that patients are willing to commit themselves to a longer and more aggressive treatment regime [56, 57]. Some dietary supplements included in the low-Phe diet, have resulted in better therapeutic control of Phe concentrations and these are now recognised as vital for PKU management. These supplements are considered as newer approaches for the management of PKU.

Newer approaches in dietary management

Tetrahydrobiopterin and other pharmacological chaperones

Stage of development: clinical application

The (6R)-L-erythro-5,6,7,8-tetrahydrobiopterin (BH4) is a cofactor in the hydroxylation of Phe to Tyr by PAH (Fig. 1). Elevated Phe concentrations in patients had been either associated with classic PKU (lack of PAH) or with inadequate synthesis or recycling of BH4 [58–60]. BH4 deficiency accounts for approximately 2 % of the high Phe concentrations detected during newborn screening [61]. Low concentrations of serum BH4, due to deficient BH4 metabolism can be detected by decreased pterins in urine or reduction in activity of enzymes involved in BH4 biosynthesis (GTP cyclohydrolase I and PTPS) and BH4 regeneration (dihydropterdine reductase) [28, 62]. BH4 supplementation in these conditions called as 'malignant hyperphenylalaninemia' was used by Danks et al. [63].

However, in certain non-PKU HPA phenotypes BH4 synthesis and regeneration is normal, but these patients have exhibited an increased PAH activity in presence of higher amounts of BH4 [64]. Such a condition is termed as 'BH4-responsive HPA' and the individuals show normal hydroxylation of Phe to Tyr through the classical PAH pathway [65, 66]. This phenotype usually demonstrates a milder PAH deficiency while those with a major PAH derangement or complete lack of PAH activity may show mild improvement [26, 67]. Several mechanisms have been proposed for the reduction in Phe concentrations, with some researchers suggesting an increase in enzyme stability [68] while others adducing an increased affinity of the higher amounts of BH4 to the catalytic domain of PAH [69].

Many pharmacological chaperones, which are small molecules improving protein stability by rectifying protein folding, have been tried in in vitro studies considering PKU as a protein misfolding disorder [70]. Several researchers have shown stabilisation of misfolded protein by BH4 therapy. Aguado et al. demonstrated the chemical chaperone effect of BH4 in stabilising mutant PAH proteins and reduction in PAH degradation rate [71]. Pey et al. carried out a detailed analysis of kinetic and binding properties of BH4 in human recombinant PAH and seven PKU mutations. BH4 prevented the degradation of protein folding variants, proving its effect as a chemical chaperone [72]. They also performed a high throughput screening with more than 1,000 pharmacological compounds and found four compounds which enhanced the thermal stability of PAH. Two compounds, namely, (3-amino-2-benzyl-7-nitro-4-(2-quinolyl)-1,2-dihydroisoquinolin-1-one) and (5,6-dimethyl-3-(4-methyl-2-pyridinyl)-2-thioxo-2,3-dihydrothieno[2,3-d] pyrimidin-4(1H)-one) have been shown to stabilise the function forms of wild type PAH and other mutants [73]. A mini review detailing the kinetic and stability analysis in phenylketonuric mutations has been published by Perez et al. [74].

Although BH4 supplementation was shown to be effective in patients with mild PKU, some form of dietary restriction may need to be perused. BH4 can be added to the modified diet as it can be taken orally [75]. Success in the clinical trials has led to Food and Drug Administration (FDA) approval of first drug in the management of PKU [76]. Sapropterin hydrochloride (Kuvan™), a synthetic BH4, is on the markets in the USA since December 2007. The product has also been approved by European Medicines Agency and is also marketed in several European countries. Phase II and phase III clinical trials have already proved the utility of this approach [77–79]. In a randomised, double-blind, placebo-controlled clinical trial, BH4 responsive patients showed a dose-dependent reduction in blood Phe concentrations. In a subgroup of patients receiving 10 mg kg^{-1} day^{-1} of sapropterin, 28 % reduction as compared with placebo group was observed [80]. Results from a recently concluded phase III clinical study to evaluate to the long-term (2 years) safety of sapropterin reported it to be a safe and well tolerated drug [81]. Sapropterin therapy may also offer relaxation in the strict dietary regimen in patients with mild PKU [82]. Combination of sapropterin with a low-Phe diet increased the stability of blood Phe concentrations [83] and may improve tolerance to dietary Phe [84]. A recent study in 45 children with PKU reported an increase in tolerance to protein diet, with subjects showing a 2.6-fold increase in Phe intake [85].

