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Erythrocyte-mediated delivery of phenylalanine ammonia lyase for the treatment of phenylketonuria in *BTBR-Pah*^{enu2} mice

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ABSTRACT

Phenylketonuria (PKU) is an autosomal recessive genetic disease caused by defects in the phenylalanine hydroxylase gene. Preclinical and clinical investigations suggest that phenylalanine ammonia lyase (PAL) could be an effective alternative for the treatment of PKU. The aim of this study is to investigate if erythrocytes loaded with PAL may act as a safe delivery system able to overcome bioavailability issues and to provide, *in vivo*, a therapeutically relevant concentration of enzyme. Murine erythrocytes were loaded with recombinant PAL from *Anabaena variabilis* (r*Av*PAL) and their ability to perform as bioreactors was assessed *in vivo* in adult *BTBR-Pah*^{enu2} mice, the genetic murine model of PKU. Three groups of mice were treated with a single i.v. injection of r*Av*PAL-RBCs at three different doses to select the most appropriate one for assessment of efficacy. Repeated administrations at 9–10 day-intervals of the selected dose for 10 weeks showed that the therapeutic effect was persistent and not affected by the generation of antibodies induced by the recombinant enzyme. This therapeutic approach deserves further *in vivo* evaluation either as a potential option for the treatment of PKU patients or as a possible model for the substitutive enzymatic treatment of other inherited metabolic disorders.

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1. Introduction

Phenylketonuria (PKU; OMIM*261600) is the most common inborn error of amino acid metabolism among Caucasians (overall incidence 1:10,000) caused by a deficiency of the enzyme phenylalanine hydroxylase (PAH; EC 1.14.16.1) (OMIM*612349) that converts L-phenylalanine (Phe) into tyrosine [1]. PAH defects result in Phe accumulation in all tissues, including brain, and lead to severe neurological and intellectual disability [1]. One of the current treatments is a rigorous life-long Phe-restricted diet, necessary to keep an optimal cognitive function [2]. Nevertheless, the diet implies some nutritional risks and an obvious psychological burden, resulting in poor patient compliance [3]. Another available treatment is sapropterin dihydrochloride (Kuvan® BioMarin Pharmaceutical Inc., Novato, CA), a synthetic cofactor of PAH approved for the treatment of PAH-deficient subjects who proved to be responsive. However, many patients, especially those affected by the most severe defects, are not responsive to Kuvan® [4] and are still in search of a medicament overcoming the limitations of diet.

Phenylalanine ammonia lyase (PAL; EC 4.3.1.24) is a non-mammalian enzyme derived from the blue-green algae *Anabaena variabilis* (*Av*) which is able to convert the essential amino acid Phe into metabolically harmless trans-cinnamic acid and trace amounts of ammonia [5]. r*Av*PAL chemically modified by polyethylene glycol (r*Av*PAL-PEG) is an enzyme substitution therapy under investigation (BioMarin Pharmaceutical Inc., Novato, CA) now in Phase III for patients whose Phe levels are not adequately controlled by dietary therapy or Kuvan® (see www.ClinicalTrials.gov; Government trial Identifiers: phase I NCT00634660; phase II NCT00925054, NCT01560286, NCT00924703 and NCT01212744; phase III NCT01819727 and NCT01889862). Although PEG has been used to modify several therapeutic molecules (mostly enzymes) thanks to its ability to attenuate the neutralizing immune response against the therapeutic agent [6], concerns about PEG metabolism and accumulation in the organism still remain [7–10], suggesting the usefulness of alternative strategies

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to widen the use of PAL treatment. In theory, to overcome these constraints the Phe-depleting enzyme may be loaded into circulating cells with the advantage of protecting it from the immune reaction while it carries out its own enzymatic activity on blood Phe. Several factors make red blood cells (RBCs) an ideal delivery system for r*Av*PAL: a) a protracted but predictable life-span; b) their biodegradability; c) their biocompatibility and non-immunogenicity; d) procedures exist for the transient opening of pores across the red cell membrane which permits non-diffusible enzymes to be loaded inside RBCs [11–14]; and e) when appropriate enzymes are entrapped, erythrocytes protect them from rapid clearance and from the action of antibodies commonly elicited in the patients upon repeated administrations [15,16].

Here we show that human and murine erythrocytes can be loaded with different amounts of r*Av*PAL and have the ability to perform as enzyme reactors to metabolize Phe *in vitro* and to reduce the systemic Phe concentration in *BTBR-Pah^{enu2}* mice. Two specific aims were pursued:

- to perform a dose range-finding investigation to determine the optimal dose of r*Av*PAL-loaded RBCs (r*Av*PAL-RBCs) able to lower Phe to safe levels;
- to assess the effectiveness of repeated administrations of r*Av*PAL-RBCs in *BTBR-Pah^{enu2}* mice to maintain low levels of blood Phe despite potential immunological host response.

2. Materials and methods

2.1. Animals

Adult homozygous *BTBR-Pah^{enu2}* and wild type (*WT*) mice were employed in this study. Their use was approved by the Ethical Committee for Animal Experiments of the University of Urbino "Carlo Bo"-Italy (Prot. CESA 3/2012). Animals were fed on Teklad global 18% protein rodent diet (Harlan Laboratories Inc., Madison, WI).

