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Mol Genet Metab. Author manuscript; available in PMC 2012 November 1.

Published in final edited form as:
Mol Genet Metab. 2011 November ; 104(3): 249–254. doi:10.1016/j.ymgme.2011.06.016.

Evaluation of orally administered PEGylated phenylalanine ammonia lyase in mice for the treatment of Phenylketonuria

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Abstract

Phenylketonuria (PKU), a Mendelian autosomal recessive phenotype (OMIM 261600), is an inborn error of metabolism causing impaired postnatal cognitive development in the absence of treatment. We used the $Pah^{enu2/enu2}$ PKU mouse model to study oral enzyme substitution therapy with various chemically modified formulations of phenylalanine ammonia lyase (*Av*-p.C503S/p.C565S/p.F18A PAL). *In vivo* studies with the most therapeutically effective formulation (5 kDa PEG-*Av*-p.C503S/p.C565S/p.F18A PAL) revealed that this conjugate, given orally, yielded statistically significant ($p = 0.0029$) and therapeutically relevant reduction (~40%) in plasma phenylalanine (Phe) levels. Phe reduction occurred in a dose- and loading-dependent manner; sustained clinically and statistically significant reduction of plasma Phe levels was observed with a treatment ranging between 0.3 IU and 9 IU and with more frequent and smaller dosings. Oral PAL therapy could potentially serve as an adjunct therapy, perhaps with dietary treatment, and will work independently of phenylalanine hydroxylase (PAH), correcting such forms of hyperphenylalaninemias regardless of the *PAH* mutations carried by the patient.

Keywords

Phenylketonuria; hyperphenylalaninemia; phenylalanine ammonia lyase; oral enzyme substitution therapy

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INTRODUCTION

PKU is an inborn error of metabolism, inherited as an autosomal recessive trait (OMIM 261600). It results from compromised activity of phenylalanine hydroxylase (PAH) (EC 1.14.16.1), the enzyme that catalyzes the irreversible conversion of phenylalanine (Phe) to tyrosine (Tyr) [1, 2]. Patients afflicted with this metabolic condition exhibit intolerance to the essential amino acid Phe in their diet, resulting in a systemic Phe concentration that may increase to neurotoxic levels and impair cognitive development [1, 2].

PKU is among the very first of the genetic diseases to show an opening for treatment; this was achieved by restriction of the dietary Phe intake [1, 2]. When newborn screening for early diagnosis was combined with treatment, the PKU landscape changed. However, it then became apparent that continual treatment, projected for a lifetime, is difficult and perhaps unsustainable, and most patients would not comply [3–5]. The imperfections of dietary treatment [5], instigated a search for a more effective treatment independent of dietary manipulation.

The recently approved synthetic cofactor to PAH, KUVAN® (sapropterin dihydrochloride (BH_4)), acting as a chaperone on the misfolded PAH protein caused by certain missense *PAH* mutations [6], has brought about partial or complete correction of hyperphenylalaninemias (HPA) in some patients [7–9]. However, patients who do not respond to BH_4 are still in search of a non-diet dependent form of treatment.

An obvious lead candidate would be enzyme *replacement* with the native PAH. However, this has presented a number of challenges, the most significant of which are its inherent instability and cofactor (BH_4) requirement to function [10, 11]. Recently, we reported on enzyme *substitution* therapy with the non-mammalian Phenylalanine Ammonia Lyase (PAL) [12, 13]. PAL, an enzyme which converts Phe into metabolically harmless *trans*-cinnamic acid and trace amounts of ammonia [14], empirically acts on the metabolic phenotype. The effect, which is independent of the *PAH* mutation's influence on the corresponding protein, has the potential to correct all such forms of HPA. PAL derived from the blue green algae, *Anabaena variabilis (Av)*, has particularly favorable biochemical and structural characteristics [15]; and when it is chemically modified by polyethylene glycol (PEG) and injected subcutaneously, the formulation displays prolonged plasma half-life *in vivo* with effectively normalized HPA in the PKU mouse model [12]. This PAL formulation has since completed phase I (Government Identifier: NCT00634660 (completed)) and entered phase II (Government Identifiers: NCT00924703 (enrolling participants by invitation only); NCT00925054 (recruiting); NCT01212744 (not yet recruiting)) clinical trials as a potential therapeutic for PKU; (http://www.bmrn.com/pipeline/peg-pal-for-pku.php; http://clinicaltrials.gov/ct2/results?term=peg+pal).