Since 1–3 % of the patients with HPA suffer from BH4 deficiency, co-factor supplements are expected to be the mainstay therapy for these patients and in patients with 'BH4 responsive HPA'. At present, the cost of BH4 therapy is relatively high ($70,000/annum) [86] coupled with its short half life (3.3–5.1 h) necessitating frequent dosing, which further accrues treatment cost [87]. Also, further studies ensuring long-term safety are needed especially during pregnancy [88]. Going forward, development of affordable forms of BH4 substitutes or sustained release dosage forms may result in the reduction in the cost of therapy. Classical dietary therapy and BH4 supplements should be contemplated in patients showing improvement in Phe concentrations.

Large neutral amino acids

Stage of development: clinical trials

Although the mechanisms by which higher concentrations of Phe lead to brain damage and neurocognitive defects remain unclear, normal cognitive behaviour has been noted in certain patients with classical PKU [89]. Despite high blood concentrations of Phe, cerebral Phe concentrations

remained normal inkling towards a possible mechanistic phenomenon in Phe transport across the blood–brain barrier [90, 91]. L-type amino acid carrier (neutral amino acid transporter 1 (LAT1)) has been implicated in the movement of Phe from blood to cerebral circulation [92]. In PKU, higher affinity and concentration of Phe competitively inhibits the transport of other large neutral amino acids (LNAAs), resulting in a brain concentration decline of these essential amino acids, mainly Tyr, methionine and tryptophan, which have been recognised to be essential for brain development [93, 94]. This reduces dopamine synthesis, coupled with reduction in protein synthesis and demyelination [95].

Higher concentrations of other LNAAs were shown to inhibit the passage of Phe to the brain through the LAT1 transporter in an animal model [96]. Peitz et al. studied the effect of LNAA supplementation in six PKU patients and observed a decline in brain Phe concentrations with additional LNAA supplementation over the control group [97]. A similar competitive inhibition of the LAT1 transporter occurs at the intestinal level as LNAAs cross the intestinal mucosa by the same carrier-mediated pathway [98]. Administration of LNAAs in the diet also tends to lower the amount of Phe absorbed from the gut as the amino acid transporters are also present in the intestinal mucosa, leading to a reduction in blood Phe concentrations [99]. A double-blind placebo-controlled clinical trial showed a 39 % reduction on blood Phe concentrations, establishing the evidence of beneficial effects of oral supplementation of LNAAs [100]. However, van Spronsen et al. suggested that decreased blood Phe concentrations might be a result of increased protein synthesis due to availability of increased quantities of essential amino acids, as the clinical studies did not take the natural protein intake into consideration and the subjects under study might have essential amino acid deficiencies [101]. LNAA supplementation has also shown improvements in cognitive functions and executive capabilities like verbal generativity. However, higher anxiety levels were reported to be associated with LNAA supplementation [102].

An important outcome from clinical studies with LNAAs was that they were of limited value in patients responding well to dietary therapy. Those unable to comply with the strict dietary regimen may benefit at large from LNAAs supplementation. LNAAs may inhibit the transport of Phe from intestines to blood and further from blood to brain, maintaining safe plasma Phe concentrations. In future, clinical toxicology studies are needed to establish the safety on long-term usage of these supplements. Other potential amino acid transporters need to be explored for their role in Phe transport. Although many clinical trials have proved their efficacy, these dietary supplements are not governed by the legislations that apply to pharmaceutical products and hence clinical use of these dietary supplements is at the discretion of the physician. Clearly, the concept of using LNAAs is sound and should have a definite role to play in the management of PKU.