2.2. rAvPAL

Recombinant *Av*PAL was prepared by the BioMarin clinical manufacturing group. Two lots of recombinant protein were used, whose specific activity (SA) were 1.8 IU/mg and 1.54 IU/mg, respectively.

2.3. Development of human rAvPAL-RBCs for preliminary in vitro studies

Human blood was obtained from the Blood Transfusion Center of the Hospital "S. Maria della Misericordia", Urbino (PU) Italy; blood was provided by healthy adult volunteers who signed an informed consent form before donation and samples were collected anonymously in heparinized tubes. The use of human blood in the present study was approved by the research ethics committee of the University of Urbino (PU), Italy. r*Av*PAL (SA 1.8 IU/mg) was loaded into human RBCs by means of hypotonic dialysis, isotonic resealing and "reannealing", essentially according to Magnani et al. [17] with these modifications: RBCs were loaded with increasing amounts of enzyme by adding r*Av*PAL in the 6–29 IU range to RBC suspensions at fixed 70% hematocrit (Ht) in physiological saline solution containing 10 mM HEPES (pH 7.4), 154 mM NaCl and 5 mM glucose (Hepes solution). Each condition (1 ml final volume) was dialyzed at +4 °C in a cellulose tube (14 kDa cut-off) *vs* 50 ml of hypotonic solution 60 mOsm measured by Osmometer Fiske Associates Model 210, Norwood, MA, USA. After dialysis the cells were approx. 90 mOsm. To determine the best loading conditions to be used during the dialysis step, RBCs were re-suspended at Ht range 40–70% in Hepes solution in the presence of fixed 29 IU r*Av*PAL. Unloaded (UL) RBCs (*i.e.* cells submitted to the same procedure without the addition of the enzyme) were used as controls. The amount of entrapped r*Av*PAL was quantified essentially by a kinetic assay as previously described [18]. Hematological parameters were measured by an automatic Coulter AcT 5 Diff Hematology Analyzer (Beckmann, Miami, FL). Percent RBC recovery was calculated from the number of RBCs submitted to the dialysis step and those recovered at the end of the loading procedure.

2.4. Evaluation of phenylalanine metabolism by rAvPAL-RBCs

Enzyme-loaded and UL RBCs were incubated in Hepes solution with 2000 μM Phe to a final Ht of 40% at 37 °C for up to 60 min. At planned intervals (0, 30 and 60 min), 40 μl (in duplicate) was spotted on filter paper (Whatman903), dried and processed for Phe determination by tandem mass spectrometry (MS/MS).

2.5. Development of murine rAvPAL-RBCs for preliminary in vitro studies

Blood was collected from CO_2 anesthetized control *BTBR-WT* mice by puncture of the retro-orbital sinus in heparinized tubes and murine RBCs were essentially processed as previously described for human RBCs. To maximize r*Av*PAL loading into RBCs, 24 IU, 42 IU and 54 IU of enzyme (SA 1.8 IU/mg) were added to RBC suspensions at 60%, 50% and 40% Ht respectively. It must be noticed that only by decreasing RBC Ht, a greater volume was available for enzyme addition. Each condition was dialyzed 1 h at 4 °C *vs* dialysis buffer optimized for murine RBC loading: 15 mM NaH_2PO_4, 15 mM $NaHCO_3$, pH 7.4, 20 mM glucose, 4 mM $MgCl_2$, 3 mM glutathione, and 2 mM ATP (85 mOsm). After dialysis the cells reached about 105 mOsm. Subsequent steps were then carried out as described for human cells and the amount of entrapped protein was determined as reported [18].

2.6. Loading procedure and in vivo efficacy of rAvPAL-RBCs: dose finding study

In this step, 42 IU r*Av*PAL (400 μl of protein solution with SA 1.54 IU/mg) was added to 600 μl of packed RBCs to obtain 1 ml of erythrocytes at 50% Ht and dialyzed as described. This procedure was carried out for 15 separate dialysis tubes. At the end, all RBC suspensions were pooled (final volume 15 ml) and allowed to equilibrate 5 min at 37 °C under gentle stirring. At this stage the cells reached 102 mOsm. After resealing and re-annealing steps, loaded erythrocytes were washed twice in Hepes solution at 450 *g* for 10 min to remove the unentrapped enzyme. The expression of phosphatidylserine (PS) on RBC surface was evaluated immediately after the loading procedure by annexin V binding (see after). r*Av*PAL-RBCs were then re-suspended in Hepes solution at 36% Ht (final volume 3.5 ml) and checked for the amount of encapsulated protein before injection into *BTBR-Pah^{enu2}* mice (mean body weight 25.64 ± 9.47 g). r*Av*PAL-RBCs were prepared at 36%, 18% and 9% Ht (corresponding to approx. 4.75, 2.37 and 1.18 IU r*Av*PAL/ml, respectively), in order to administer the scheduled doses to the mice in the same volume (250 μl).

Three different doses of enzyme-loaded RBCs were administered to three cohorts of *BTBR-Pah^{enu2}* mice by i.v. injection so that each cohort received 0.25 IU ($n = 5$ mice), 0.5 IU ($n = 6$ mice) and 1 IU ($n = 3$ mice) of r*Av*PAL, respectively. The efficacy of the treatment was evaluated by biochemical monitoring of blood Phe, sampling blood at time 0, and then 1, 2, 5, 8, 12, 16, and 21 days after a single injection. Blood (40 μl) was collected from the submandibular vein by special animal lancets (Goldenrod 5.5 mm, Braintree Scientific Inc., Braintree, MA) after 2 h of food deprivation, spotted on filter papers and analyzed by MS/MS for Phe levels.

