

Large neutral amino acids in the treatment of phenylketonuria (PKU)

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Summary Large neutral amino acids (LNAA) have been used on a limited number of patients with phenylketonuria (PKU) with the purpose of decreasing the influx of phenylalanine (Phe) to the brain. In earlier studies on mice with PKU (ENU²/ENU²), LNAA were given and a surprising decline in blood Phe concentrations was observed. The formula used in the mouse experiment (PreKUnil) lacked lysine. Therefore, a new formulation of LNAA (NeoPhe) was developed, introducing changes in the concentration of some amino acids and adding lysine, so that such a mixture could be used in humans. The new formula was found to be effective

in reducing blood Phe concentration in mice by about 50% of the elevated levels. Patients with PKU were given LNAA and blood Phe concentrations were determined in an open-label study. Three centres—in Russia, the Ukraine and the USA—took part in the study. NeoPhe was given at 0.5 g/kg per day in three divided doses to eight subjects with PKU and at 1.0 g/kg per day to three patients, for one week. The NeoPhe resulted in decrease of elevated blood Phe by 50% in both groups. The preliminary data from this study are encouraging and a double blind placebo-controlled trial will be required to show long-term efficacy and tolerance of LNAA in the treatment of PKU.

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Abbreviations

CSF	cerebrospinal fluid
5-HIAA	5-hydroxyindolacetic acid
LNAA	large neutral amino acid
Phe	phenylalanine
PKU	phenylketonuria
VIL	valine, isoleucine and leucine

Introduction

Phenylalanine-restricted diet for the treatment of PKU started following the early trials of Bickel and colleagues (1953) about 50 years ago. Experience gained from treating PKU showed the efficacy of dietary treatment in preventing mental retardation, even with some diet relaxation as children got older. Recently, 'Diet for life' has been advocated beyond childhood years (Azen et al 1991; Fisch et al 1997; Gleason et al 1992; Holtzman et al 1986; Michals et al 1985; Walters et al 2002). When blood Phe concentrations are not brought down to what is considered a therapeutic concentration, problems with poor school performance, impaired executive

functioning, changes in the white matter of the brain and loss of intelligence quotient may be encountered (Burgard et al 1997; Diamond 2001; Fisch et al 1995; Griffiths et al 1995; Lou et al 1985; Michals et al 1988; Pietz et al 1998; Ris et al 1994; Schmidt et al 1994; Scriver and Kaufman 2001; Seashore et al 1985; Smith et al 1978, 1991; Thompson et al 1990, 1994; Walters et al 2002). Therefore, in order to prevent blood concentrations of Phe exceeding a certain acceptable concentration, alternative modes of therapy for PKU are being sought, so that some reduction of blood Phe concentrations continues throughout the life of patients with PKU (Scriver and Kaufman 2001).

Clinics have adopted their own criteria for acceptable concentration of blood Phe for adults with PKU. In order to have uniformity in the treatment of PKU and to have a range of acceptable blood Phe concentrations in the USA, the National Institutes of Health (NIH) convened a consensus conference and issued guidelines for treating PKU. Blood Phe concentration of 120–360 $\mu\text{mol/dl}$ were recommended for young children 0–13 years; for those of 13 years and older the concentration are allowed to go higher, 900 $\mu\text{mol/dl}$ (NIH 2001). In Europe the guidelines for adults are different and blood Phe concentrations of 1200 $\mu\text{mol/dl}$ are acceptable (MRC 1993).

The first approach to lowering blood Phe concentrations and compliance with dietary treatment was attempted by companies who make formulas for PKU. In order to improve treatment, PKU formulas were made more palatable and were offered in a variety of flavours and textures such as gels, bars, tablets and other forms. The new formulas are more acceptable to adults, but still compliance has been limited.

