



Short Communication

Lipid nanoparticle delivers phenylalanine ammonia lyase mRNA to the liver leading to catabolism and clearance of phenylalanine in a phenylketonuria mouse model

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ABSTRACT

Phenylketonuria (PKU) is a genetic disorder affecting around 1 in 12,000 live births (1), caused by a mutation in the phenylalanine hydroxylase (PAH) gene in the liver which facilitates the catabolism of phenylalanine (Phe). Without a functional copy of PAH, levels of Phe in the blood and tissues rise, resulting in potentially life-threatening damage to the central nervous system. (2) Treatment options for PKU are limited, and center around adherence to a strict PKU diet that suffers from poor patient compliance. There are two approved drugs available, one of which must be used in conjunction with the PKU diet and another that has serious immunological side effects. Here we demonstrate that the LUNAR® delivery technology is capable of delivering mRNA for a replacement enzyme, the bacterial phenylalanine ammonia lyase (avPAL), into the hepatic tissue of a PKU mouse, and that the enzyme is capable of metabolizing Phe and reducing serum levels of Phe for more than five days post-transfection. We further demonstrate the ability of LUNAR to deliver a plant-derived PAL protein with a similar impact on the level of serum Phe. Taken together these results demonstrate both the capability of LUNAR for the targeted delivery of PAL mRNA into hepatic tissue in vivo, replacing the defective PAH protein and successfully reducing serum Phe levels, thereby addressing the underlying cause of PKU symptoms. Secondly, that plant-based PAL proteins are a viable alternative to bacterial avPAL to reduce the immunogenic response.

1. Introduction

Phenylketonuria (PKU) is an inherited autosomal recessive metabolic disorder and one of the most common inborn errors of metabolism, affecting around 1 in 12,000 live births [1]. PKU is caused by defects in the phenylalanine hydroxylase (PAH) gene, which mediates the conversion of phenylalanine (Phe) to tyrosine (Tyr) in the liver. Without a functional copy of the PAH gene, Phe accumulates in the blood and body tissues. The excess Phe is toxic to the central nervous system, and when left untreated, can result in mental retardation and other neurological deficits [2]. Therapy for PKU is centered around a highly restrictive, low protein PKU diet. However, non-compliance due to poor palatability is common, along with growth retardation due to nutritional deficiencies [3].

There are two FDA approved therapeutics for PKU. Sapropterin dihydrochloride (Kuvan®) [4,5] is a synthetic form of tetrahydrobiopterin (BH4), the natural cofactor for the enzyme PAH. Treatment with Sapropterin can activate any residual PAH enzyme, but as it relies upon the presence of functional PAH, it is only effective in a subset of patients known as BH-4 responsive [6] and must be used in conjunction with the PKU diet [5]. Pegvaliase (Palynziq®), is a pegylated form of the *Anabaena variabilis* derived phenylalanine ammonia lyase (avPAL) protein, an enzyme that catalyzes the degradation of Phe to ammonia and trans-cinnamic acid (tCA). Pegylation serves to reduce the immunogenicity of the bacterial PAL protein. However, almost all patients (93.5%) experienced hypersensitivity adverse events (HAE), and a significant proportion of patients (4.6%) suffered Anaphylaxis during phase 3 trials [7], leading the FDA to approve Pegvaliase with a boxed warning,

