Novel Approaches to the Therapy of Phenylketonuria

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Key Words

Phenylketonuria • Phenylalanine hydroxylase • Phenylalanine • Kuvan™ • Large neutral amino acids • Glycomacropeptide • Gene therapy • Targeted enzyme replacement therapy

Abstract

Since its discovery by Følling in 1934, phenylketonuria (PKU) has been the subject of numerous research studies spanning many disciplines including human genetics, biochemistry, development, neurobiology and others. During the last 75 years, different mutations in the genes responsible for the disease have been mapped, the biochemical basis has been elucidated and the flawed mechanisms leading to the clinical outcome have been revealed. Moreover, the newborn screening programs based on the Guthrie test and the subsequent biochemical analysis in newborn children, in addition to the low-protein diet, have all made a tremendous impact on the way inherited inborn errors of metabolism have been looked upon in these modern times. In fact, this simple yet most effective treatment for PKU – the low-protein diet – is considered a breakthrough in treatment of the disease enabling the retention of low levels of phenylalanine in the blood, thus preventing the high and persistent phenylalanine levels from damaging normal brain development. However, despite the simplicity and high effectiveness of the

low-protein diet treatment, the incomppliance of some PKU patients and the consequences that are associated with it have led scientists to constantly seek new and alternative methods for treatment. In this review, we discuss major advances in PKU therapy, including suggested new cures. We remain optimistic that one or more of the potential cures reviewed here will evolve into future medications for PKU patients.

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Introduction

One of the first metabolic disorders to be characterized was phenylketonuria (PKU). This disease was first described in 1934 by the Norwegian biochemist and physician Asbjørn Følling as an inherited metabolic disorder characterized by severe intellectual impairment, motor problems and skin abnormalities [1]. PKU is a metabolic disorder predominantly resulting from an abnormal form of the enzyme phenylalanine hydroxylase (PAH; EC 1.14.16.1). The PAH enzyme catalyzes the irreversible hydroxylation of the amino acid (AA) phenylalanine (phe) to tyrosine (tyr) in the liver. This step is considered the rate-limiting step in the catabolic pathway of phe [2].

Thus far, more than 550 mutations have been found in the PAH gene, many of them leading to a dysfunctional

enzyme (www.pahdb.mcgill.ca). Inherited in a classic recessive process, PKU has an approximate incidence of 1 in 15,000 newborns worldwide, which varies based on the ethnic background of different populations. PAH is a liver-specific enzyme with a crucial role in the maintenance of a low and steady level of phe in the plasma. Elevated levels of phe in the blood are known to cause permanent neurological damage and mental retardation by affecting multiple processes in the brain including reduced concentrations of other AAs, protein synthesis, dopamine biosynthesis and myelination. Currently, children diagnosed with PKU are immediately prescribed a low-protein diet for life in order to lower and maintain normal phe levels in their plasma [3].

PAH enzyme deficiency affects the brain through elevated blood levels of phe. When phe levels rise, brain concentrations of other large neutral AAs (LNAAs) are reduced as a result of competition for transport across the blood-brain barrier [4]. Phe has a more direct effect on tyr and tryptophan concentrations in the brain. This finding is based on evidence that phe acts as a competitive inhibitor on the brain tyr and tryptophan hydroxylases [5]. These AAs function, in addition, as precursors for the monoamine neurotransmitters. As a consequence, a reduction in precursor levels leads to a reduction in the biosynthesis of the neurotransmitter dopamine [5]. Another cause for brain damage is the reduction of protein synthesis in the brain attributed to two reasons: the reduction in the AAs tyr and tryptophan, and the prevention of the normal phosphorylation of the ribosomal protein S6 caused by the high levels of phe which affects the initiation of protein synthesis [5]. Another important mechanism for brain damage in PKU is attributed to the abnormalities in myelination and demyelination. These abnormalities are apparently the result of a combination of increased myelin turnover and decreased myelin production, which are both secondary responses to an increase in brain phe [6, 7].

Since the pathology of the disease originates from high and persistent blood and consequently brain phe levels, therapies for PKU aim at lowering and maintaining low phe levels throughout life.