A recent addition to therapy is the use of (6*R*)-L-erythro-5,6,7,8-tetrahydrobiopterin (BH_4) for treatment of PKU. Kure and colleagues (1999) reported that patients with mild PKU responded to BH_4 by significant reduction in their blood Phe. Subsequent studies (Blau and Scriver 1997; Blau and Trefz 2002; Erlandsen et al 2004; Kure et al 1999; Lassker et al 2002; Lindner et al 2003a, b; Matalon et al 2003, 2004; Muntau et al 2002; Spaapen et al 2000; Trefz et al 2000, 2001; Weglage et al 2002) confirmed the findings of Kure and colleagues. It seems that patients with mild missense mutations in one allele may show significant decline in their blood Phe concentrations when given BH_4 . From our experience with BH_4 , about 50% of patients may benefit from BH_4 but only about 10% of patients will require BH_4 as a monotherapy and the other 40% of the patients who respond to BH_4 will require dietary restriction of Phe in addition to BH_4 treatment (Matalon et al 2002, 2004).

Interest in the possibility that large neutral amino acids (LNAA) could lower brain Phe was initiated by the work of Oldendorf and colleagues (Oldendorf and Szabo 1976). In order to cross the blood–brain barrier, amino acids require a transporter protein. The large neutral amino acids

and the cationic amino acids (phenylalanine, tyrosine, tryptophan, threonine, isoleucine, leucine, valine, methionine, lysine, arginine and histidine) share a common transporter to the brain and compete with one another (Choi and Pardridge 1986; Hargreaves and Pardridge 1988; Pardridge 1977, 1982; Pardridge and Oldendorf 1975). Pardridge (1982) showed that the transport of LNAAs on the carrier protein and the movement of amino acids across the blood–brain barrier depend on the affinity of each amino acid for the carrier protein. So far, studies with PKU have concentrated on the blood–brain barrier and the reduction of entry of Phe into the brain compartment.

Large neutral amino acids and cationic amino acids cross the intestinal mucosa by means of a carrier protein similarly their crossing of the blood–brain barrier, except that the affinity of the amino acids for the intestinal carrier is determined by a K_m two orders of magnitude higher than that of the blood–brain carrier. In this study we document the lowering of blood Phe concentration in humans and mice with PKU using a mixture of LNAAs supplied in the diet, suggesting that competition at the intestinal carrier protein can be achieved.

Materials and methods

The mixture of LNAAs (NeoPhe) was obtained from Prekulab, Korsor, Denmark. The composition of NeoPhe is shown in Table 1 and is compared to PreKUnil, which is not suitable for long-term use in humans because of potential lysine deficiency if it is taken with limited protein intake. In NeoPhe, lysine and histidine are added and leucine is increased. Mice with PKU ($n = 7$), genotype $\text{ENU}^2/\text{ENU}^2$ (classical PKU), were purchased for the University of Texas Medical Branch (UTMB) laboratory from Jackson Laboratories, Bar Harbor, ME, USA. The ENU^2 mutant mouse strain was produced at the University of Wisconsin by exposure

Table 1 Comparison of PreKUnil and NeoPhe^a (LNAA) composition per tablet

L-Amino acid	PreKUnil (mg)	NeoPhe (mg)
Tyrosine	128	195
Tryptophan	128	51
Methionine	35	32
Isoleucine	35	35
Threonine	35	32
Valine	35	35
Leucine	35	130
Histidine	0	30
Lysine	0	30
Arginine	35	30
Total	466	600

^aPrekulab, Denmark

of founder animals to *N*-ethyl-*N'*-nitrosourea, which caused a F263S point mutation, resulting in classical PKU in homozygous animals. Mice used in this study were genotyped to verify that they were homozygous for the F263S mutation according to the method of McDonald and Charlton (1997). The group of mice at UTMB, Galveston and at Wichita State University were given 16.7% NeoPhe blended into their normal chow diet. This was done following ICAUC approval. On average, mice eat approximately 6 g of chow daily. The addition is 1 g of LNAA to 5 g of chow, which contains about 50 mg of Phe, so the ratio of LNAA to Phe is very high. Blood Phe was determined four times in one week before NeoPhe and twice in one week while on NeoPhe, and twice following cessation of treatment.