Glycomacropeptide

Stage of development: research

A recent improvement to increase the palatability and lack of satiety with the classic low protein diet for PKU has been glycomacropeptide (GMP), a 64-amino acid glycophosphopeptide, derived from goat milk during cheese production. This protein contains minimal amount of Phe (only about 2.5–5 mg/g of protein) as compared with other natural protein foods which contain 2.4–9 % of Phe by weight [103]. As the taste of conventional amino acid formulae is the biggest hurdle in the dietary compliance, GMP serves as a potential alternative with better organoleptic properties. Also, it has been reported that the concentrations of LNAAs like isoleucine and threonine are 2- to 3-fold higher than conventional formulae [104], with limiting amounts of histidine, leucine, tryptophan and Tyr. These LNAAs are expected to further reduce the intestinal absorption of Phe and contribute further to reduction of blood Phe concentrations.

Ney et al. showed that patients had a more satisfied feeling with a GMP-based breakfast as compared with the conventional amino acid formulae, as measured by a dip in postprandial ghrelin concentrations, an appetite stimulating hormone [105]. This allows patients to space their amino acid intake throughout the day, offering better utilisation for protein synthesis [106]. Although a recent clinical study comparing GMP to the conventional diet showed no significant decrease in the serum Phe concentrations, patient acceptance was markedly greater [107]. In another report published recently, a patient on GMP diet showed 13–14 % reduction in Phe concentrations as compared with classical amino acid diet, over a period of 10 weeks [103]. Still in infancy, research efforts in developing newer GMP food supplements may potentially lead to better patient acceptance.

Emerging trends in PKU management

Two potential treatment approaches in the management of PKU have come to fore recently. Possibly replacing the defective gene sequence for PAH is the ideal way for correcting the metabolic derangement in PKU. On the other hand, supplementation of the enzyme PAH or its substitute PAL through various efficient delivery systems has also been studied. Both approaches have shown promises in animal models and/or clinical trials.

Gene therapy

Stage of development: research

Ultimate treatment strategy for any genetic disorder would aim to solve the anomaly at the basic level. In PKU, liver transplant although being a desirable option, is not viable due to lack of donors and remains the choice only in very rare cases [108]. The possibility of replacing the mutated DNA sequence coding for PAH gene with a wild-type, non-mutant, copy of PAH gene, spells hope for PKU patients. Since the determination of the nucleotide sequence for the entire enzyme in 1985 [2] and further research on its expression at cellular level [109], past two decades have seen intensive research efforts in the PKU mouse models using different modes of gene transfer.

Specific vector-mediated DNA integration in liver or muscle has been investigated, which has been supported by the availability of various kinds of vectors, including recombinant adenovirus [110, 111] and recombinant adeno-associated virus vectors (rAAV) [112–114]. Adenovirus vectors, although successful in correcting the liver PAH activity, have been reported to suffer from the drawback of immune rejection of adenovirus-transduced hepatocytes. On the other hand, rAAV vectors have demonstrated the ability to resist immune rejection and resulted in longer lasting liver PAH expression without the need for chromosomal integration [9, 115]. They have also been reported to be safe [116].

Liver-directed gene transfer using various adeno-associated virus (AAV) vectors showed promise despite varying degrees of transduction efficiencies and gene expression levels within different serotypes. A compilation of various serotypes has been reported recently by Jacobs and Wang [115]. Although several serotypes, AAV1, AAV2, AAV5, AAV7, AAV8 and AAV9, were reported in literature for correction of various inborn errors of metabolism, only AAV2, AAV5 and AAV8 have been used in phenylketonuria.