Potential Therapies for PKU

Today, more than 40 years since the implementation of PKU screen tests for newborns, most PKU patients still rely on a low-protein diet as the only source of treatment of the disease [8]. This approach, still considered a break-

through in fighting metabolic deficiencies, holds some major drawbacks. Generally, as the amount of phe a PKU patient can tolerate is very low, the PKU diet restricts any of the protein-rich foods such as different types of meat and dairy products as well as high-protein foods derived from plants such as grains. Additionally, artificial sweeteners based on Aspartame should be avoided as phe is released once this compound is metabolized. Since many of these ingredients are major components of a normal everyday diet, the compliance of patients with the PKU diet throughout life is a demanding responsibility. Unfortunately, this demand for a strict lifestyle frequently results in the inability to comply. On the other hand, patients that do comply suffer from low levels of other metabolites as a result of their restrictive diet [9]. Although this low protein diet keeps phe levels around normal values in the blood, some researchers claim that the IQ of PKU patients kept strictly on this diet are slightly lower than those of the average population, implying that they are still subjected to minor brain damage [10, 11]. In addition, pregnant women suffering from PKU must revert to this strict diet in order to ensure normal fetus development [12]. Hence, even for PKU, a metabolic deficiency that is considered treatable, researchers are constantly searching for new effective approaches to treatment. We will review some of the more promising approaches in the following pages.

The Diet/Supplements Approach

H₄ Biopterin Supplementation

KuvanTM is the first drug to be approved for PKU treatment by the FDA. The drug is a synthetic copy of the H₄ biopterin (BH₄) cofactor termed sapropterin dihydrochloride (Kuvan, Bio-Marin Corp., Novato, Calif., USA). This supplementation therapy is designed to act at the enzymatic level, probably by increasing the binding affinity of PAH to its phe substrate, thus leading to the stabilization of the tertiary structure of the enzyme. At the biochemical level, the BH₄ cofactor is oxidized during the conversion of phe into tyr. BH₄ is a reduced pterin with a significant role in the anchoring of the substrate inside the catalytic domain of the enzyme [13–15]. About 1–3% of hyperphenylalaninemia patients suffer from BH₄ deficiency arising from a deficiency in one of the enzymes involved in the synthesis of this cofactor [16]. In these patients, as well as in up to one third of PKU patients with mutations in PAH, it was found that oral administration of BH₄ is sufficient to reduce phe blood levels [17, 18].

These patients, referred to as BH₄-responsive, are probably carriers of PAH mutations with residual PAH enzyme activity, so that the addition of high concentrations of the cofactor corrects defects in the stability or kinetics of the residual mutant enzyme [19]. The approval of Kuvan for treatment of PKU is based on the finding that oral administration of this sapropterin can eliminate the need for dietary therapy altogether in those with defects in BH₄ synthesis, or enable a modest relaxation in the diet in BH₄-responsive PKU patients with PAH mutations [20]. When analyzing the effects of sapropterin administration in a number of clinical trials, it was shown that this drug could significantly increase tolerance of dietary phe in BH₄-responsive PKU patients over several weeks [21]. As Kuvan was recently approved for treatment in PKU, this drug has yet to be evaluated for its long-term effects in patients in terms of both efficiency and safety.

Large Neutral Amino Acids

An additional promising approach for PKU therapy is based on the observation that an increase in the levels of other LNAAs given as a nutritious supplement can prove beneficial by decreasing the blood and brain levels of phe in PKU patients [4, 22]. In this case, the mechanism of action by which this supplementation works is the competitive transport of LNAA across the intestinal mucosa and then through the blood-brain barrier via the L-type AA carrier [23]. Due to the higher affinity as well as the larger concentrations of this AA in PKU, phe is favored in the competition across the transporter resulting in an increase in blood and brain phe levels along with a decrease in the concentration of other LNAAs. In fact, as described earlier, it is believed that one mechanism by which brain damage is caused in PKU is the decreased concentration of some of these AAs essential for brain metabolism. With this observation in mind, it was proposed that by increasing the concentrations of LNAAs, blood and brain levels of phe could potentially be reduced along with an increase in the availability of other LNAAs vital for normal brain function. Studies in LNAA supplementations are still underway showing promising results. Indeed, by using a double-blind placebo control trial, one of the most recent studies has shown a significant reduction of 39% in phe blood levels of treated patients compared to a decline of about 5.4% in the placebo group [22]. Yet, despite these encouraging results, it is still hard to predict the long-term effects of such a treatment as additional trials will be needed in order to confirm the safety of these supplements [23].