Patients with PKU were recruited from three centres: the Institute of Clinical Genetics, Kharkiv State Medical University, Kharkiv, Ukraine; the Department of Clinical Genetics, Institute of Pediatrics and Child Surgery, Moscow, Russia; and the Department of Pediatrics, University of Texas Medical Branch, Children's Hospital, Galveston, Texas, USA. The patients enrolled in the open-label study had to have PKU and be old enough to swallow pills. Each patient signed an institutionally approved informed consent prior to enrolment and all the patients were genotyped earlier.

Patients in the Ukraine and Russia were treated with Tetraflex early in life. Tetraflex is similar to other formulas in Europe. The patients in the study were not in optimal dietary control, and Phe intake exceeded 500 mg/day. The patients from the US clinic were older and were on 'vegetarian' diet; their Phe intake exceeded 1000 mg/day.

There were 11 patients, 4 male and 7 females. Eight patients (mean age 20.5 years) received 0.5 g/kg per day and 3 patients (mean age 16.5 years) received 1.0 g/kg per day of NeoPhe divided into three doses and taken before meals. Patients were instructed to continue their diet as they did prior to enrolling in the trial. Baseline Phe was determined on four separate occasions and at zero time and post NeoPhe at one week. Blood Phe was also determined one week after NeoPhe treatment. Blood Phe was assayed using filter paper and tandem mass spectroscopy (Pediatrix, Bridgeville, PA, USA).

Paired *t*-tests were used to assess changes from baseline measurements with the T-Test procedure using SAS statistical software (*SAS/STAT 9.1 User's Guide*; SAS Institute, Inc., Cary, NC, USA).

Results

The mean blood Phe concentration for mice ($n = 7$) was determined four times during nine days on normal chow. The average concentrations were used as a baseline for each mouse. The average Phe concentration of two blood samples while on NeoPhe was used for post-LNAA testing. Blood

Phe concentrations were decreased by 53%, from an average of 1444 to an average of 678 $\mu\text{mol/dl}$. This decline in blood Phe is statistically significant ($p < 0.0001$) as shown in Fig. 1. The identity of each mouse is shown on the right of the graph. Blood Phe decreased on average by 766 $\mu\text{mol/dl}$ ($\text{SD} = 197$). Since mice could not manipulate their intake and constantly ate the same diet, the decrease in their blood Phe concentrations clearly shows 'proof of principle' that LNAA in NeoPhe can reduce blood Phe concentrations.

Blood Phe concentration in the 8 patients (Fig. 2) taking 0.5 g/kg per day of LNAA decreased from an average of 957.4 $\mu\text{mol/dl}$ to an average of 458.4 $\mu\text{mol/dl}$, a decline of 52%, which is statistically significant ($p = 0.004$). There were three patients (Fig. 3) who took 1 g/kg per day of LNAA. Their baseline average was 1230 $\mu\text{mol/dl}$ compared to an average of 549.0 $\mu\text{mol/dl}$, an average decline of 55%.

All patients experienced a decrease in blood Phe concentrations from baseline after NeoPhe. The average decrease was 601 $\mu\text{mol/dl}$ ($\text{SD} = 370$), and when analysed together ($N = 11$) this drop in blood Phe was highly significant ($p = 0.0003$).

When treatment was discontinued, blood Phe concentrations increased to pre-trial levels.

Discussion

Lack of adherence to diet in the treatment of PKU has resulted in neuropsychological deficits, even with early detection and treatment (Burgard et al 1997; Diamond 2001; Fisch et al 1995; Griffiths et al 1995; Lou et al 1985; Michals et al 1988; Pietz et al 1998; Smith et al 1978; Thompson et al 1990). This created the need to reassess treatment strategies and prompted the National Institutes of Health (NIH) in the USA to convene a consensus conference so that recommendations for treatment guidelines could be reached (NIH 2001). The difficulty in attaining the goal of blood Phe of 120–600 $\mu\text{mol/dl}$ in adolescents was highlighted at the conference, and was further documented by the report of Walters and colleagues (2002). There have been ongoing attempts to find other modalities for therapy of PKU. A promising method for lowering blood Phe concentrations in patients with mild PKU is supplementation with BH_4 (Blau and Trefz 2002; Kure et al 1999; Lindner et al 2003a, b; Matalon et al 2002, 2004; Muntau et al 2002; Trefz et al 2000, 2001; Weglage et al 2002). Studies with BH_4 indicate that approximately 50% of patients with PKU have a positive response to BH_4 . Clinical trials of long-term treatment with BH_4 are now in progress.