Given that PKU patients require lifelong therapy, and that a benign and non-invasive formulation would be preferable [16, 17], we continued our investigation of oral formulations which target the enterorecirculation of Phe in the intestine before its reabsorption.

We have already provided proof of principle for oral enzyme substitution therapy with PAL [13]. More recently, we showed that the PEGylated, triple mutant *Av*-p.C503S/p.C565S/p.F18A PAL exhibited improved resistance against protease digestion *in vitro* [18]. Here we report on the short-term pharmacodynamic (PD) profiles of orally administered, chemically modified, *Av*-p.C503S/p.C565S/p.F18A PAL as therapeutic agents in PKU.

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MATERIALS AND METHODS

PAL Species and Mutant Variants

We tested two different species of PAL protein isolated from *Anabaena variabilis (Av),* and *Rhodosporidium toruloides (Rt).* For the *Av*PAL we tested two variants of the wild type derived by site-directed mutagenesis (*Av*-p.C503S/p.C565S and *Av*-p.C503S/p.C565S/p.F18A [12, 15, 18]); for the *Rt*-PAL we tested the wild type only.

Synthesis of recombinant PAL

Av-p.C503S/p.C565S PAL, was kindly provided by BioMarin Pharmaceutical Inc. (Novato, CA, USA), and *Av*-p.C503S/p.C565S/p.F18A PAL and *Rt*-PAL were generated based on the protocol described earlier [13, 15, 18].

Expression, purification and measurement of the specific activity of the recombinant PAL

Histidine tagged *Av*-p.C503S/p.C565S/p.F18A PAL, incorporating C503S, C565S, and F18A mutations was expressed and purified based on the previously described protocol [15, 18]. Enzymatic activity was assayed for the formation of *trans*-cinnamic acid, as monitored by optical absorption, as described earlier [13].

PEGylation of PAL

Sunbright ME-050HS N-hydroxysuccinimide (NHS) activated 5 kDa linear, 10 kDa branched and 20 kDa linear polyethylene glycol (PEG; Nippon Oil and Fat (NOF) America Corporation, NY, USA) was used for the PEGylation of *Av*-p.C503S/p.C565S/p.F18A PAL. PEGylation of the protein samples was performed as described by Sarkissian et al. [12].

PTD-Av-p.C503S/p.C565S/p.F18A PAL fusion proteins

Protein transduction domains (PTDs), such as TAT-peptide (YGRKKRRQRRRG) from HIV-1 viruses, have been shown to be capable of transducing fusion payloads across cell membranes [19, 20]. In particular, PTD-4 (YARAAARQARAG), a TAT-peptide variant designed to reinforce the helical and amphipathic nature of the cationic peptide, was shown to improve *in vitro* transduction efficiency by 33-fold [21]. In this project, we generated a PTD-*Av*-p.C503S/p.C565S/p.F18A PAL variant by introducing a segment coding for the PTD-4 sequence to the 5′-terminal of the *Av*-p.C503S/p.C565S/p.F18A PAL construct. Expression and purification of the His-tagged labeled PTD-*Av*-p.C503S/p.C565S/p.F18A PAL fusion protein was performed using an identical strategy to the *Av*-p.C503S/p.C565S/p.F18A PAL protein.

Dextran sulfate/chitosan PAL nanoparticles

Av-p.C503S/p.C565S/p.F18A PAL was formulated into dextran sulfate/chitosan nanoparticles based on a protocol modified from that reported by Sarmento et al. [22]. Briefly, 1 ml of protein solution was mixed with 20 ml of 0.15 % w/v dextran sulfate solution, pH 4.0. The solution mixture was added dropwise using a 27 gauge hypodermic needle into 20 ml of 0.1 % w/v medium molecular weight chitosan in 1 % v/v acetic acid solution, pH 5.0. During the addition process, the chitosan solution was stirred at a constant speed of 600 rpm at room temperature. Upon dropping the protein mixture solution into the chitosan solution, the protein/dextran mixture rapidly gelates into discrete protein/dextran/chitosan nanoparticles. This suspension of particles was further stirred at 600 rpm for an additional 30 min at room temperature. The particles were harvested by centrifuging at 1750 rpm for 30 min at 15 °C. The supernatant of the centrifugation was then measured using the Bradford Assay to determine the loading efficiency of the protein of interest into the

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nanoparticles. Finally, the particles were washed with deionized water before being harvested by centrifugation.