A Pah^{enu2} (PKU) mouse model (PAH-deficient strain), produced by chemical mutagenesis using *N*-ethyl-*N*-nitrosourea (ENU) [117] was used in these studies. Earliest conceptual evidence in this arena was provided by the work of Cristiano et al. in 1993 [118]. Fang et al. reported normalisation of serum Phe concentrations within 1 week and only 10–20 % of enzymatic activity was good enough to restore Phe concentrations [111]. Long-term correction of HPA using rAAV vector for transfecting murine PAH cDNA in PKU mouse showed significant improvement in locomotor activity and exploratory behaviour in a 12-month-old mouse [114]. The effect, however, persisted only in male mice, while females returned to the pretreatment levels. In these studies with rAAV vectors, two major shortcomings have been recognised. Firstly, a higher dose of AAV vector is needed to correct the PKU phenotype and secondly, there is a gender-dependent effect, with female mice requiring higher doses [119].

Pseudotyped rAAV vectors with AAV5 and AAV8 capsids from other species have been found to be more effective. Portal vein injection of AAV2/8 serotype has been demonstrated to stabilise serum Phe concentrations in Pah^{enu2} mouse models for up to 17 weeks [112]. Ding et al. pseudotyped the vectors with capsids from AAV serotype 8, resulting in a sex-independent decrease in Phe concentrations in PKU mouse [120]. Rebuffat et al. investigated the hepatic PAH activity and Phe clearance upon intramuscular injection of rAAV2 pseudotype 1, 2 and 8 vectors. It was reported that long-term effects only existed with pseudotype 1 and 8. With type 8, Phe concentrations remained within the therapeutic range in males throughout the period of 1 year, whereas it started to rise at 8–10 months in females, although the increase was marginal and managed by either giving synthetic BH4 or administering a vector of another type to prevent immune response [121]. Yagi et al. constructed an AAV8 pseudotyped vector using a self complementary AAV genome. This resulted in efficient restoration of Phe concentrations over a period of 1 year, with a gender-independent effect [122]. This has been the longest period reported among the studies intending to correct the genetic defect.

Another interesting approach has been the application of gene therapy to muscle. However, as muscle is not the primary organ of Phe hydroxylation, the enzyme system has to be comprised of the additional enzymes and co factors in addition to PAH. Additional nucleotide sequences coding for BH4 synthesis and regeneration need to be added [123]. Ding et al. recently proposed an integrated Phe hydroxylating system composed of PAH as well as BH4 synthetic enzymes (guanosine triphosphate cyclohydrolase I and 6-pyruvoyltetrahydrobiopterin synthase). This was achieved by using a recombinant triple-cistronic AAV2 pseudotype 1 vector. A sustained and longer reduction in blood Phe concentrations was noticed with increase in pigmentation of skin [124]. An advantage of this approach is the ease of access for vectors as compared with liver-directed gene therapy [35].

Recent advances in novel drug delivery systems, particularly using inhalational and transdermal routes have been explored for delivering genes. Transdermal protrusion array device which was used to successfully deliver luciferase reporter plasmids resulted in the expression of cells in the skin. They were also used to silence the reporter gene expression in skin [125]. Pearton et al. used silicon microneedles followed by application of pDNA hydrogels for mediating gene expression [126]. In addition, spray-dried formulations

of small interfering RNA have been prepared with the intention of delivering this molecule via the inhalational route [127]. Such approaches may in future be applied for delivering gene therapeutics directed towards liver or muscle in the management of PKU.

Although there are some ethical issues surrounding the application of gene therapy, the concept is far more convincing. The conceptual evidences from animal studies for gene therapy of PKU have laid the foundation for what is expected to be the lasting solution for PKU patients. A consensual effort from several disciplines to ensure the efficacy and safety of this approach may have a profound outcome in the management of PKU.

Enzyme therapy

Stage of development: clinical trials

Majority of metabolic diseases result from anomalies in the enzymatic function in the metabolic pathways [128]. Enzyme replacement/substitution which offers a promising alternative has been investigated for various lysosomal storage disorders like Gaucher disease [129, 130], Fabry disease [131], mucopolysaccharidosis [132, 133] and Pompe disease [134]. Enzyme replacement therapy (ERT) employing enzyme glucocerebrosidase has been approved by FDA in the management of Gaucher disease [135]. Enzyme replacement in PKU is an appealing prospect as majority of the 560 mutations identified in the *PAH* gene lead to a dysfunctional enzyme [128].