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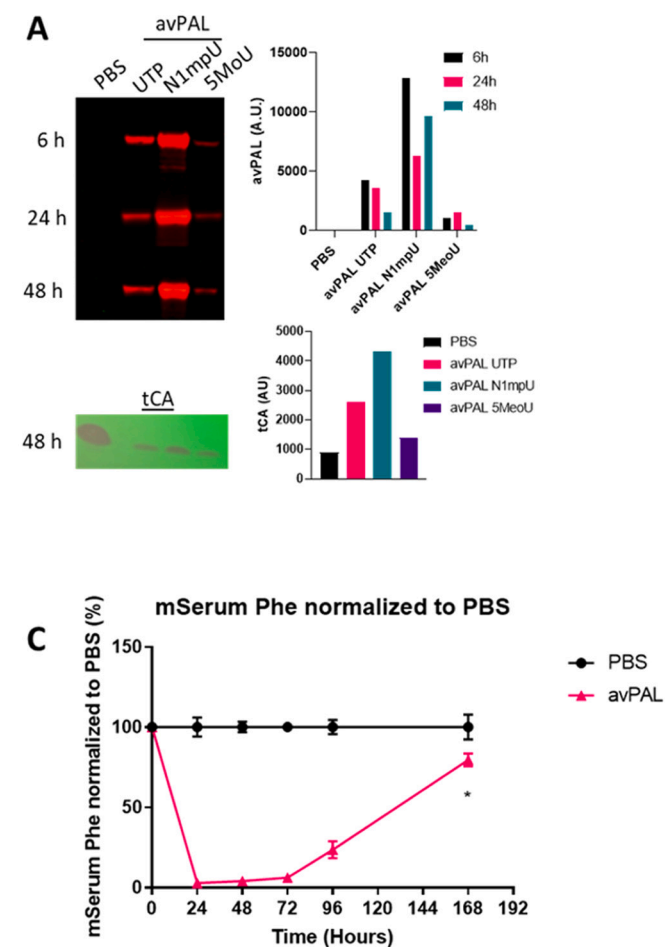
making the product available only under a Risk Evaluation and Mitigation Strategy [8]. Patients taking Pegvaliase must titrate up to the full dose over a 12-month period, are required to carry auto-injectable adrenaline (epinephrine), and prophylactic use of H1 and H2 blocking antihistamines prior to initiating treatment is recommended [9].

There is clearly an unmet need for more effective treatment options for patients suffering from PKU. Here we discuss the progress of one such therapy, using Arcturus Therapeutics' LUNAR technology for a liver tissue-specific delivery of bacterial and plant-derived PAL mRNA into hepatic tissue both in cell culture and in a mouse knock-out model repairing the broken link in the Phe catabolism pathway and addressing the underlying cause of PKU symptoms.

2. Results

2.1. In vitro and in vivo expression of PAL protein

In vitro studies show positive expression for all three of the PAL protein variants, as shown in the western blot and accompanying thin layer chromatography (TLC) (Fig. 1a.) PAL protein expression was evident in the cells after 6 h and was still present at the 48-h time point. TLC results also show the presence of the Phe metabolite tCA, indicating that the PAL protein is biologically active and metabolizing Phe to tCA. As can be seen in the bar charts (Fig. 1a) the avPAL N1 in lane 3 shows the highest level of protein expression, and the greatest amount of tCA, accordingly avPAL N1 was moved forward to knock-out mouse model studies.



Two versions of avPAL N1, wildtype and the clinically used double mutant (C503S/C565S) were delivered into a PKU mouse model using the LUNAR delivery system to determine both the dose-response and in vivo stability of the mRNA, together with the duration of expression and the activity of the resultant PAL protein. Fig. 1b., shows the expression level of the PAL protein in vivo at 1 mg/kg and 3 mg/kg dosing levels. Both versions of the protein show dose-dependent expression, with similar expression levels at the higher dose, but lower expression levels of the mutant (C503S/C565S) PAL protein at the 1 mg/kg dose.

To determine the activity of the PAL protein in vivo the level of serum Phe was measured at 24-h time points out to 96 h, and again at 168 h. The results shown in Fig. 1c. confirm that the wild type PAL protein was biologically active and metabolizing Phe at 96 h and beyond. Serum levels of the Phe metabolite hippurate (HA) (Fig. 1d), measured at the same time points, reveal a concordant increase in the level of HA out to the 96-h time point.