2.7. Loading procedure and in vivo efficacy of rAvPAL-RBCs: repeated administrations

For the repeated administration studies, 42 IU r*Av*PAL (SA 1.54 IU/mg) was added to packed RBCs (85.5 ± 7.1% Ht) to obtain 1 ml at 49 ± 2% Ht, and dialyzed 75 min at 4 °C (dialysis buffer for murine use 81.75 ± 2.31 mOsm), obtaining an osmolality of 108.5 ± 5.9 mOsm. The following

resealing steps, as well as the assay of encapsulated protein activity, were carried out as described above.

Packed loaded RBCs were re-suspended in Hepes solution at approx. 20% Ht in order to administer 0.67 ± 0.07 IU/mouse in a final volume of 180 ± 43 μl (step 1) or approx. 200 μl (step 2), an amount of enzyme according to that suggested by the "dose finding" *in vivo* study. In both steps, *BTBR-WT* ($n = 5$) and *BTBR-Pah^{enu2}* ($n = 5$) mice were used as controls and received repeated injections of Hepes solution following the same schedule as the RBC-treated mice.

Phe was evaluated by MS/MS analysis of blood spots and samples were also drawn from control healthy and *BTBR-Pah^{enu2}* mice. Blood samples (100 μl) for anti-r*Av*PAL IgG analysis were collected in heparin from the submandibular vein at different times from RBC infusion: time 0 (before each administration) then 9–10 and 13–14 days post i.v. (step 1); only at time 0 (before each RBC injection) for step 2. In both steps, plasma was collected 21 days post last infusion, too.

2.8. Tandem mass spectrometry

RBC suspensions and mouse whole blood were collected on Schleicher&Schuell 903 grade filter paper, dried under ambient conditions and stored at 4–8 °C in plastic bags. The analysis of Phe in the dried blood spots (DBS) was performed using a previous method [19] with some modifications. Three millimeter diameter dots were punched out and eluted in 100 μl of methanol/water (80:20) solution containing labeled amino acid internal standards (CIL, Andover, MA, USA). The samples were shaken for 30 min at 30 °C. Then 65 μl of supernatant was dried under nitrogen flow at 45 °C. The residues were treated with 50 μl of 3 M hydrochloric acid in n-butanol solution at 60 °C for 30 min. After derivatization, the samples were dried under nitrogen flow at 45 °C and recovered in 70 μl of acetonitrile/water (80:20) containing 0.1% formic acid. Twenty microliters was injected into a LC-MS/MS system (API 2000, Sciex, Toronto, Canada). A Series 200 micro pump (PerkinElmer, Norwalk, CT, USA) and a Series 200 autosampler (PerkinElmer) were used for solvent delivery and automated sample loading. The mobile phase was acetonitrile/water (80:20) at a flow rate of 50 μl/min. Neutral loss scan of 102 Da fragment was used for the detection of Phe. The total acquisition time was 2 min.

2.9. Evaluation of plasma anti-rAvPAL IgG levels

IgG levels were evaluated by standard indirect ELISA. Plasma was obtained from blood samples and tested by standard indirect ELISA employing r*Av*PAL as antigen (1 μg/ml in 50 mM carbonate buffer, pH 9.7). Samples were serially diluted in the range of 1:50–1:200 for pre-treatment plasma and 1:400–1:409,600 for post-treatment plasma and tested in duplicate. The immune complexes were revealed by a chromogenic reaction.

2.10. Annexin V staining

Annexin V was used as a probe to detect cells that have exposed phosphatidylserine on the surface, an event associated with cell death or membrane damage. Positive RBCs were counted by flow cytometry as previously described [20].

2.11. Statistical analysis

Blood Phe levels were analyzed by 2-way ANOVA (dose finding study) or by one-way ANOVA (repeated administration studies).

3. Results

3.1. In vitro studies

3.1.1. Development of human rAvPAL-RBCs and Phe metabolism

Human RBCs loaded with increasing quantities of protein were obtained both by adding increasing amounts of enzyme during the encapsulation procedure (Table 1, a–d) and by varying RBC Ht (range 70–40%, Table 1, d–g) in the presence of a fixed amount of r*Av*PAL (29 IU). Both strategies were effective in modulating the encapsulated protein. The most efficient procedure provided approx. 10 IU/ml packed cells, when the highest amount of protein (29 IU) together with the lowest RBC Ht (40%) was used. RBC recovery and hematological parameters were also investigated: by decreasing the RBC Ht, cell recovery is reduced; however, at the end of the loading procedures, a recovery higher than 50% could always be achieved for all the tested dialysis conditions. RBC corpuscular indices (MCV, MCH, MCHC) of r*Av*PAL-RBCs are in agreement with those of native cells (reference values) for the 70% Ht conditions, whereas the differences are more pronounced when Ht was reduced during dialysis. Accordingly, RDW values, too, differ from reference, particularly at the lowest Ht.