Barium alginate PAL microspheres

Conventionally, alginate microspheres are formed by ionotropic interaction with divalent calcium ions. It has been shown that the strength and the release rate of molecules entrapped within the microspheres relate to the choice of divalent ion used [23], with barium ions having the strongest interaction [24]. Therefore, in order to prepare PAL-entrapped microspheres with the highest theoretical physical protection, barium alginate PAL microspheres were prepared using an emulsification protocol modified from those reported earlier [25]. Briefly, 550 µl of 2 % sodium alginate solution added with 230 µl of protein solution of appropriate concentration. The solution mixture was then gradually added to 11 ml of hexane with 300 mg Span 85 and continuously stirred at 1000 rpm. 1.2 ml of Tween 80 solution was then added and stirred for an additional 5 min at 1000 rpm. Finally, 10 ml of 0.2 % chitosan solution with 0.2 M barium chloride solution were gradually added to the solution mixture and stirred at 800 rpm for 25 min to ensure complete evaporation of hexane. The formed microspheres were then washed twice with 50 ml of deionized water before harvesting by centrifugation.

Amorphous Silica PAL particles

The particles were prepared based on the strategy and protocol described in our earlier paper [18].

PKU mouse model

The studies described here were conducted in accordance with the guidelines and policies of the Canadian Council on Animal Care and received approval from the Animal Care Committee Review Board McGill University, where the procedures were performed.

The $Pah^{enu2/enu2}$ (ENU2) homozygous mutant mouse [26] was used for the *in vivo* studies. An orthologous counterpart for human PKU, it carries a missense mutation (c.835T->C) in exon 7 (p.F263S) [27] of the *Pah* gene, has no hepatic Pah activity, and on normal diets displays a 10 to 20-fold elevated blood Phe level (see http://www.pahdb.mcgill.ca - Information – Mouse) which can be manipulated by dietary changes to Phe intake.

Animals at 3–9 months of age and between 25–40 g bodyweight were used for these studies.

In vivo evaluations of PAL formulations

Efficacy of the modified PAL enzyme was assessed in ENU2 mice by measuring plasma Phe concentrations before and after scheduled administrations of PAL (Supplementary Table 1).

All animals were housed individually for the duration of the study. To establish euphenylalaninemia, the animals were supplied (*ad libitum*) with a Phe-free diet from Harlan Teklad (97152), plus water containing 30 mg/L L-Phe for 3 consecutive days prior to all dosings. The animals were weighed on day 3 and the weights were then used to standardize a subcutaneous (lower back) injections of L-Phe [0.15 mg/g (body weight)] on day 4 at 11:00 a.m., for reproducible HPA. Group designated formulations were administered, via gavage, directly to the stomach of the animals; starting at 1 hr post-L-Phe challenge (day 4), and were repeated as indicated in Supplementary Table 1. Two controls were included: one positive (PAL enzyme with aprotinin formerly shown to lower Phe levels [13]) and one negative bovine serum albumin (BSA) control in 0.5 M Tris, pH 8.5 with or without chemical modification, equivalent in protein concentration to each of the

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tested PAL variants. Blood samples were collected from the tail vein immediately prior to initial gavage and on subsequent scheduled times as indicated in Supplementary Table 1. Protocols were designed to test the effect of chemical modifications of *Av*-p.C503S/p.C565S/p.F18A PAL molecules via: (a) PEGylation, (b) coating of PAL nanoparticles, (c) barium alginate PAL microsphere production, (d) amorphous silica PAL particle production, and (e) PTD-PAL fusion proteins. Formulations that showed statistical reduction in plasma Phe levels were further tested for the effects of dose response and loading dose.

The animals were observed for morbidity, mortality, activity and general health during the course of the experiments, and monitored for an additional 24 h after the completion of the study.

Analytical

Measurement of plasma Phe concentration—We collected blood from the tail vein into heparinized tubes, extracted plasma, deproteinised with sulphur salicylic acid and measured Phe levels by HPLC (BIOCHROM 30 series Amino Acid Analyzer with Lithium Citrate buffer).

Statistical methods—The change of plasma Phe levels from baseline to hour 4, 6, or 7 (post Phe challenge) was analysed using analysis of variance (ANOVA). For studies where each subject served as their own control, subjects were treated as blocks in the ANOVA. All studies included treatment as a factor in the ANOVA, and one study also included sex as a factor. Contrasts were used to compare each treatment to its respective control.