2.2. Comparison of bacterial and plant-derived PAL

The results of further experiments to compare the transfection, expression, and biological activity of three plant-derived PAL proteins versus bacterial avPAL are shown in Fig. 2. Western blot results at the 48-h and 72-h time points (Fig. 2a) show similar levels of protein expression in cell culture for all four PAL proteins. Phe levels (Fig. 2b) were also reduced to a comparable level by all four of the PAL variants, and the presence of tCA was established by TLC (Fig. 2c).

The three plant PAL variants plus avPAL were transfected into PKU

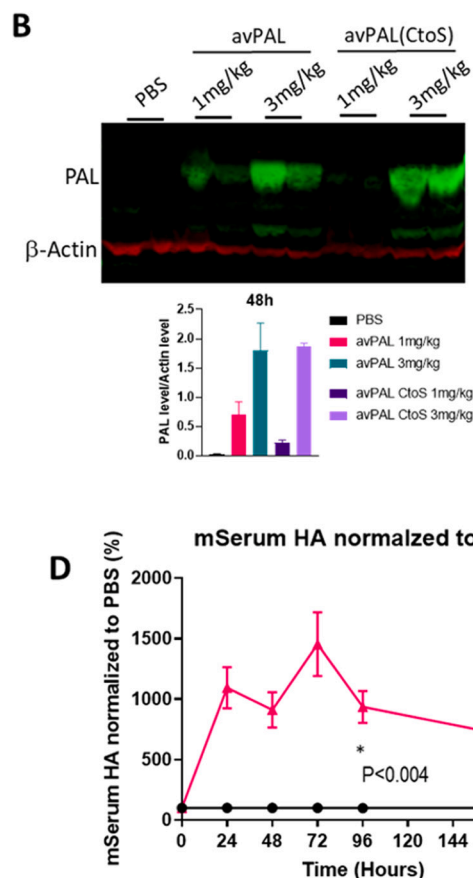


Fig. 1. Bacterial avPAL mRNA expression and efficacy in vitro and in PKU mouse model. (A) In Vitro expression levels of avPAL variants, and confirmation of biological activity by the presence of the Phe metabolite tCA. (B) Dose-dependent expression of avPAL protein in the mouse model. (C, D) Confirmation of biological activity of avPAL protein in a PKU mouse model through the reduction in serum Phe levels (C) and increase in the level of the Phe metabolite HA (D).