As concerns the ability of human r*Av*PAL-RBCs (Table 1, d–g) to metabolize Phe, the cells proved to be very efficient even at the lowest dose of encapsulated enzyme (2.4 IU/ml packed RBCs), condition at which 80% Phe was metabolized after 30 min incubation at 37 °C. At the same time point, Phe totally disappeared in the presence of the highest dose of loaded enzyme (10.1 IU/ml packed RBCs). After 1 h at 37 °C, Phe was completely metabolized even by RBCs loaded with 4.4 IU/ml packed cells; at this incubation time, Phe was below 200 μM in all the tested conditions.

3.1.2. Development of murine rAvPAL-RBCs and Phe metabolism

To provide the necessary information for preclinical investigations, the best experimental condition to load r*Av*PAL in murine RBCs was determined. The results reported in Table 2 show that, like human cells, mouse RBCs could be loaded with increasing amounts of r*Av*PAL by varying RBC Ht and protein concentration. However, when compared to human RBCs, the recovery percentage was much lower. The corpuscular indices revealed values for murine r*Av*PAL-RBCs not significantly different from reference values (for dialysis at 60% and 50% Ht); it

Table 1
Optimization of r*Av*PAL loading into human RBCs.

RBC dialysis Ht	Added r*Av*PAL (IU)	Loaded r*Av*PAL (IU/ml RBCs)	RBC recovery (%)	MCV (μm^3)	MCH (pg)	MCHC (g/dl)	RDW (%)
a) 70%	6	1	81	84	24.9	29.6	21.6
b) 70%	12	1.3	77	89	26.2	29.4	20.8
c) 70%	24	1.5	75	88	26.3	29.7	20.9
d) 70%	29	2.4	66	81	23.9	29.5	24.3
e) 60%	29	3.3	72	83	23	27.7	23.4
f) 50%	29	4.4	62	82	21.4	26.1	25.6
g) 40%	29	10.1	50	82	16.1	19.6	35.4
Reference values for human erythrocytes (range)[a]				80–97	26.5–33.5	31.5–35	10–15

[a] Reference ranges are the minimum and maximum values observed in human blood before being submitted to the loading procedure. Results are from a single experiment.

Table 2
Optimization of r*Av*PAL loading into murine RBCs.

RBC dialysis Ht	Added r*Av*PAL (IU)	Loaded r*Av*PAL (IU/ml RBCs)	RBC recovery (%)	MCV (μm^3)	MCH (pg)	MCHC (g/dl)	RDW (%)
a) 60%	24	3.15	28	43	16.6	38.3	18.4
b) 50%	42	8.06	29	40	18.5	34.6	18.8
c) 40%	54	15.62	18	42	9	21.3	20.1
Reference values for murine erythrocytes (range)[b]				48	16.8–18.1	34.7–37.7	14.5–15.2

[b] Reference ranges are the minimum and maximum values observed in murine blood before being submitted to the loading procedure. Results are from a single experiment.

should be highlighted that the samples dialyzed at 40% Ht showed values not comparable to native cells, confirming that this condition is too strong for the fragility characteristics of murine RBCs. Phe metabolism was evaluated as for human cells; murine RBCs loaded with different r*Av*PAL doses (3.15, 8.06 and 15.62 IU/ml packed cells) were able to metabolize 72–80% of Phe after 1 h of incubation at 37 °C.

3.2. In vivo efficacy of rAvPAL-RBC treatment: dose finding study

This study was performed to test the ability of different doses of r*Av*PAL-RBCs to reduce blood Phe concentration in *BTBR-Pah^{enu2}* mice. Based on the results *in vitro*, condition b shown in Table 2 was selected. A bulk loading procedure was used, at the end of which 2.34 ml of RBCs at 54% Ht loaded with 16.7 IU of r*Av*PAL was obtained (corresponding to 13.2 IU r*Av*PAL/ml packed RBCs). Differences between loaded RBCs of *in vitro* and *in vivo* studies are likely due to intrinsic cell variability among animals. The cells positively stained with annexin V increased from 0.3% in basal conditions to 1.5% after the procedure. The evaluation of the *in vivo* effect of r*Av*PAL-RBCs yielded the results shown in Fig. 1.

Phe levels before treatment were 1186 ± 342, 1221 ± 149 and 1292 ± 211 μM in groups that received 1, 0.5 and 0.25 IU/mouse respectively, and were not significantly different ($p > 0.05$). All the doses of enzyme caused Phe to dramatically decrease in the first hours after treatment, with peak values of respectively 54.8 ± 90.5, 5.6 ± 6 and 57.1 ± 56 μM after 24 h. However, with 0.25 IU r*Av*PAL, Phe slowly returned toward basal levels reaching on day 8 roughly 50% of the pre-treatment values. For the two highest doses, the effect remained maximal for the first 5 days after infusion, with slow return to basal levels (both approx. 21% of their respective basal values on day 8). On day 8, the effect of these two doses was statistically superior ($p < 0.05$) to that of the lowest dose while between them there was no significant difference. On day 12, Phe was similar in the three groups (74%, 73%, and 68% of basal values, with 0.25, 1 and 0.5 IU/mouse, respectively). Moreover, Phe levels were significantly lower ($p < 0.05$) than basal values through 8 days post treatment in every group.