RESULTS

In vitro comparison of various chemically modified PAL variants

The post-modification specific activity of the modified variants of *Av*-p.C503S/p.C565S/p.F18A, *Av*-C503S/C565S and *Rt*- PAL preparations used in our studies are described in Table 1.

In vivo response in Phe concentration to the various chemically modified PAL variants

The subsequent results are data extracted from the protocols listed in Supplementary Table 1.

We reported earlier on the favourable biochemical and structural characteristic of PAL derived from the blue green algae *Anabaena variabilis* (*Av*) [15] and the beneficial therapeutic properties of the intravenously administered variant *Av*-p.C503S/p.C565S PAL [12]. Here we show the PD profiles of an orally administered chemically modified triple mutant *Av*-p.C503S/p.C565S/p.F18A PAL *in vivo*, which we previously [18] described as having improved resistance against protease digestion *in vitro*.

Significant reduction of plasma Phe levels was previously achieved by oral dosings of protease-resistant PAL formulations [13]. The most efficacious of these formulations (*Rt*-PAL with aprotinin (40mg/ml)) provided us with proof-of-principle for oral enzyme substitution therapy; it was used as a positive control in our studies and compared with unprotected, aprotinin protected and/or chemically modified formulations of either *Av*-p.C503S/p.C565S or *Av*-p.C503S/p.C565S/p.F18A PALs.

Table 2 describes the *in vivo*, short term (up to 6 hours post initial oral formulation dosing) effect of BSA or *Rt*-PAL + aprotinin (40mg/ml) controls vs. unprotected, aprotinin-protected

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and/or chemically modified formulations of *Av*-p.C503S/p.C565S or *Av*-p.C503S/p.C565S/p.F18A PAL treatments, on plasma Phe concentrations in the PKU mice.

Oral administrations of a number of the chemically modified PAL variants, as described in Table 2, indicated short term clinically significant clearance of plasma Phe in the mouse model.

Of the PAL formulations described above, we observed significant short term *in vivo* reduction of plasma Phe levels by: *Rt*-wild type + aprotinin ($p = 0.0005$, F = 28.57, df = 1, 9); *Av*-p.C503S/p.C565S PAL + aprotinin ($p < 0.0001$, F = 62.02, df = 1, 9); *Av*-p.C503S/p.C565S/p.F18A PAL+ aprotinin ($p = 0.0172$, F = 7.06, df = 1, 16); 20 kDa PEGylated-*Av*-p.C503S/p.C565S/p.F18A PAL + aprotinin ($p = 0.0212$, F = 7.76, df = 1, 9) and 5 kDa PEGylated-*Av*-p.C503S/p.C565S/p.F18A PAL ($p = 0.0029$, F = 12.28, df = 1, 16), as compared with the following formulations: 20 kDa PEGylated-*Av*-p.C503S/p.C565S PAL ($p = 0.1673$, F = 2.26, df = 1, 9); 10 kDa PEGylated-*Av*-p.C503S/p.C565S/p.F18A PAL ($p = 0.2235$, F = 1.60, df = 1, 16); 20 kDa PEGylated-*Av*-p.C503S/p.C565S/p.F18A PAL ($p = 0.0657$, F = 3.90, df = 1, 16); Hydrogel protected nanoparticles of *Av*-p.C503S/p.C565S/p.F18A PAL ($p = 0.8270$, F = 0.05, df = 1, 16); Barium Alginate Microspheres of *Av*-p.C503S/p.C565S/p.F18A PAL ($p = 0.7128$, F = 0.14, df = 1, 9); Amorphous Silica particles of *Av*-p.C503S/p.C565S/p.F18A PAL ($p = 0.4704$, F = 0.55, df = 1, 16); and PTD-*Av*PAL TM fusion *Av*-p.C503S/p.C565S/p.F18A PAL + aprotinin ($p = 0.1324$, F = 2.43, df = 1, 24) which did not show significant reduction when compared to their respective controls.