Improvements in Diet Treatment for PKU

The phe-free AA formula is currently a major component in the everyday diet of PKU patients. As an alternative to this formula, glycomacropeptide (GMP) has been recently suggested to replace this nutrition supplement. GMP is a 64-AA glycoposphopeptide formed during cheese production which in its pure form contains no phe in addition to a number of other AAs. Since phe is absent from this glycoposphopeptide and since it could easily be made available as a side product in cheese production, GMP can probably be used as a low phe protein nutrient source for PKU patients. Indeed, a clinical study which compared the known AA formula-based diet to a new GMP diet in 11 PKU patients, showed no significant difference in postprandial plasma phe concentration [24]. This study also showed an improvement in other measured factors in addition to a preference of the participating subjects for the new diet. As the compliance to the low-protein diet is harsh because of minimal dietary options, additional nutrient supplements such as GMP may make this everyday struggle easier for the PKU patients and their families. An additional outcome of the current low-protein diet is that it might subject PKU patients to reduced levels of additional nutrients. A number of studies found that children with PKU have low dietary intakes of long-chain polyunsaturated fatty acids (LC-PUFA). These reductions can further cause subtle neurological deficits which have been shown to improve upon addition of n-3 LC-PUFA to the diet [25]. These studies, though not considered major breakthroughs in PKU treatments, continue to improve the clinical outcome of PKU children, and thus should be further pursued.

The 'Enzyme' Solution Approach

Cell Therapy and Organ Transplantation

Metabolic deficiencies which result from dysfunctional liver enzymes can potentially be cured by the replacement of the liver by transplantation. Indeed, in one reported case where a PKU child required orthotopic liver transplantation because of concomitant, unrelated end-stage chronic liver disease, phe levels were restored to normal levels under a regular diet [26]. However, this treatment will subject the patient to a wide variety of rejection complications that would require lifelong need for immunosuppression [26–28]. As an alternative to a transplantation of the whole liver, it was suggested that perhaps a population of donor-derived wild-type hepatocytes could serve by converting the high levels of phe to

tyr. An additional proposal has been raised with regard to the ability of the human kidney to serve as the active site for phe conversion as PAH is normally expressed in this organ [19].

These therapies are currently only suggested ideas which have yet to be assessed by research before such interventions could be pursued.

Gene Therapy

Amongst all potential treatments for metabolic diseases, the ultimate treatment for genetically inherited diseases such as PKU would be the replacement of the mutated DNA sequence with a normal wild-type gene. In this context, different strategies have been examined over the last years. The possibility of replacing the mutated PAH sequence in the *Pah enu2* mouse model has been tested through the use of different gene transfer techniques. One important approach is based on the replacement of the mutant gene in the original site of expression and action – the liver. The ultimate goal of liver-directed gene transfer (as with any gene transfer) is to reach site-specific integration into the DNA sequence, without subjecting treated patients to the hazards of nonspecific DNA integration. Several vectors have been tested with the aim of integrating the wild-type DNA sequence into the genome of mice [19]. One of the more successful attempts first demonstrated a transient correction of the PKU phenotype in the PKU mouse model by intravascular delivery of a recombinant replication-defective adenovirus expressing murine PAH cDNA [29]. This attempt was then followed by a recent study which reported the success of complete and persistent phenotypic expression of murine PAH [30]. The persistent correction was achieved through the use of a bacteriophage integrase system to attain site-specific genome integration. The authors reported that blood phe levels dropped from 1,600–1,800 to 650–850 μM following an initial injection of integrase gene construct. Treated mice blood levels further decreased by additional construct injections until reaching a 90–120 μM level, a measure within the normal range for healthy mice. This level remained stable until week 20, when the mouse livers were examined for PAH activity [30]. In addition to liver-directed gene therapy, other targets for this technique have been tested showing that the use of this method in humans would probably fail if the PAH gene was targeted at the bone marrow [31], but could prove potentially beneficial if the PAH gene was targeted efficiently at the muscle together with additional constructs expressing the first two enzymatic steps in the BH_4 synthetic pathway. Addition of sequences from