3.3. In vivo efficacy of rAvPAL-RBC treatment: repeated administrations

We assessed the effect of repeated administrations of r*Av*PAL-RBCs with a dose of at least 0.5 IU/mouse (minimum administered dose). The aims were: a) assessment of efficacy of three subsequent infusions of r*Av*PAL-RBCs at 18 day-intervals and evaluation of the most appropriate time-lag between the subsequent injections in order to keep blood Phe in a potentially safe range (step 1); b) assessment of efficacy of seven administrations of loaded RBCs at 9–10 day-intervals (step 2); and c) exploration of the specific immune response against r*Av*PAL in mice treated with r*Av*PAL-RBCs (step 1 and step 2). The main properties of loaded RBCs used in these studies are shown in Table 3.

3.3.1. Efficacy of three rAvPAL-RBC injections at 18–19 day-intervals in BTBR-Pah^{enu2} mice

Five *BTBR-Pah^{enu2}* mice were treated with three i.v. injections of 0.67 ± 0.07 IU/mouse r*Av*PAL-RBCs (an amount of enzyme according to that suggested by the "dose finding" study) at 18–19 day-intervals. The results reported in Fig. 2a show that the injected r*Av*PAL-RBCs are able to decrease blood Phe levels to values near normality 4–5 days after each treatment, while, after 13–14 days, Phe returns to the initial values. Data were also graphed by Box-and-Whiskers plot (Fig. 2b) and suggest 9–10 days as the longest possible time interval between administrations capable of maintaining blood Phe at concentrations significantly lower than pathological.

Phe excess inhibits metabolic pathways dependent upon tyrosine, such as melanin production. By reducing Phe, this pathway is restored as illustrated by the fur darkening of a mouse involved in step 1 (Fig. 3), potentially representing a decrease in Phe-mediated pathology.

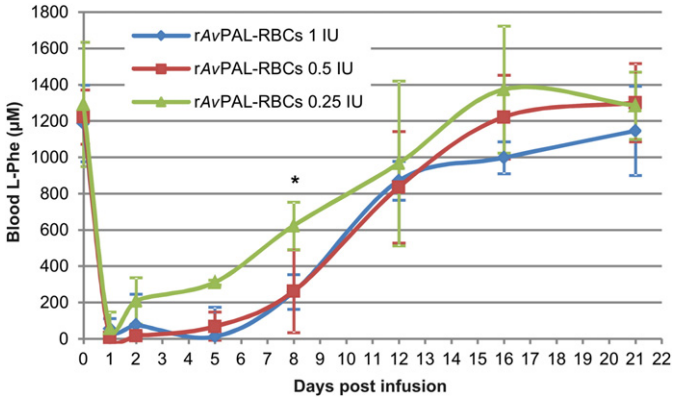

Fig. 1. Blood Phe concentrations before and after r*Av*PAL-RBC injection. Mice received 1 ($n = 3$), 0.5 ($n = 6$) and 0.25 ($n = 5$) IU r*Av*PAL-RBCs. The treatment efficacy is not significantly different between 0.5 IU and 1 IU r*Av*PAL, whereas a significantly lesser effect was observed in mice receiving 0.25 IU, compared to the other two doses, 8 days post treatment (by 2-way ANOVA followed by Tukey's test, *$p < 0.05$). All doses were significantly effective until 8 days post treatment (by 2-way ANOVA followed by Dunnett's test, $p < 0.05$ *vs* pre-treatment).

Table 3
Features of murine r*Av*PAL-RBCs of the *in vivo* repeated administration study, step 1 and step 2.

	Loaded r*Av*PAL (IU/ml RBCs)	RBC recovery (%)	MCV (μm^3)	MCH (pg)	MCHC (g/dl)	RDW (%)	Annexin V (%)
Step 1 ($n = 3$)	17.2 ± 5.69	49 ± 10.45	37 ± 1.83	12.2 ± 1.47	32.88 ± 2.95	17.53 ± 0.47	5.03 ± 1.55
Step 2 ($n = 7$)	17.97 ± 4.2	45.79 ± 11.3	37.14 ± 1.86	12.69 ± 2.12	33.79 ± 4.34	18.01 ± 0.91	4.6 ± 1.1
Control erythrocytes (range)[c]			48–50	16.5–22.2	33.7–45	13.6–15	1.3 ± 0.4

[c] Reference ranges are the minimum and maximum values observed in overall murine blood before being submitted to the loading procedure. Data are means ± SD. n = number of independent loading procedures.