Since PAH is the endogenous enzyme involved in Phe metabolism, it comes as a natural choice for enzyme based therapeutics in the management of PKU. Although propitious, protein-based therapeutics face several challenges such as immune rejection by circulating antibodies, short biological half life, rapid renal clearance and degradation by proteolytic enzymes [136]. Research efforts have thus been focussed on developing truncated forms of PAH, which retained their catalytic activities [137] or developing PEGylated forms to circumvent the immune response [138]. This has been encouraged by the recent approval of several PEGylated protein products by FDA [136]. Gamez et al. PEGylated three recombinant forms of PAH and found them to be superior to their non-derivatised predecessors [138].

Targeted delivery of protein drugs to a specific tissue has been utilised for long in eliminating pathogens or cancerous cells. Eavri and Lorberboum-Galski used a novel targeted approach by tagging PAH to certain small peptides or homing ligands called as protein transduction domains [128]. They constructed PAH-fusion proteins with HIV transactivator of transcriptor peptide and human hepatocyte growth factor and noticed that these internalised in the liver and reduced Phe concentrations in a healthy C57BL mouse model. They postulated that this reduction will be even stronger in PKU mouse models since Phe concentrations are several order higher in magnitude. Also, this approach helps to reduce the circulation time of the enzyme in the blood, thus reducing the possibility of immune rejection. However, large-scale isolation and purification of PAH and its short half life is a concern [139, 140]. Moreover, PAH is not an autocatalytic enzyme and requires several co-factors to function [141]. This makes the therapy quite complex and challenging as maintaining a multi-component enzyme system in a stable state is a herculean task.

Another viable option is to use an enzyme substitute that works in a similar fashion, but does not have complex co-factor requirements. PAL (EC 4.3.1.5), a non-mammalian enzyme is usually found in plants [142]. It is a key enzyme in plant metabolism and mainly involved in defence processes [143]. It can also be isolated from fungi [144] and yeast [145–147], where it plays a catabolic role in providing nutritional sources of carbon and nitrogen [148]. It is an autocatalytic protein that can convert excess of systemic Phe to *trans*-cinnamic acid (t-CA) and ammonia via a non-oxidative deamination process [145] (Fig. 4). *Trans*-cinnamic acid can be converted to benzoic acid in the liver and excreted in the urine as hippurate. The levels of ammonia generated are metabolically insignificant [149, 150]. A shortcoming over PAH ERT is that Tyr will have to be supplemented in the diet as PAL metabolises Phe to t-CA instead of Tyr [36]. Also, PAL is highly antigenic and is cleared rapidly from the body [151]. So a suitable drug delivery system to deliver this therapeutic enzyme must heed attention to these concerns.

Immobilised PAL was first used in 1978 by Ambrus et al. in PAL reactors made of nylon used as arteriovenous shunts in dog and monkey, whereby they demonstrated decrease in blood Phe concentrations over a short period of time. Such a system appeared to be complicated for regular therapeutic use in young children, who are the recipients of PKU therapy. Intravenous injection, although invasive, seems to be an ideal route for proteolysis prone enzymatic drugs. However trials with intravenous ERT in cases of other metabolic disorders, such as acatalasemia, resulted in immune response and accumulation of the drug [152].

Oral administration of drugs is the most convenient and accepted route of drug administration [153] but poses numerous challenges for protein drugs [154]. Earliest of the studies using microencapsulated PAL were reported by Bourget and Chang who immobilised the enzyme in semi-permeable microcapsules [139, 155]. They demonstrated a decrease in serum Phe concentrations in experimentally induced PKU rats. A concern in microencapsulation of PAL has been the low encapsulation efficiencies in the