Dose Response

In the aprotinin-free series of oral PAL formulations, the 5 kDa PEGylated-*Av*-p.C503S/p.C565S/p.F18A PAL conjugate was the most effective in reducing plasma Phe levels as measured over 6 hours post initial drug administration. Therefore, we used this formulation to study dose-response and observed a uniform profile with sustained reduction of plasma Phe levels when treatment ranged between 0.3 IU and 9 IU: 0.3 IU (3 doses of 0.1 IU) ($p = 0.0317$, F = 5.43, df = 1, 18); 0.9 IU (3 doses of 0.3 IU) ($p = 0.0142$, F = 7.37, df = 1, 18); 3 IU (3 doses of 1 IU) ($p = 0.0072$, F = 9.17, df = 1, 18); 9 IU (3 doses of 3 IU) ($p = 0.0029$, F = 12.28, df = 1, 16) also see Table 3. At higher doses (also at higher protein concentration) the reduction remained significant as compared with the controls (27 IU (3 doses of 9 IU) ($p = 0.0045$, F = 10.89, df = 1, 16) and 18 IU (3 doses of 6 IU) ($p = 0.0104$, F = 8.42, df = 1, 16)), however, these reductions were not enhanced with the increase in dosage, as the effects were not significantly different from that of 9 IU (3 doses of 3 IU) and comparison of these three treatments ($p = 0.8307$, F = 0.19, df = 2, 16)), also see Table 3.

Frequency of Administration

The effect of oral 5 kDa PEGylated-*Av*-p.C503S/p.C565S/p.F18A PAL conjugate on plasma Phe is altered by dosage frequency where the higher loading frequency is required for efficacy [6 IU (2 doses of 3 IU) ($p = 0.7951$, F = 0.07, df = 1, 24) and (3 IU (1 doses of 3 IU) ($p = 0.6599$, F = 0.20, df = 1, 24)]; whereas, the equivalent overall dose of the latter, administered at a frequency of 3 doses [3 IU (3 doses of 1 IU) ($p = 0.0072$, F = 9.17, df = 1, 18)] demonstrated both statistically and clinically significant reductions (Table 3).

Gender effect

There was no significant difference between the response of males versus females to oral PAL therapy ($p = 0.1780$, F = 1.78, df = 3, 24) and both sexes experienced similar clinically beneficial reductions in plasma Phe levels.

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Health status and mortality

General health condition, grooming, and behavior did not change during any of the PAL formulation administrations. Five percent of treated animals suffered from mild complications associated with gavage related regurgitation, otherwise all remaining animals were in excellent health upon study completion.

DISCUSSION

A semi-synthetic diet to reduce Phe intake [28–30], when combined with early postnatal diagnosis by newborn screening [31], almost fully neutralizes the phenotypic effects of PKU-causing mutations at the *PAH* locus. However, the dietary treatment is difficult and compliance uniformly declines as patients enter adolescence [3–5]. An alternative to diet therapy, especially for these older patients, would be an asset, hence our continuing interest in enzyme substitution therapy for the treatment of PKU.

Enzyme therapy with PAL (a non-mammalian protein) was selected as a substitute [12, 13] for the native PAH, because PAL, unlike PAH, is inherently stable and does not require a cofactor to function [11]. PAL, an autocatalytic protein, converts the excess systemic Phe to *trans*-cinnamic acid with metabolically insignificant levels of ammonia as a by-product. *Trans*-cinnamate is a harmless product that has no embryotoxic effects in laboratory animals [14], and it is converted in the liver to benzoic acid, which is excreted in the urine as hippurate [32]. Small amounts of cinnamate and benzoic acid are also excreted [33].

PAL has the potential to correct the harmful metabolic phenotype in human patients and it might eliminate or reduce the need for dietary therapy in the treatment of PKU. Enzyme substitution with genetically and chemically modified PALs [12, 13, 15, 18, 34–36] effectively lowers Phe levels in both vascular space and brain tissue of the ENU2 mouse, as well as demonstrating reversal and benefit in other endophenotypes [12]. The PEGylated-*Av*-p.C503S/p.C565S PAL formulation is currently in phase II clinical trials as a potential injectable therapeutic for PKU (http://www.bmrn.com/pipeline/peg-pal-for-pku.php). However, since enzyme substitution therapy requires repeated life-long parenteral administration, we focused our current efforts on developing a PAL therapeutic that would be less invasive and could prevent long-term complications and immunological problems that may occur with an injected enzyme [37].

If oral PAL therapy can deplete the Phe pool in the intestine, whether from dietary or from the endogenous run out of free Phe from bound pools [38], then HPA can be reversed without systemic introduction of the enzyme. Previously, we provided proof of physiological principle, where the PAL enzyme, protected from inactivation by digestive enzymes, significantly lowered plasma Phe levels in the ENU2 mouse model [13]. There, the most effective formulation combined the protease inhibitor aprotinin with the PAL enzyme to protect it from degradation by intestinal digestion, allowing the PAL to remain active in the intestinal lumen long enough to degrade Phe. However, as aprotinin is not-specific in its inhibition, as a co-administrant with PAL, it will alter the degradation and digestion of all proteins passing through the intestinal tract, potentially resulting in malnutrition in patients chronically exposed to this compound. In addition, aprotinin possesses various biological functions, including inhibition of Ca^{2+}-activated K^{+} channels, high specificity to plasmin, as well as several physiologically significant activities [39]. Finally, the use of aprotinin has been implicated with severe adverse events as well as allergic reactions [40]. Therefore, while proof of physiological principle for oral PAL therapy was provided, this earlier formulation did not merit further development as a therapeutic agent for the treatment of PKU.