the BH_4 enzymatic pathway are essential in this case, as the muscle does not contain measurable BH_4 essential for PAH activity [32]. However, despite substantial advances in the field, gene therapy is unfortunately not considered applicable for use in humans due to technical problems that have yet to be resolved [33, 34].

Enzyme Replacement Therapy

An encouraging new approach for therapy of metabolic deficiencies has appeared in the last few years. This approach, termed enzyme replacement therapy (ERT) is based on the substitution of the mutated metabolic enzyme by a recombinant protein substitute. ERT is now used regularly, for treatment of the metabolic deficiency Gaucher disease type I, which is characterized by an accumulation of the lipid glucocerebroside within the lysosomes of the monocyte/macrophage system. This accumulation results from the deficient enzyme called glucocerebrosidase that is responsible for the degradation of this lipid. ERT therapy developed for this disease is based on a mannose-terminated (modified) glucocerebrosidase, which targets itself at tissue macrophages expressing this mannose receptor and acts as a substitute for the defective enzyme [35]. The success of ERT in Gaucher disease has paved the way for the treatment of additional metabolic deficiencies which have been regarded incurable thus far.

Currently, two possible enzyme therapy alternatives for PKU have been suggested and tested to different extents. The first is treatment with the phenylalanine-ammonia lyase (PAL) enzyme. This treatment is based on a non-human metabolic pathway using the PAL enzyme which is derived from a variety of species including fungus, plants and ciannobacteria. PAL is capable of degrading the phe levels in the blood by converting this AA to another derivative called trans-cinnamic acid which can be further converted to benzoic acid, an unharmed metabolite that could ultimately be cleared from the body through the kidneys [36]. PAL is an autocatalytic enzyme that does not require a cofactor for the degradation of phe. The rationale in administration of the PAL enzyme claims that by converting phe to trans-cinnamic acid (in the intestine if given orally or under the skin when injected subcutaneously), the levels of this AA in the blood will drop to harmless values.

This candidate, although considered promising, has stumbled over some obstacles on the way to its approval for PKU therapy due to the fact that the enzyme is either degraded in the intestine or attacked by the immune system, and thus is unable to lower blood phe to the required

levels [37]. Efforts have been made to protect the enzyme from degradation by site-directed mutagenesis and chemical modification with polyethylene glycol (PEG) while maintaining specific activity. These attempts have proven partly beneficial to date [38]. Recently, different variants of engineered PEG-PAL were further assessed in a PKU mouse model showing a reduction in phe blood levels over a 90-day trial period in mice treated with one of the tested variants [39]. While these results are encouraging, further evaluation of this potential treatment will show if PAL could serve in long-term treatment of PKU.

Targeted ERT

The possibility of targeting a protein at a specific tissue or cell in order to eliminate it has existed for some time. The concept is to attach a targeting moiety to a toxic protein to produce a chimeric protein, and in this way deliver the molecule to a selected target, namely a cell expressing a specific receptor for this targeting moiety [40]. This chimeric protein enters the cell through endocytosis and acts through different mechanisms, relying on the toxic protein to eliminate the cell. Chimeric proteins are constructed by fusing cDNA sequences of the targeting domain to the toxic domain, thereby producing a fusion protein with two different biological domains. Various targeting domains have been used for the production of chimeric proteins, among them are growth factors, cytokines and specific variable regions of antibodies [41, 42]. These targeting domains have been fused to a wide variety of highly toxic proteins derived from certain plants or bacteria [41, 42]. In 1999, a chimeric protein named Ontak[®] was approved for clinical use in cutaneous T-cell lymphoma [43]. This chimeric protein is believed to be the first of many other such proteins which are currently being constructed and tested [44, 45]. For PKU, we have decided for the first time to utilize the idea of targeting a protein, not to eliminate a specific cell, but rather to bestow a functional activity in the treatment of PKU. Treatment of metabolic deficiencies by ERT, as mentioned before, has already been proven effective in the Gaucher disease type I [35]. Yet, in order to prove efficient, the recombinant enzyme must be delivered effectively to the original tissue and the site of action of this enzyme. Since PKU results from a mutation in a liver enzyme, it is essential to target the PAH at hepatocytes in order to substitute for this deficiency. With this in mind, we searched for a selective liver ligand to be fused to the PAH enzyme.