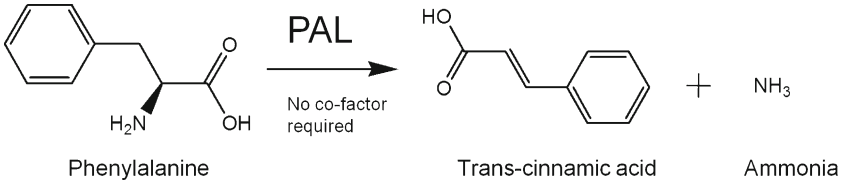

Fig. 4 Metabolic deamination of L-phenylalanine to tran-cinnamic acid and trace ammonia in presence of phenylalanine ammonia lyase (*PAL*). The process does not require a co-factor as opposed to Phe hydroxylation by PAH. This has led to PAL entering clinical trial phase, and the results are anticipated in early 2012

polymers used, with researchers reporting as low as 23 % [156]. Shah et al. demonstrated a method to increase the encapsulation efficiency and activity in cellulose nitrate microcapsules. Purified protein was radio-iodinated and after concentrating the polymer in solution, optimizing the stirring speed and aqueous: organic phase ratio, the reformulated microcapsules attained encapsulation efficiencies in the range of 80 % [157].

Until 1990, inducing HPA by injecting substrates (*p*-chlorophenylalanine/Phe) that inhibit PAH activity was used in most of the studies [158]. This did not ideally represent human PKU condition as the results could be affected by these inhibitors. A revolutionary innovation was the development of chemical mutagenesis using ethylnitrosourea (ENU)-induced PKU in mouse by McDonald et al. [117, 159]. Most of the studies then onwards have employed this PKU mouse model for a better estimation of the efficiency of delivery systems. Safos et al. were the first to report the efficiency of oral microencapsulated PAL in PKU mouse model [160].

The slow progress in this area until the turn of the twenty-first century was due to lack of the quantity of enzyme available to researchers. Most of the research was focussed on PAL isolated from *Rhodotorula glutinis* [145], which yielded low amounts of PAL. Efforts in increasing the yield revolved around developing mutated yeast strains (*Rhodotorula rubra* and *Rhodotorula graminis*) which expressed higher levels of PAL [147, 161]. Expression in *Escherichia coli* of entire PAL gene from *Rhodosporidium toruloides* was carried out by Orum and Rasmussen, which paved a way for large scale isolation of PAL and intensified the research in enzyme replacement management of PKU [162]. Sarkissian et al. developed a PAL gene construct with a high expression promoter in *E. coli* [163] to fabricate an oral formulation (PAL protected in *E. coli* and with aprotinin, a protease inhibitor) as well as intraperitoneal formulations, which showed significant improvements in Phe profiles in PKU mouse. They also reported a heteroallelic mouse model to characterise mild, moderate and severe PKU conditions and have a better correlation for studying various classes of PKU [164].

Albeit the better results obtained in these studies, the pharmacological effect was not sustained due to protein degradation, elimination or immunogenic response by generated antibodies. Wang et al. reported a structural modification approach by controlled proteolysis of PAL. They also reported PEGylation of PAL to reduce the immunogenicity and prolong the action of the drug. Prolonged reduction in Phe concentrations were noticed compared with non-PEGylated PAL at equivalent doses [165]. Similar PEGylation approach was reported by Gamez et al., with benefits of retention of catabolic activity of PAL [166]. Ikeda et al. used PAL derived from parsley (*Petroselinum crispum*) and PEGylated it with PEG_2 [2,4-bis(O-methoxypolyethyleneglycol)-6-chloro-striazine]. Similar results of higher circulation time and reduction in plasma Phe were observed [167].

In the quest for more efficacious alternatives of PAL, Moffitt et al. identified PAL in the genomes of two cyanobacterial species, *Anabaena variabilis* and *Nostoc punctiforme* [168]. They demonstrated that these cyanobacterial PALs were 20 % smaller than eukaryotic PALs but presented similar selectivity and kinetic activity for Phe. They were found to be structurally similar to PAL derived from parsley and yeast. However, it was observed that truncated forms wild type *A. variabilis* PAL (AvPAL) did not crystallise and aggregate readily. Wang et al. engineered AvPAL double-mutant PAL from bacteria *A. variabilis* which was shown to be superior to wild type AvPAL [169]. The AvPAL double-mutant C503S/C565S has been reported to be the most thermally stable and offered highest resistance to protease among the PAL derived from different species of cyanobacteria, fungi, yeasts and parsley [170]. Although AvPAL does not possess high specificity, it has been reported to be highly stable, which is a desirable virtue for protein therapeutics [170]. PEGylation of AvPAL resulted in negation of the immune response and a dose-dependent reduction of Phe concentration were observed in brain and blood over a period of more than 3 months. Hypopigmentation and weight loss associated with PKU were also alleviated [170].