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In order to determine a preferred PAL candidate for oral administration, *Rt*-PAL, *Av*-p.C503S/p.C565S PAL (both formerly shown to be efficacious in reducing plasma Phe levels parenterally [12, 13, 36]) and *Av*-p.C503S/p.C565S/p.F18A PAL, all administered with a concomitant dose of 40 mg/ml aprotinin, were compared for their efficacy in plasma Phe reduction (Table 2). Despite the fact that *Av*-p.C503S/p.C565S PAL had a much lower affinity to the Phe as compared to *Rt*-PAL [15], when administered at one-third the dose of the *Rt*-PAL formulation, it yielded a 37.6 % reduction in plasma Phe, as compared with the 26.8 % reduction for the latter. Given the structural similarity and superior protease stability of a further variant of *Av*PAL, *Av*-p.C503S/p.C565S/p.F18A PAL, we expected the *in vivo* activity to be comparable to *Av*-p.C503S/p.C565S PAL. We measured a reduction of 61 % plasma Phe, 5 hours post dosing with the highest administrable dose for this formulation. Therefore, *Av*-p.C503S/p.C565S/p.F18A PAL was chosen as a lead molecule for subsequent chemical modifications.

We recently described several chemically modified formulations of *Av*-p.C503S/p.C565S/p.F18A PAL conjugate with improved *in vitro* resistance against protease digestion and prolonged degradation of Phe [18]. Here we examined the *in vivo* efficacy of these formulations, as well as determined the dose response to a selected formulation with significant impact on plasma Phe reduction.

Conjugating PEG molecules to the ε-amino group of lysine residues (where trypsin targets the protein for digestion) in *Av*-p.C503S/p.C565S/p.F18A PAL results in *in vitro* resistance to digestion by trypsin at a supra-physiological dose, and modestly influences resistance against chymotrypsin digestion administered at the same dose [18]. However, since PEGylation is effective in at least partial protection against intestinal proteases, 5 kDa, 10 kDa, and 20 kDa PEG-*Av*-p.C503S/p.C565S/p.F18A

PAL conjugates were tested for *in vivo* efficacy. All three formulations were administered to the PKU mouse models at their highest possible concentrations/doses. Of these, the 5 kDa PEG-*Av*-p.C503S/p.C565S/p.F18A PAL conjugate could be delivered at a significantly higher concentration due to its relatively lower viscosity. *In vivo* studies with the PKU mouse model revealed that the 5 kDa PEG-*Av*-p.C503S/p.C565S/p.F18A PAL conjugate yielded a statistically significant ($p = 0.0029$) and therapeutically relevant reduction (ie. a 425 ± 66 μM decrease from 1052 ± 74 μM pre-treatment values) in plasma Phe levels. In contrast, *Av*-p.C503S/p.C565S/p.F18A PAL PEGylated with 10 kDa or 20 kDa PEG molecules did not yield statistical significant reductions of plasma Phe. The 20 kDa PEG-*Av*-p.C503S/p.C565S/p.F18A PAL was then administered with 40 mg/ml aprotinin to examine whether the PEGylation reaction was the cause of the poor kinetics. Not surprisingly, the reduction of the plasma Phe was similar to the unPEGylated *Av*-p.C503S/p.C565S/p.F18A PAL administered with the same protease inhibitor (Table 2).

We also investigated an *Av*-p.C503S/p.C565S/p.F18A PAL hydrogel protected nanoparticle formulation [22], a barium alginate microsphere formulation [25], an amorphous silica particle formulation [18], as well as a fusion protein formulation with PTDs. Despite exhibiting varying levels of *in vitro* activities, *in vivo* activities for these formulations were unremarkable and did not produce statistically significant data.

Dosage and frequency of oral drug administration are of particular interest since lowering numbers in both aspects could reduce the potential cost and improve the overall ease of treatment. Accordingly, our lead formulation, 5 kDa PEG-*Av*-p.C503S/p.C565S/p.F18A PAL, was further examined for dose-response in the PKU mouse model (Table 3).