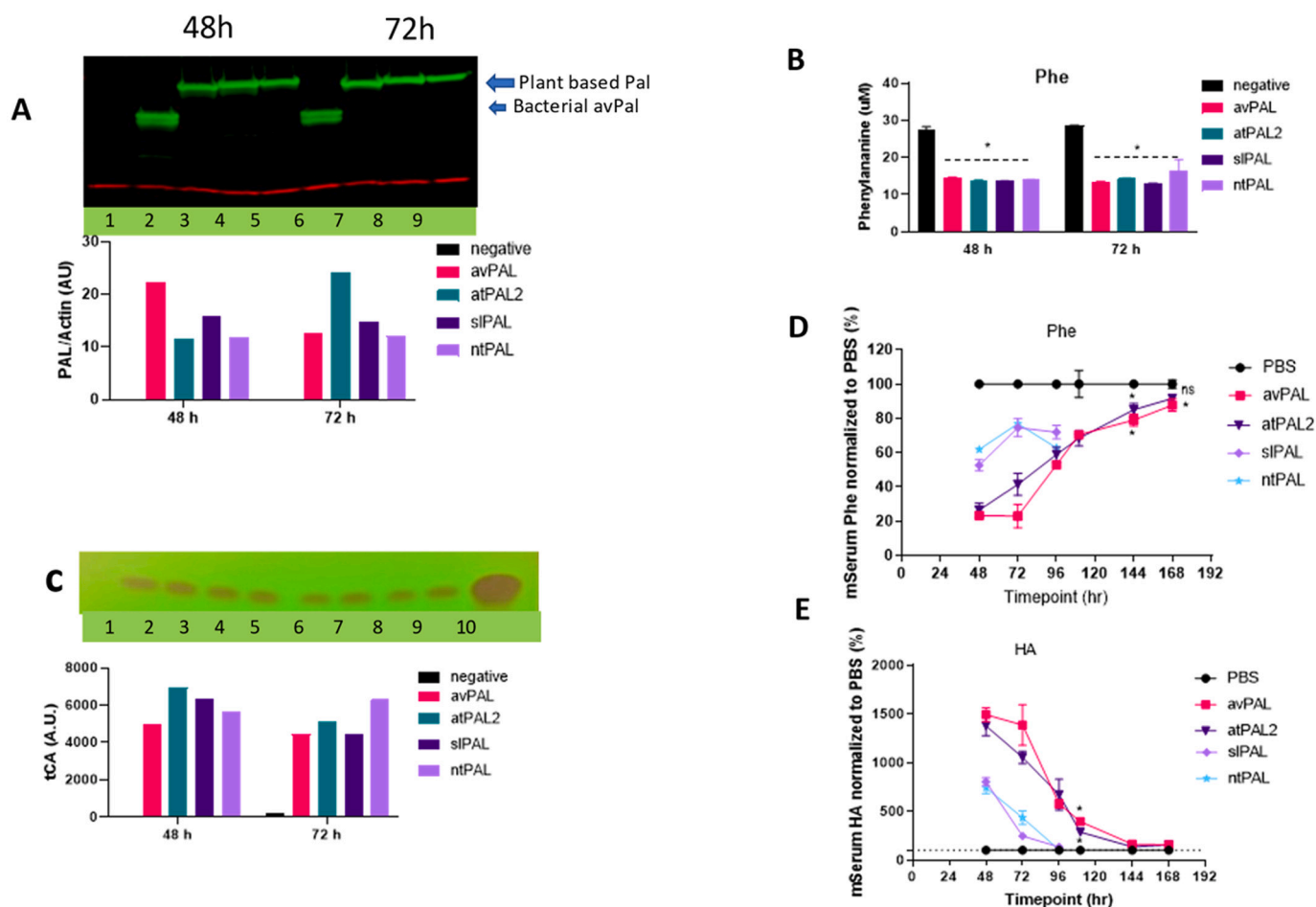


Fig. 2. Plant-based PAL mRNA expression and function in vitro and in vivo. (A) Comparison of in vitro expression of bacterial and plant-derived PAL proteins. Plant-derived PAL has a higher MW than bacterial PAL, hence the difference in band position. (B) Levels of Phe, and (C) levels of the Phe metabolite tCA for each of the four PAL protein variants in vitro. Blood serum levels of (D) Phe, and (E) the Phe metabolite HA in PKU mice following transfection with each of the four PAL protein variants.

mice using the LUNAR technology and serum levels of Phe and HA were again measured at time points from 48 h to 168 h post-transfection. Fig. 2d shows serum Phe levels for all four PAL variants, demonstrating the stability and biological activity of the PAL proteins out to the 120-h time point and beyond. Fig. 2e shows the serum levels of HA in the same mice and measured at the same time points. Demonstrating comparable stability and biological activity of the avPAL and the atPAL plant protein out to the 120-h time point and beyond.

3. Discussion

3.1. Transfection efficiency, mRNA stability, and biological activity

The results demonstrate the capability of the LUNAR delivery system to convey PAL mRNA into target hepatic tissue, and that the resultant PAL protein remains biologically active at clinically relevant levels for at least 5 days post-transfection. Once transfected into PKU mice the PAL protein is able to fill the missing gap in the Phe metabolism pathway, facilitating the breakdown of Phe to tCA, and that the remaining steps of the metabolic pathway are able to complete the breakdown of tCA to HA. The intracellular expression of the PAL protein could be an alternative to the Pegvaliase recombinant protein treatment since it could reduce the chances of an immunogenic response.