The idea of utilizing the competition of LNAA with Phe on the carrier protein for the blood–brain barrier has been entertained for some time. The transport of LNAA to the brain is mediated by a carrier protein with the lowest K_m

Fig. 1 Mean blood Phe concentration with standard deviation of ENU²/ENU² mice (*n*=7) on normal chow (pre-LNAA) and on 16.7% NeoPhe. The ID of each mouse is given at the right. The reduction in blood Phe level is 53% from baseline with *p*-value <0.0001

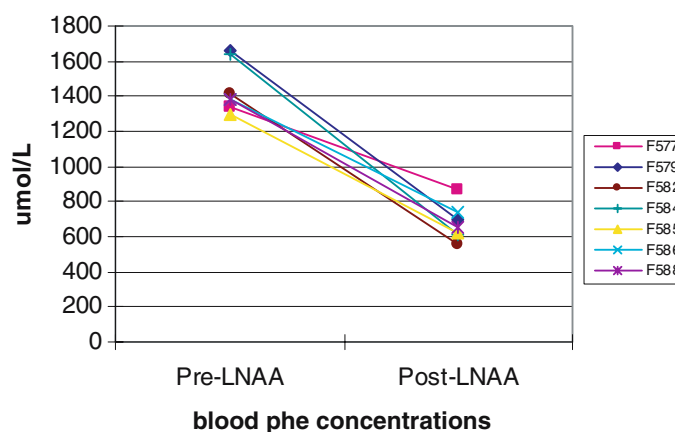


Fig. 2 Blood Phe response to 0.5 g/kg NeoPhe in 8 patients with PKU, showing 52% average decline in blood Phe concentration

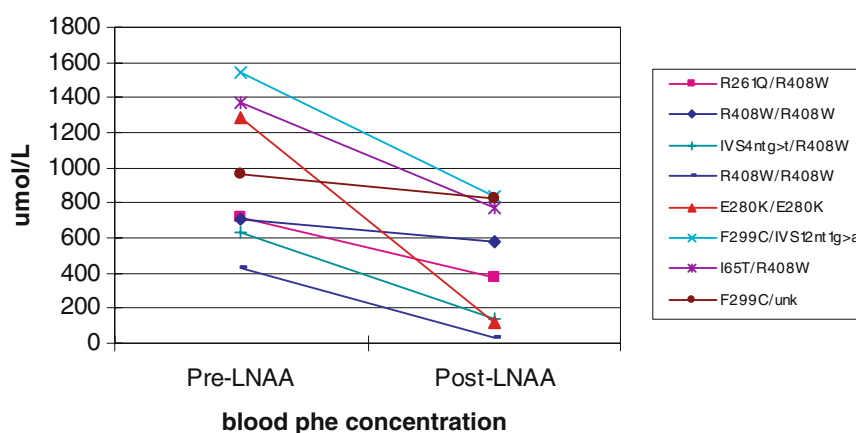
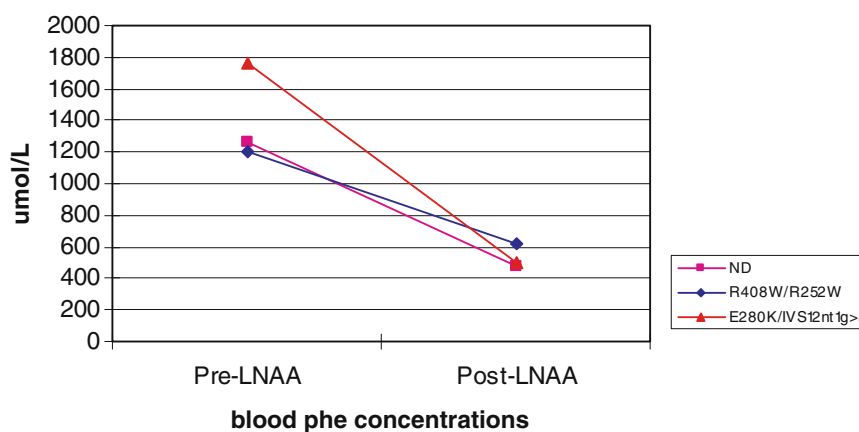