A uniform dose-response profile (Table 3) with sustained clinically and statistically significant reduction of plasma Phe levels was observed with a treatment ranging between

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0.3 IU and 9 IU, orally administered over 3 equal doses, 2 hours in succession. In addition, drug administration frequency (Table 3) affects the efficacy of oral 5 kDa PEG-*Av*-p.C503S/p.C565S/p.F18A PAL, where the 3 doses of 1 IU demonstrates significant plasma Phe reductions as compared with a single dose of 3 IU. This observation suggests that while the 5 kDa PEG-*Av*-p.C503S/p.C565S/p.F18A PAL has a definitive effect in reducing plasma Phe levels, the effect is not lasting long enough, and would require the administration of the agent at regular intervals.

The absence of a gender effect, where both sexes experienced similar reductions in plasma Phe levels, was measured with various *Av*-p.C503S/p.C565S/p.F18A PAL formulations. Since the enteral route is believed to make use of completely different physiological mechanisms [38] for Phe degradation than parenteral administration [12], orally administered PAL may also bypass the gender effect observed with long-term exposure to injectable PEG-PAL therapy [12]. This may then expand the benefits of oral treatment by providing similar long-term treatment dosing regimens for males and females.

Finally, since the general health conditions, grooming, and behavior did not change with clinically significant Phe reduction, and the animals were in excellent health upon study completion (data not shown), oral administration of chemically modified PAL variants can potentially serve as a non-invasive therapeutic option for PKU patients.

We have examined the *in vivo* activities of several variants and formulations of PAL derived from non-mammalian origins. Of particular interest is the 5 kDa PEG-*Av*-p.C503S/p.C565S/p.F18A PAL which exhibited a promising dose-response profile upon administration via the oral route.

Oral PAL therapy could potentially serve as an adjunct therapy, perhaps with dietary treatment, and will work independently of phenylalanine hydroxylase (PAH), correcting such forms of hyperphenylalaninemias regardless of the *PAH* mutations carried by the patient.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This research project and C.N. Sarkissian were supported by the NIH grant [Grant U01 NS051353]. T.S. Kang was supported by the National University of Singapore, Overseas Postdoctoral Fellowship. A. Gámez was supported by a research contract from “Ramón y Cajal” program by Ministerio de Ciencia e Innovación and Fundación Ramón Areces. We thank BioMarin Pharmaceutical Inc. for kindly providing the *Av*-p.C503S/p.C565S PAL, Kristiina Aro and Andrea Lawrance for overseeing the animal care facility and for providing related technical advice and assistance, Keo Phommarinh for technical assistance with plasma Phe analysis, Lin Wang for advice in the preparation of *Av*-p.C503S/p.C565S/p.F18A PAL, Ellen Maki for conducting the statistical analyses, and Angela Walker for assistance with manuscript preparation and submission.

Abbreviations

PKU	phenylketonuria
HPA	hyperphenylalaninemias
Phe	phenylalanine
PAL	phenylalanine ammonia lyase
***PAH* or *Pah*–gene; PAH or Pah–enzyme**	phenylalanine hydroxylase

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PD	pharmacodynamic
Av	*Anabaena variabilis*
ENU2	PKU mouse model $Pah^{enu2/enu2}$
NOF	Nippon Oil and Fat
PTD	Protein transduction domains

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Table 1

Specific Activity of chemically modified PAL variants

	Specific activity(IU/mg)
Rt-PAL + aprotinin	0.15
Av-p.C503S/p.C565S PAL + aprotinin	0.08
20 kDa PEGylated-*Av*-p.C503S/p.C565S PAL	0.16
Av-p.C503S/p.C565S/p.F18A PAL+ aprotinin	0.65
5 kDa PEGylated-*Av*-p.C503S/p.C565S/p.F18A PAL	1.08
10 kDa PEGylated-*Av*-p.C503S/p.C565S/p.F18A PAL	0.55
20 kDa PEGylated-*Av*-p.C503S/p.C565S/p.F18A PAL	0.55
20 kDa PEGylated-*Av*-p.C503S/p.C565S/p.F18A PAL+ aprotinin	0.33
Hydrogel protected nanoparticles of *Av*- p.C503S/p.C565S/p.F18A PAL	0.76
Barium Alginate Microspheres of *Av*- p.C503S/p.C565S/p.F18A PAL	1.46
Amorphous Silica particles of *Av*- p.C503S/p.C565S/p.F18A PAL	0.34
PTD-*Av*PAL TM fusion *Av*-p.C503S/p.C565S/p.F18A PAL + aprotinin	0.79