3.2. Plant-derived PAL proteins

Experiments to compare bacterial with plant-derived PAL demonstrate comparable levels of transfection efficiency between the bacterial avPAL mRNA and the three different plant-derived PAL mRNAs. Intracellular stability of the mRNA and biological activity for at least one of these plant-derived PAL proteins (atPAL) is comparable to bacterial avPAL as shown through the reduction in serum Phe, and the increase in serum HA for both avPAL and atPAL (Fig. 2d & 2e). Demonstrating a comparable level of in vivo performance is an important consideration as plant-derived PAL proteins could offer the potential for reduced immunogenic side effects. Furthermore, LUNAR PAL mRNA could offer an increased duration of efficacy compared to PAH mRNA [11].

4. Conclusion

Delivery of PAL mRNA with the LUNAR system into the liver of PKU mice shows that this technology could be an effective new therapy for the treatment of PKU disease. The ability of LUNAR to accurately deliver its mRNA payload to a specific cellular target with very low off-target effects has already been demonstrated in animal models [10,11]. Here we have shown that both bacterial and plant-derived PAL mRNA is stable, and that the resultant PAL protein will facilitate the breakdown of the Phe which normally accumulates in patients suffering from the disease, and that it is able to remain stable and biologically active for over 5 days in vivo, a sufficient duration to make this an effective and

tolerable therapy via injection.

5. Materials and methods

5.1. *In vitro* transcription (IVT) for synthesis

Mouse codon-optimized PAL sequences (*Anabaena variabilis* or *Trichormus variabilis* PAL (Q3M5Z3), *Arabidopsis thaliana* atPAL2 (P35510), *Solanum lycopersicum* slPAL (P35511), *Nicotiana tabacum* ntPAL (P25872)) were cloned into plasmids containing a T7 promoter, 5'UTR, in frame with a Myc tag coding sequence, 3'UTR and polyA tail. The cloned portions of all plasmid constructs were verified by DNA sequencing. Plasmids were linearized immediately after the poly(A) stretch and used as a template for *in vitro* transcription reaction with T7 RNA polymerase. The RNA was synthesized with 100% substitution of UTP with N1-methyl-pseudo-UTP or 5-Methoxy-UTP when indicated. The reaction for RNA was performed as previously described [10] with proprietary modifications to allow highly efficient co-transcriptional incorporation of a proprietary Cap1 analogue and to achieve high quality mRNA molecule transcription. RNA was then purified through silica column (Macherey Nagel) and quantified by UV absorbance. For the *in vivo* experiments, the RNA quality and integrity were verified by 0.8–1.2% non-denaturing agarose gel electrophoresis as well as Fragment Analyzer (Advanced Analytical). The purified RNAs were stored in RNase-free water at -80°C until further use.

5.2. Cell culture PAL mRNA transfections for PAL expression and activity

Transfections were performed using a transfection reagent, Lipofectamine MessengerMax (Thermo Fisher Scientific) according to the manufacture instruction. Mouse Hepa1–6 cells (ATCC) were plated in 96 well plate the day before transfection. DMEM medium containing 10% FBS was replaced immediately before beginning the transfection experiment. Medium was collected at desired time points post transfection and 100 μl fresh medium was added into each well. Medium was kept at -80°C until Phe or tCA analysis was performed. After deproteinizing media samples with 10Kda MWCO spin filter, Phe was quantified following Phenylalanine Assay Kit (Sigma).

Cells were also collected at the same time points for protein analysis by western blot.

5.3. tCA quantification by thin layer chromatography (TLC)

Conditioned media was collected from Hepa1–6 cell culture and proteins precipitated with ethyl acetate and 0.1% formic acid. After centrifugation, the upper layer was extracted and dried out. The precipitate was resuspended in methanol and dotted in TLC glass membranes for trans-cinnamic acid (tCA) detection, in parallel to a control of pure tCA. Mobile phase was chloroform:methanol:formic acid (85:15:1).