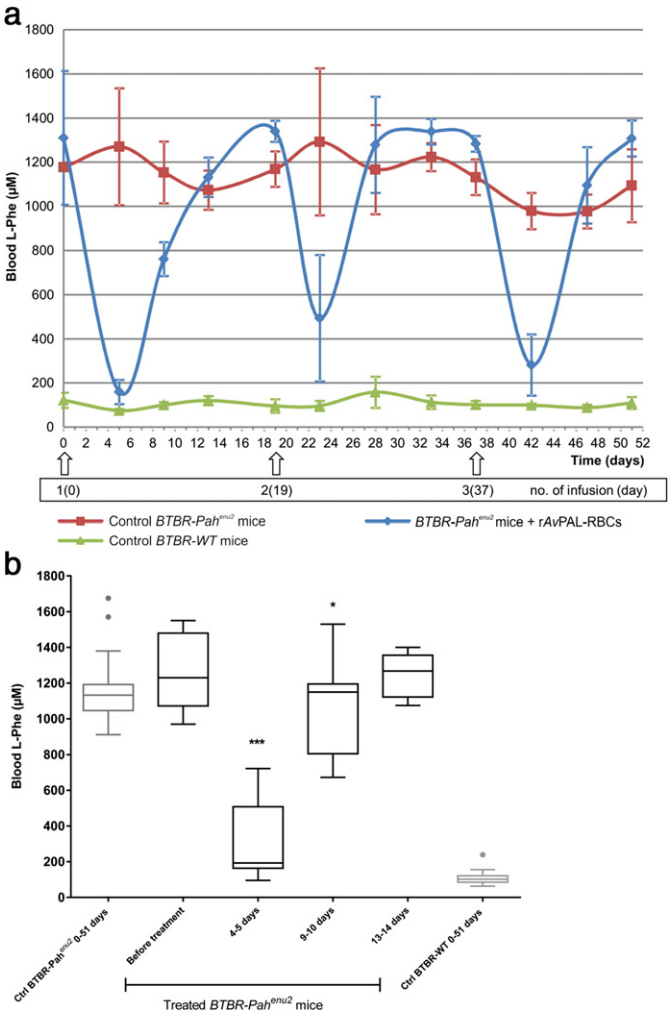

Fig. 2. Blood Phe levels in control mice and *BTBR-Pah^{enu2}* mice treated with r*Av*PAL-RBCs at 18–19 day-intervals. (a) Time-course representation of mean Phe values ± SD of control ($n = 5$ for both control groups) and treated mice ($n = 5$). (b) Box-and-Whiskers plot of Phe values in control (Ctrl) and treated mice; the effect was significant by one-way ANOVA followed by Dunnett's test ($p < 0.05$ *vs* before treatment) up to 9–10 days post RBC injections. Control mice received i.v. injections of Hepes solution.

Noteworthy, as revealed by the evaluation of anti-r*Av*PAL plasma IgG (Fig. 4), the presence of antibodies did not modify the ability of loaded RBCs to reduce blood Phe. In fact, as expected, the first infusion had minimal effect on IgG production, while subsequent injections resulted in a strong and increasing boost in the immune response.

3.3.2. Efficacy of seven rAvPAL-RBC injections in BTBR-Pah^{enu2} mice

Eight *BTBR-Pah^{enu2}* mice received seven infusions of 0.67 ± 0.07 IU/mouse r*Av*PAL-RBCs at 9–10 day-intervals. Blood Phe and anti-r*Av*PAL plasma IgG were monitored during the whole experimental period. Results reported in Fig. 5a and b show that the seven r*Av*PAL-RBC injections were able to maintain blood Phe to values between those of healthy control mice (115.30 ± 50.40 μM) and those of *BTBR-Pah^{enu2}* mice (1137.99 ± 127.19 μM) for the duration of the study. All the infusions were able to significantly decrease Phe to a similar extent, reaching values similar to those of healthy mice 4–5 days after each treatment and returning to 65% of pre-treatment values 9–10 days after each i.v., thus confirming what previously seen (step 1). In addition, when the efficacy of the treatment was evaluated for the whole duration of the experiment (from day 0 to day 70) by estimating the AUCs, a reduction of 51.6% in blood Phe was observed.

Like the 3-dose study, mice treated more frequently with r*Av*PAL-RBCs restored fur pigmentation, indicating a restoration

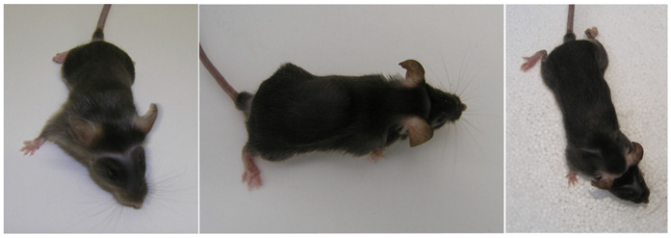

Fig. 3. Fur pigmentation of a *BTBR-Pah*^{enu2} mouse involved in step 1 of the "Repeated administration" study. Pictures were taken at time 0 before infusions (left), 9 days after the 2nd i.v. (middle) and 20 days after the 3rd i.v. of r*Av*PAL-RBCs (right).

of tyrosine metabolism. In this study, anti-r*Av*PAL plasma IgG levels generally increased after each infusion (Fig. 6), peaking at a similar titer as seen in step 1. The presence of anti-r*Av*PAL antibodies did not modify the efficacy of enzyme-loaded RBCs in removing blood Phe after several administrations.

4. Discussion

We have demonstrated that r*Av*PAL-RBCs act as bioreactors able to decrease blood Phe *in vitro* and *in vivo* in *BTBR-Pah*^{enu2} mice, a widely used animal model of human PKU. Clinical studies have shown that RBCs are ideal carriers to deliver therapeutic enzymes in circulation and can protect them from premature inactivation both by plasma proteases and by neutralizing antibodies, when repeated administrations are needed [15,16]. Starting from these results, the same strategy was employed here to treat PKU. In preliminary *in vitro* studies, the best conditions to load r*Av*PAL in human and murine RBCs were developed. The results showed that it is possible to load different amounts of enzyme by varying both the RBC hematocrit and the enzyme concentration during the dialysis step. Up to now, RBCs loaded with different quantities of protein were obtained only by adding the latter in increasing amounts during the dialysis [21]. Therefore, this is the first evidence of the possibility to modulate the final protein content by acting on Ht values too.