Recently, new mechanisms for protein delivery have been proposed. One new promising approach involves

the fusion of proteins with small peptides called protein transduction domains (PTDs) [46]. These small peptides facilitate the penetration of fused proteins through membranes in a large variety of eukaryotic cells. Although the mechanism responsible for the internalization process is not clearly understood, involving neither receptor and clatherin-mediated endocytosis nor phagocytosis, these new PTDs are considered promising delivery tools [46]. Out of many PTDs discovered so far, the HIV trans-activator of transcription (TAT) peptide is the most widely studied. TAT is an 11-amino-acid (residues 47–57) arginine and lysine-rich portion of the Tat protein encoded by HIV-1 virus [47]. TAT fusion proteins were shown to be delivered efficiently into cultured cells, intact tissue and live tissues when injected into mice [48, 49]. Proteins delivered by this peptide retained their activity after internalization. A study conducted on the pharmacokinetics of TAT- β -galactosidase fusion protein revealed that the liver is the main target for this fusion protein [50]. Based on this notion, a new TAT-PAH fusion protein was constructed in order to target PAH to the liver.

Additionally, in the search for a selective liver-targeting domain, we found the hepatocyte growth factor (HGF) to be a promising candidate. HGF is synthesized by mesenchymal cells, and is regarded as a potent paracrine growth factor with mitogenic and morphogenic activities [51, 52]. The liver is the main target of HGF, and biological responses to this growth factor are mediated by the Tyr-kinase receptor encoded by the Met oncogene (cMet) [53, 54]. Research conducted on the binding properties of the HGF revealed that the α -chain alone can bind to the cMet receptor. However, this fragment will not induce phosphorylation of the cMet receptor. Hence, mitogenic, motogenic and morphogenic responses will not be activated [55]. Moreover, it was found that smaller fragments of this α -chain consisting of the N-amino terminal and the first kringle domain can bind to the cMet receptor and can be cleared from the cell surface through endocytosis [55]. We have used these α -chain-based fragments as liver-selective ligands and constructed liver-targeted PAH molecules aimed to deliver this enzyme to its natural location and site of biological activity, thus producing the NK1-PAH and NK2-PAH fusion proteins.

All PAH fusion proteins were expressed using an *Escherichia coli* bacterial expression system. Following expression, PAH-based proteins were tested for their PAH enzymatic activity by an in vitro activity assay. By testing the in vitro enzymatic activity, we were able to show a dose-dependent activity. We found that although the ad-

dition of the TAT peptide to the N-terminus of the PAH lowers the $V_{\max}$ of the enzyme, it does not affect the K_m . Thus, the addition of short protein sequences, such as the TAT, to PAH, most probably does not change the affinity of the enzyme to its substrate.

Following the internalization of PAH-based proteins into target cells by Western blot analysis and confocal microscopy, we observed that the internalization of PAH into cells can be demonstrated only when this protein is fused to a delivering moiety. PAH alone cannot enter cells. We found that PAH internalization is more efficient when this protein is fused to fragments of HGF than when it is fused to TAT. This could imply that delivery of fused proteins by receptor-mediated endocytosis is most probably favored over delivery by PTDs. In addition, we found that the internalization of the PAH-based proteins is time dependent.