Kang et al. developed an oral formulation for AvPAL by three different strategies. At first, they utilised a site-directed mutagenesis of a specific site on the enzyme (double-mutant AvPAL) to produce a triple mutant AvPAL (TM-AvPAL), which has an increased resistance to proteolytic enzymes of the gut. In the second approach, TM-AvPAL was used in a microencapsulation process to entrap the enzyme in silica

gel matrix to shield it from the adverse environment in the stomach and intestines. The third approach involved classical PEGylation of the AvPAL lysine residues to reduce their susceptibility to trypsin [171]. They have recently demonstrated the efficacy of PEGylated TM-AvPAL in PKU mouse model, observing 40 % reduction in Phe concentrations, terming it as the most effective PAL formulation [172]. Clinical trials in humans using this PEGylated formulation are in progress. The ongoing trials are aimed at evaluating the human dose, safety, efficacy and tolerability of regular subcutaneous administration of recombinant AvPAL [34]. One of the trials has recently concluded but the results are yet to be released.

Other approaches have also been investigated for developing suitable ERT dosage forms for PAL. Liu et al. transformed PAL cDNA from parsley in *E. coli*. A 2.2 Kb fragment of PAL cDNA was then subcloned and expressed in *Lactococcus lactis* [173], which is known to be a bacteria beneficial to small intestine, making it ideal for an oral dosage form. They used several vectors for cloning and demonstrated different expression levels. Two formulations, original *L. lactis* bacteria and enteric coated microcapsules, were used in two groups of rats and all of them lowered Phe concentrations, more so in the enteric coated treatment group, showing that the bacteria formulation was susceptible to gastric environment. Zhang et al. have encapsulated *L. lactis* in Ca-alginate microparticles showing retention of 92.9 % activity [174].

One of the concerns for protein drugs still remains in their vulnerability to harsh acidic and enzymatic challenges in the gastric environment or the high rate of immunogenic response generated when administered through the parenteral route. Transdermal and inhalational routes have provided viable alternatives for delivering protein drugs such as enzymes and vaccines [175, 176]. Dubey and Kalia demonstrated the iontophoretic delivery of ribonuclease T1 through intact skin [175]. There have been several reports showing the delivery of insulin through inhalational routes [177, 178]. All these are encouraging prospects and further research in development of efficient drug delivery systems and exploration of other routes of enzymatic delivery may bring hope to PKU patients as well.

Conclusions

Although PKU patients still face the difficulty in adjusting to the unsavoury diet and lack of efficient therapeutic systems, the future of PKU therapeutics appears bright. Looking back, considerable success has been achieved in the management of PKU ranging from the neonatal screening methods to modifications and continual improvements in the dietary components. Tetrahydrobiopterin supplements have improved clinical profiles in BH4-dependent PKU patients and the future calls to find economical alternatives for this important co-factor. Development of diets based on LNAAs and glycomacropeptide, though a minor component in PKU management, could potentially reduce the restrictions in the classical PKU diet. On the other hand, many studies have been done on gene and enzyme replacement therapies. Complex enzyme systems tagged to recombinant vectors (e.g. pseudotyped rAAV vectors) have already provided proof of concept for the feasibility of genetic correction. Enzymatic therapy may become a viable therapeutic option when effective drug delivery systems can be developed. Translational research with inputs from both clinicians and drug delivery scientists will help to address the current limitations. Conclusively, the prospects of PKU management exhibit considerable promise.

Conflict of interests The authors declare no conflict of interests.

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