Human and murine r*Av*PAL-RBCs showed a pronounced ability to metabolize Phe as expected from the Phe uptake kinetics [22]. Therefore, differences in Phe metabolism were due to the different amount of loaded enzyme. To evaluate the preclinical efficacy of the strategy, the experimental conditions were set at three enzyme doses (1, 0.5 and 0.25 IU r*Av*PAL/mouse). As far as *in vivo* efficacy is concerned, all the doses of enzyme delivered by RBCs were able to dramatically decrease blood Phe in the first hours after treatment with no differences among doses. The lowest one (0.25 IU/mouse) however, appeared to

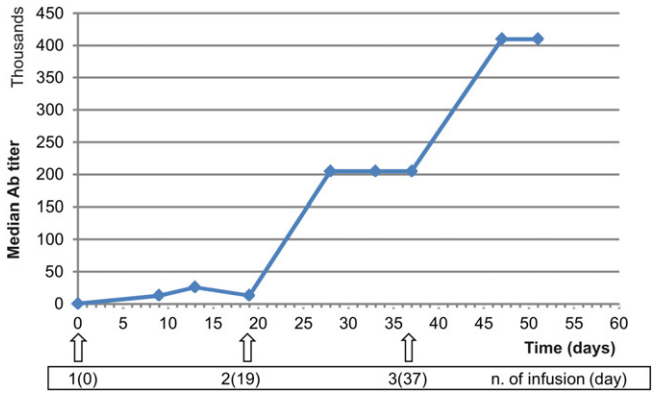

Fig. 4. Median antibody titers of *BTBR-Pah*^{enu2} mice treated with r*Av*PAL-RBCs at 18–19 day-intervals (step 1, $n = 5$). Blood samples were collected at time 0 before each infusion and at time 9–10 days and 13–14 days post infusion. Samples were collected on day 21 after the last treatment, too. On the basis of the results so far obtained, a longer term study with more frequent dosing was performed.

have a shorter duration of action, as Phe started to return toward basal concentrations on day 2 and reached roughly 50% of the pre-treatment values on day 8. The other two doses (0.5 and 1 IU/mouse) seemed to be both supramaximal. These results suggest that the duration of action of the treatment is determined by the clearance of loaded erythrocytes from circulation. In fact, the half-life of loaded murine RBCs has been repeatedly reported to be in the range of 6–11 days [23–25], slightly reduced in comparison with native cells (range 12–14 days [23,26]), due to a minimal loading-induced damage. These data overall demonstrate that blood Phe levels can effectively be modulated *in vivo* by injection of r*Av*PAL-RBCs and, moreover, they allow the selection of a proper schedule of repeated treatments for long term control of hyperphenylalaninemia in *BTBR-Pah*^{enu2} mice. As expected, once r*Av*PAL is administered through RBCs three times every 18–19 days, the presence of plasma anti-r*Av*PAL IgG does not modify the enzyme activity. Similar rates of Phe reduction were observed among injections following the repeated administrations in spite of the increasing levels of anti-r*Av*PAL IgG. The subsequent seven infusions of r*Av*PAL-RBCs (0.67 ± 0.07 IU/mouse) at 9–10 day-intervals proved to be able to reduce blood Phe to levels significantly lower than their corresponding starting values during the whole experiment (70 days). Moreover, a reversal of hypopigmentation was observed, confirming what was previously reported by Sarkissian [27] in which *BTBR-Pah*^{enu2} mice were treated with weekly s.c. injections of r*Av*PAL-PEG (4 IU for 10 weeks and then 2 IU for 6 weeks). This result was probably due to the reduced inhibition of tyrosinase caused by the excess of blood Phe in PKU disease [28]. It has to be highlighted that in the present work the reversal of hypopigmentation was observed following less frequent injections of a lower dose of enzyme.

In our studies, comparisons with free and/or r*Av*PAL-PEG were not performed since authoritative and detailed literature in this field had already been produced [27] showing the advantages of PEGylation. Moreover, Sarkissian *et al.* clearly indicated how the administration of decreasing doses of the PEGylated enzyme permits to reduce immunogenicity, thus emphasizing the importance of protection against the immune reaction. In our study, a better efficacy (Phe range 100–900 μM) was attained by the i.v. administration of approx. half the dose every 9–10 days compared to the lowest dose employed by Sarkissian *et al.*, which resulted in blood Phe in the range 500–1200 μM [27].

In addition, a very recent work by Longo et al. [9] (clinical trial NCT00925054) shows that a single s.c. injection of r*Av*PAL-PEG in PKU patients induced the production of antibodies both against PEG and r*Av*PAL and caused some moderate hypersensitivity adverse events, even though the treatment was generally fairly well tolerated. While this important study confirms the efficacy of a single s.c. bolus of r*Av*PAL-PEG in reducing blood Phe (at a dosage of 0.1 mg/kg) in adult PKU patients, the effect of repeated dosing as concerns immunological reaction and enzymatic activity remains to be explored. In the experimental setting we settled, the erythrocyte-based approach seems to overcome some problems associated with s.c. injection of the enzyme while ensuring a persistent therapeutic effectiveness.