Fig. 3 Blood Phe response to 1.0 g/kg NeoPhe in 3 patients with PKU, showing 55% average decline in blood Phe concentrations



for Phe. The Michaelis–Menten constant (K_m) for each large neutral amino acid is dictated by the equation:

$$K_m(\text{app}) = K_m \left(1 + \sum [\text{aa}] / K_m^{\text{aa}} \right) \quad (1)$$

The K_m apparent (app) of a given amino acid deviates from the absolute K_m in the presence of a competing amino acid. The K_m equation predicts that if the plasma concentration of

an LNAA is much less than its value of K_m , then that amino acid will not compete effectively for the carrier protein. In PKU, blood Phe is much higher than other LNAAs so Phe can displace other amino acids and readily cross the blood–brain barrier (Pardridge 1982). Therefore, Phe is favoured to cross the blood–brain barrier rather than other LNAA (Choi and Pardridge 1986; Hargreaves and Pardridge 1988; Moller et al 1997; Oldendorf and Szabo 1976). The lower concentrations of tyrosine, tryptophan and branched-chain amino acids that have been reported in patients and mice

with PKU (Smith and Kang 2000) are the result of the competition of Phe with other LNAAs to cross the blood–brain barrier because of its higher concentrations in blood.

Clinical trials with tyrosine on patients with PKU started with the work of Lou and colleagues (1985), who gave 160 mg/kg of tyrosine to patients and reported increased attention span and neurotransmitter synthesis, as judged by neurotransmitter metabolites in the CSF. Subsequent studies by Pietz and colleagues (1995) giving 100 mg/kg tyrosine/kg to 24 early-treated PKU patients for 4 weeks showed no beneficial effects in neuropsychological tests. The addition of tryptophan to the diet of patients with PKU resulted in increase in 5-HIAA in CSF, while Phe concentration were unaltered. No effect on neuropsychological performance or vigilance was observed (Neilsen 1987). Berry and colleagues (1982, 1990) used branched-chain amino acids to inhibit the influx of Phe to the brain. Valine 150 mg/kg, isoleucine 150 mg/kg, and leucine 200 mg/kg (VIL) were used in these studies. The patients on VIL had a substantial lowering of Phe in the CSF, but tyrosine concentrations also were lower. Since VIL is not a complete mixture of LNAAs and does not contain tyrosine and tryptophan, which are precursors for neurotransmitters, it is not considered an adequate mixture of amino acids for the treatment of PKU (Hommes 1989).

The first study of LNAAs supplementation in the treatment of PKU was conducted using formulas of LNAAs without lysine, such as PreKUnil (Dotremont et al 1995). Four patients were treated for one month using a formula with 0.8 g/kg LNAAs and a low-protein diet, 0.6 g/kg. The treatment led to negative nitrogen balance due to lysine deficiency, indicating that such a formulation was not adequate for treatment of PKU.

A different study by Pietz and colleagues (1996) used Phe loading in six male patients with PKU, who were given Phe 100 mg/kg with and without LNAAs. When treated with LNAAs, the influx of Phe to the brain was decreased as measured by magnetic resonance spectroscopy (MRS). Moats and colleagues (1999, 2000), using MRS to measure brain Phe, studied patients on 0.6 g/kg of LNAAs and showed a decrease in brain Phe concentration following the treatment with LNAAs. The use of MRS to measure brain Phe concentration is technically difficult and it is not an accepted method for routine follow-up on treatment in PKU. A method that relies on blood determination of Phe concentration would be the preferred method, since this is an accepted practice.