5.4. PKU animal model

Pah^{em2}/*J* homozygous mice were obtained from The Jackson Laboratory. Prior to dosing, animals were group-housed (up to 5/cage); from the time of dosing, animals were single-housed. Mice were housed in microisolator caging in ventilated racks. Environmental controls for the animal room generally targeted a temperature range of $23 \pm 3^{\circ}\text{C}$ and a relative humidity range of $50 \pm 20\%$ with a 12-h/12-h light/dark cycle. Throughout the study, the mice were offered Teklad Global 18% protein rodent diet (Envigo RMX, Inc.) and water ad libitum. All animals were males aged 2–4 months on the day of dosing.

Mice were held in a pathogen-free environment and all mouse studies were approved by the Explora Biolabs Institutional Animal Care and Use Committee (IACUC) and performed under Animal Care and Use Protocols.

5.5. Blood collection

Prior to dosing (" 0 h "), and at the indicated time points post-dose blood was collected from each animal by retro-orbital bleeding. For each time point, blood was collected into K_2EDTA -containing tubes and processed to plasma by centrifugation. The resulting plasma was stored at -80°C until transferred for evaluation.

5.6. Plasma Phe and hippuric acid (HA) concentration measurements

All *in vivo* plasma samples were assessed for Phe and HA concentrations by LC-MS/MS at JadeBio (La Jolla, CA). In brief, for each sample, plasma was diluted in water and protein was precipitated by combining with methanol containing each of the internal standards (^{13}C -6-Phe and ^{13}C -6-HA). The precipitate was pelleted, and the supernatants were transferred to a fresh microtiter plate and subjected to LC-MS/MS for chromatographic separation.

5.7. PAL protein quantification in mouse liver and mouse cells by western blot

Protein extraction from liver tissue was done using Precellys Lysing Kit tubes and RIPA buffer including a cocktail of protease inhibitors. After lysing the tissue using Precellys 24, samples were briefly sonicated and centrifugated and the supernatant was kept for standard western blot analysis. Protein extraction from Hepa1–6 cells was done by sonication for 4 cycles of 30 s on ice with a 1-min interval in RIPA buffer containing a cocktail of protease inhibitor (Complete, Roche). Before loading, samples were normalized, loading the same amount of protein for each sample in the same western blot.

Immunoblot was performed on PVDF Membranes following LI-COR Quantitative Western Blot system. PAL was detected using Rabbit Anti-Myc polyclonal antibody (AbCam Cat.9106) and Donkey Anti-Rabbit IRDye 680 RD (LICOR) as the primary and secondary antibodies. Beta-actin (β -Actin; a housekeeping protein used as a loading control) was detected using Mouse Anti-Actin antibody (AbCam Cat. 6276) as the primary antibody and Donkey Anti-Mouse IgG-800CW (LICOR) as the secondary antibody. After secondary antibody incubation and washing, membranes were scanned and analyzed by Odyssey system to obtain western images and band intensity quantification.

5.8. Lipid nanoparticle formulations

Lipid nanoparticle formulations preparations have been described previously [11].

5.9. Statistical analysis

Where appropriate, values are expressed as means \pm SEM. Groups were compared by nonpaired two-tailed heteroscedastic *t*-tests using GraphPad Prism software. A *p* value <0.05 was considered significant.

Data availability

Study data are included in the article. Any information regarding LUNAR technology or mRNA sequences can be directly addressed to Arcturus Therapeutics.

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Author contributions

S.L. and R.D.T. performed *in vitro* experiments and quantified and

analyzed in vivo samples; K.K., J.P. and A.D. produced and purified mRNA; J.A.G., T.A.G., T.D. and M.S. performed and supervised in vivo studies; J.H.K., J.B.V. and P.P.K. prepared and supervised lipid nanoparticle formulations for in vivo studies; R.D.T. and K.T. supervised studies, interpret data and prepare the manuscript; K.T. designed mRNA sequences and P.C. developed LUNAR technology.

Declaration of Competing Interest

All authors are employees of Arcturus Therapeutics. Data presented in this article is part of a filed patent.

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