The administration of PAL by carrier RBCs as a therapy for PKU was first proposed by Sprandel *et al.* in 1990 [29] with a short-term study

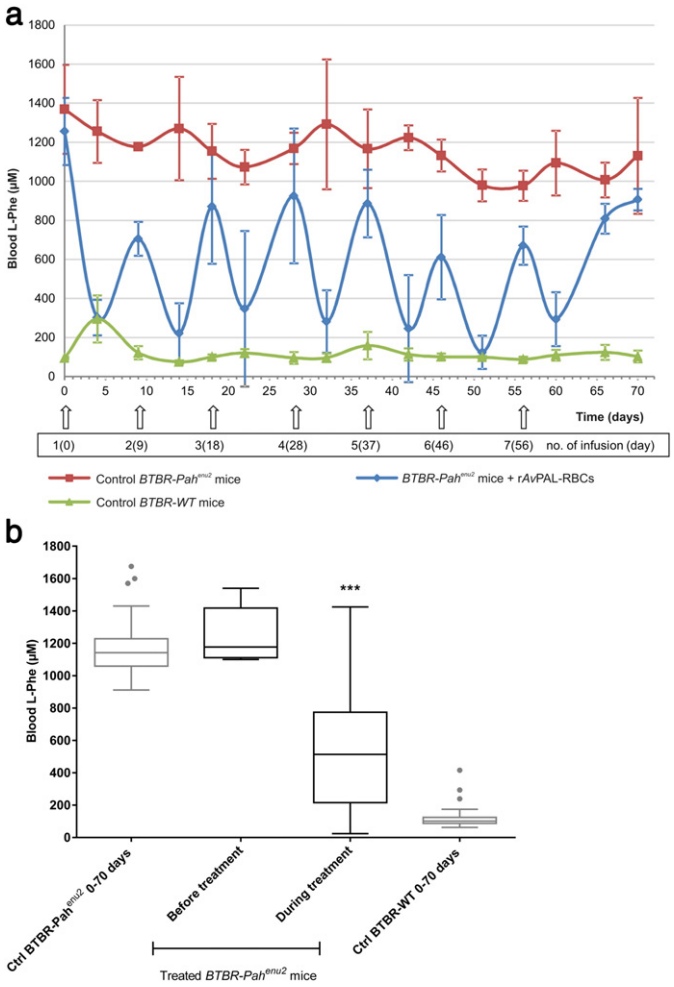

Fig. 5. Blood Phe levels in *BTBR-Pah*^{enu2} mice treated with r*Av*PAL-RBCs at 9–10 day-intervals. a) Time-course representation of mean Phe values ± SD of control ($n = 5$ for both control groups) and treated mice ($n = 8$). b) Box-and-Whiskers plot of Phe values in control (Ctrl) and treated mice; for the whole duration of the study (10 weeks), r*Av*PAL-RBC injections maintained Phe at levels significantly lower than their starting condition (by non parametric ANOVA followed by Dunn's test, $p < 0.05$). The set named "During treatment" comprises all values from day 4 post 1st i.v.to day 14 post 7th i.v. Control mice received i.v. injections of Hepes solution.

demonstrating the effectiveness of the strategy. Recently, PAH-loaded RBCs were proposed for the same purpose [30] but, unfortunately, they were unable to lower Phe when administered to *BTBR-Pah*^{enu2} mice, thus revealing the superiority of r*Av*PAL-RBCs in reaching the objective.

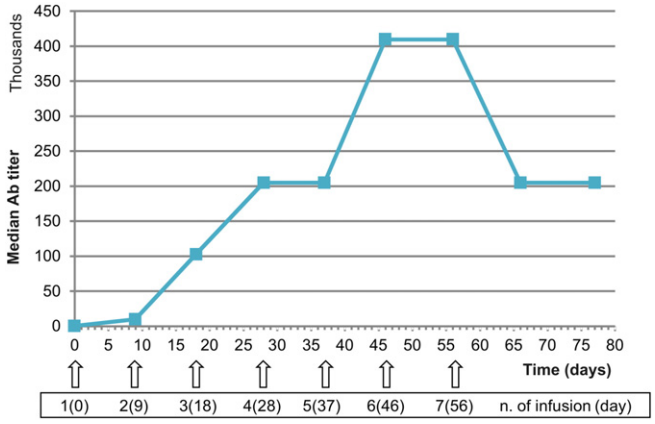

Fig. 6. Median antibody titers of *BTBR-Pah*^{enu2} mice treated with r*Av*PAL-RBCs at 9–10 day-intervals (step 2, $n = 8$). Blood samples were collected at time 0 before each infusion and on days 10 and 21 after the last infusion.

Moreover, it is noteworthy that an electromedical device (named Red Cell Loader©) capable of processing, in aseptic and pyrogen-free conditions, a small volume of autologous erythrocytes to be re-infused into the same donor, is already in clinical use (www.erydel.com). In fact, clinical studies have been performed employing dexamethasone 21-P-loaded-RBCs generated by the "Red Cell Loader" equipment for the treatment of different diseases [31–35].

5. Conclusions

Our data indicate r*Av*PAL-RBCs as a potential treatment for PKU patients. As demonstrated here, proteins can be loaded into RBCs, thus opening new perspectives for the development of enzyme replacement therapies for disorders involving enzymatic deficiencies. Other inborn errors of metabolism, which share with PKU a similar pathophysiological mechanism and are characterized by a progressive blood accumulation of toxic compounds [36], could benefit from the biotechnological approach here described.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jconrel.2014.08.012.

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