In the GI tract, LNAAs are also transported by a carrier protein with a K_m that is two orders of magnitude higher than that of the CNS carrier protein. Lysine and arginine are also transported on the same carrier protein (Hidalgo and Borchardt 1990; Larsen et al 1964; Pardridge 1982). According to the experiments of Hidalgo and Borchardt (1990), using human intestinal-epithelial cells (Caco-2-cells in monolayers with a buffer containing 10 $\mu\text{mol/L}$ Phe), significant

inhibition of Phe transport requires 100-fold (1 mmol/L) LNAAs, as dictated by the K_m equation for affinity of LNAAs to the GI carrier protein. For example, at such concentrations, leucine inhibits Phe transport by 55%, tyrosine by 45% and the cationic amino acid lysine by 50%.

Under physiological conditions, competition of LNAAs with Phe is not likely to occur in the GI tract. However, by increasing the concentration of LNAAs and lysine while Phe is unchanged or reduced, competition with the GI transporter can be achieved. When tested on mice with PKU mouse chow with 16.7% LNAAs, a statistically significant decrease in blood Phe concentration was observed. This suggests that a competition with the transport of Phe can be attained with high concentrations of LNAAs in the GI tract, satisfying the concentrations required by the K_m for the GI transporter. Earlier, studies with PreKUnil resulted in lowering of blood and brain Phe in mice (Matalon et al 2003.)

The same mixture of LNAAs (NeoPhe) was used in patients with PKU, and also resulted in lower blood Phe concentration in the treated patients, suggesting that the mixture leads to increased concentrations of LNAAs in the GI tract competition with Phe transport.

The data presented indicate that the inhibition of transport of Phe can occur in the GI tract using LNAAs at the concentrations used in the study. It is also possible that better utilization of amino acids and protein synthesis is increased (anabolic effect). In mice we have not seen weight gain. In patients the trial was too short to document anabolic effects. Since this is a preliminary study, future experiments should focus on the mechanism of lowering of blood Phe concentration. The decline in blood Phe in PKU patients taking LNAAs is easy to measure and it is being reported for the first time. It is possible that if natural protein is somewhat limited (less Phe), more effective competition of LNAAs with Phe should be further enhanced, leading to lower blood Phe concentrations.

The 11 patients with PKU in this report were mostly classical PKU patients, as seen by the blood concentration of Phe and their genotype. Only two patients in this group responded to BH_4 , while the others had no decline in blood Phe concentrations following loading with BH_4 . None of these patients was on BH_4 during the study. These findings indicate that LNAAs can be effective in reducing blood Phe in all PKU patients. It is possible that patients who respond partially to BH_4 and still require Phe restriction will benefit from combination of BH_4 and LNAAs.

Strategies similar to the lowering of blood Phe concentration with LNAAs can be developed for the treatment of the tyrosinaemias. In this case Phe and tyrosine (and methionine in some cases) can be excluded from the LNAAs preparation. Maple syrup urine disease (MSUD) can be treated similarly by exclusion of leucine, isoleucine and valine, limiting their absorption from the GI tract. Limiting methionine

in homocysteinuria while increasing the other LNAAs may prove helpful in lowering blood homocysteine concentrations. Similarly, leucine absorption can be decreased by increasing the composition of other LNAAs in the GI tract. All such treatments need to be carefully planned and monitored in specialized metabolic centres, with the goal that diet in such diseases can be relaxed so that better compliance is achieved and metabolic crises are less frequent.

The data from this study suggest that double-blind placebo-controlled clinical trials should be conducted with LNAAs. Trials with similar formulas should be conducted in specialized centres, with the understanding that these pills are only a supplement and not a complete diet. Energy, micronutrients and other essential nutrients should be provided by the daily diet.

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