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Table 2

The *in vivo* effect of orally administered BSA versus chemically modified PAL variants, on plasma Phe concentrations, in the PKU mouse model

	Total Dose(I.U.)[a]	Pre-dosing plasma[Phe] (uM ± s.e.m.)	Time of measure(hour post initial dosing)	Reduction in plasma [Phe] (pre- versus post-initial dosing)		
				(uM ± s.e.m.)	p value	compared with control treatment*(uM)
BSA-Control – highest concentration (46.0 mg/ml)	0	959.2 ±44.3	6	−44.6 ±99	NA	NA
BSA-Control – lowest concentration (5.5 mg/ml)	0	812.6 ±35	5	122 ± 26	NA	NA
			6	15.3 ± 44	NA	NA
Rt-PAL+ aprotinin	2.88	1147.6± 108	3	303.5 ±30	0.0005	251.8
Av-p.C503S/p.C565S PAL + aprotinin	0.99	1251.7±68	3	468.6 ±15	<0.0001	371.1
20 kDa PEGylated-*Av*- p.C503S/p.C565S PAL	0.99	1159.7± 80	3	239.0 ±62	0.1673	70.8
Av-p.C503S/p.C565S/p.F18A PAL+ aprotinin	11.34	826.3 ±26	5	496.7 ±129	0.0172	432.3
5 kDa PEGylated-*Av*- p.C503S/p.C565S/p.F18A PAL	14.34	1052.4± 74	5	425.5 ±66	0.0029	470.2
10 kDa PEGylated-*Av*- p.C503S/p.C565S/p.F18A PAL	9.54	750.4 ±91	5	286.9±115	0.2235	222.5
20 kDa PEGylated-*Av*- p.C503S/p.C565S/p.F18A PAL	7.59	866.6 ±42	5	385.8 ±180	0.0657	321.4
20 kDa PEGylated-*Av*- p.C503S/p.C565S/p.F18A PAL + aprotinin	6.03	853.1 ±159	5	503.3 ±119	0.0212	354.6
			6	311.9 ±88	0.6024	89.3
Hydrogel protected nanoparticles of *Av*-p.C503S/p.C565S/p.F18A PAL	11.34	722.0 ±99	5	100.6 ±36	0.8270	36.1
Barium Alginate Microspheres of *Av*-p.C503S/p.C565S/p.F18A PAL	4.35	816.5 ±79	5	178.5 ±93	0.7128	55.7
			6	−36.2 ±187	0.7181	−51.5
Amorphous Silica particles of *Av*- p.C503S/p.C565S/p.F18A PAL	3.18	1029.7± 61	6	−53.1 ±95	0.4704	−117.7
PTD-*Av*PAL TM fusion *Av*- p.C503S/p.C565S/p.F18A PAL + aprotinin	17.25	1002.1±42	6	441.5 ±38	0.1324	289.9

[a] Dose was formulation dependant and was determined based on the highest volume administrable to the ENU2 mouse.

* These values take into account the time equivalent, plasma [Phe] of control treated mice exposed to the same regimen of dosing.

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Table 3

Dosage and frequency profiles of orally administered 5 kDa PEG-*Av*-p.C503S/p.C565S/p.F18A PAL in the PKU mouse model

			Reduction in plasma [Phe] (pre- versus 6 hours post-initial dosing)		
Number of Doses	**I.U./dose**	**Pre-dosing plasma [Phe] (uM ± s.e.m.)**	**(uM ± s.e.m.)**	**p value**	**compared with control treatment* (uM)**
3	0.1	825.3 ± 65	193.5 ± 50	0.0317	332.9
3	0.3	1086.2 ± 75	226.43 ± 29	0.0142	365.8
3	1	945.6 ± 71	268.5 ± 106	0.0072	407.9
3	3	1052.4 ± 74	425.5 ± 66	0.0029	470.2
2	3	1038.9 ± 79	110.0 ± 60	0.7951	−51.5
1	3	1027.1 ± 80	445.9 ± 189	0.6599	87.4
3	6	998.7 ± 24	344.7 ± 104	0.0104	389.4
3	9	1044 ± 75	398.1 ± 106	0.0045	442.8

* These values take into account the time equivalent, plasma [Phe] of control treated mice exposed to the same regimen of dosing.