Elevated PAH activity was found in the lysates of all cell lines treated with TAT-PAH fusion protein, as well as in the lysates of liver primary cultures. PAH activity in cell lysates varied according to the duration that the cells were incubated with TAT-PAH. We found that this activity peaked after 3 h of incubation with the fusion protein, and returned to control levels between 24 and 72 h. We also found that treatment of cMet-expressing cells with NK1-PAH and NK2-PAH fusion proteins led to elevated PAH activity in the lysates of these cells. This effect was not observed in cells not expressing this receptor. Thus, NK1-PAH and NK2-PAH fusion proteins are specific in their targeting abilities and represent new targeting molecules for the delivery of the PAH enzyme into hepatocytes, the main site/cell for the natural activity of this enzyme.

Biological effects of PAH-based fusion proteins were tested. These experiments revealed that the PAH-based proteins were not toxic to the cells. We could also show that NK1-PAH and NK2-PAH did not contribute to the proliferation of the treated cells and that they did not affect cell scattering. Thus, NK1-PAH and NK2-PAH fusion proteins are specific for targeting the PAH wild-type enzyme into hepatocytes without having undesired side effects.

TAT-PAH was injected into C57BL mice in order to test the *in vivo* effect of this fusion protein. These experiments revealed that plasma phe levels were dramatically decreased in mice treated with TAT-PAH fusion proteins. The decrease in phe levels of TAT-PAH-treated mice was not a result of PAH activity in the plasma, since we found that the plasma has an inhibitory effect on the PAH enzymatic activity. Hence, this decrease in phe levels is,

most likely, a result of TAT-PAH being taken up by the liver leading to lowered levels of phe in the plasma of treated mice [56].

Taken together, this work demonstrates that PAH-based fusion proteins can be expressed in a bacterial expression system. These proteins retain all the characteristics of the PAH enzyme such as the binding affinity to the phe substrate, the dose-dependent conversion of phe into tyr and the ability to form multimeric complexes. Yet, by fusing these enzymes to different targeting domains, these enzymes attain one additional important characteristic – the ability to be delivered into target cells. These proteins were proven to internalize into different cells, and, once inside, proved to reduce phe concentrations by converting this AA into tyr. When plasma phe levels *in vivo* were reduced, it was shown that PAH enzymatic activity is not restricted only to different cell lines, but that this approach can work *in vivo*, in the context of a whole organism. Additionally, since HGF was used for the first time as a delivering moiety of fusion proteins, this work demonstrated that this factor may also be used as a platform for the delivery of different proteins in a variety of liver-associated diseases. Most importantly, these results demonstrated for the first time a proof of concept for targeted ERT for the treatment of PKU.

Since the research objective of this preliminary study was to establish a proof of concept for targeted enzyme therapy for PKU, this study should be complemented with an analysis in the PKU mouse model. Further evaluation of these proteins should include a long-term administration of PAH-based fusion proteins in the PKU mouse model, followed by the analysis of (1) plasma phe and tyr levels; (2) uptake of the PAH into different tissues; (3) PAH activity in the different tissues; (4) possible immunogenic effects of these proteins, and (5) the fate of the metabolized phe. By using a long-term administration of PAH-based fusion proteins, we will be able to evaluate the potential of these fusion proteins for the treatment of PKU.

Conclusions

Tremendous advances have been made in our understanding of the genetics, biochemistry and clinical aspects of metabolic diseases in the last few decades. For PKU, one of the first inborn errors of metabolism to be discovered, many of the mutations have been sequenced while others are being added constantly, the biochemical

mechanism has been elucidated, and the clinical outcome is clear. Most importantly, with the aid of a newborn screening test together with controlled diet, this disease is treatable. Yet, the poor compliance of PKU patients with the low-protein diet together with complications associated with it has led researchers to seek new alternatives for treatment. The past decade has been highly productive in pioneering additional potential medications for PKU including the recent introduction of Kuvan, a

new drug approved for treatment in some PKU patients. In addition, other potential treatments have reached advanced stages of development, some of which could hopefully serve the rest of the PKU community in the future. In addition to the direct benefit to PKU patients, new potential treatments for this disease will surely aid in the development of new medications for a large variety of other metabolic diseases.

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