Molecular Genetics and Metabolism Reports 3 (2015) 80–87

Contents lists available at ScienceDirect

Molecular Genetics and Metabolism Reports

journal homepage: http://www.journals.elsevier.com/molecular-genetics-and-metabolism-reports/

Physiological competition of brain phenylalanine accretion: Initial pharmacokinetic analyses of aminoisobutyric and methylaminoisobutyric acids in *Pah*$^{enu2\ -/-}$ mice

Kara R. Vogel [a,1], Garrett R. Ainslie [a,1], Brian Phillips [b,c], Erland Arning [d], Teodoro Bottiglieri [d], Danny D. Shen [b,c], K. Michael Gibson [a,*]

[a] Section of Experimental and Systems Pharmacology, College of Pharmacy, Washington State University, Spokane, WA, United States
[b] Department of Pharmaceutics, University of Washington School of Pharmacy, Seattle, WA, United States
[c] Department of Pharmacy, University of Washington School of Pharmacy, Seattle, WA, United States
[d] Institute for Metabolic Disease, Baylor Research Institute, Dallas, TX, United States

ARTICLE INFO

Keywords:
Phenylketonuria
Large neutral amino acids
Phenylalanine
LAT-1 transporter
Aminoisobutyric acid
Methylaminoisobutyric acid
Pharmacokinetics

ABSTRACT

Objective: Initial studies on the use of non-physiological amino acids (NPAAs) to block the accretion of Phe in the brain of *Pah*$^{enu2\ -/-}$ mice revealed that 2-aminoisobutyrate (AIB) and *N*-methyl-2-aminoisobutyrate (MAIB) were promising lead compounds whose pharmacokinetic parameters warranted investigation.
Methods: Control and *Pah*$^{enu2\ -/-}$ mice received intraperitoneal NPAA treatments as test compounds (150, 300 and 500 mg/kg, 1 or 7 days) followed by collection of sera, liver and brain. LC–MS analysis was developed to quantify both AIB and MAIB in all matrices, and pharmacokinetic parameters for distribution, partitioning, accumulation and MAIB demethylation were determined.
Results: MAIB was partially converted to AIB in vivo. AIB and MAIB partitioned similarly from sera to the brain and liver, with an approximate 10-fold higher accumulation in the liver compared to the brain. In comparison to MAIB, AIB accumulated to approximately 3 to 7-fold higher concentration in the brain. Analysis of the brain and liver revealed a trend toward decreased Phe with increased MAIB serum concentration.
Conclusions: Our data support further pharmacokinetic characterization of MAIB and AIB in preparation for additional preclinical safety, toxicity and tolerability studies of both AIB and MAIB.

1. Introduction

Restriction of dietary protein intake in phenylketonuria (PKU) patients has been convincingly shown to prevent the most serious neurological impairments [1,2]. Nonetheless, accumulating evidence indicates that patients still exhibit deterioration in physical, cognitive and behavioral parameters, even for those whose dietary control is considered optimal [3]. A number of novel therapeutic modalities for PKU have appeared in the last several years, aimed at relaxing the degree of dietary protein restriction. These include sapropterin (Kuvan^{R}: Biomarin Corp.) to augment phenylalanine hydroxylase activity, glycomacropeptide intervention (a foodstuff devoid of phenylalanine (Phe)), and dietary supplementation with large neutral amino acids (LNAAs), the latter employed to compete with phenylalanine (Phe) for entry into the brain [4]. The last approach, however, faces the challenge of overcoming the high affinity of Phe for the large neutral amino acid transporter (LAT-1) at the blood–brain barrier interface [5]. As noted by Camp and colleagues in their consensus paper, "new drugs that are safe, efficacious, and impact a larger proportion of individuals with PKU are needed" [1].

Our laboratory seeks to develop a novel pharmacotherapeutic approach to PKU that employs non-physiological amino acids (NPAAs) to block the accretion of Phe in the brain [6,7] (Fig. 1). In pilot studies, we hypothesized that selected NPAAs such as DL-norleucine (NL), 2-aminonorbornane (NB; 2-aminobicyclo-(2,1,1)-heptane-2-carboxylic acid), 2-aminoisobutyrate (AIB), and N-methyl-aminoisobutyrate (MAIB) could effectively compete with Phe for BBB transport and reduce brain Phe in *Pah*$^{enu2\ -/-}$ mice, a relevant murine model of PKU [6]. All of these altered brain Phe in this model, yet all manifested effects on other LNAAs (tyrosine (Tyr), methionine (Met), and branched chain amino acids, including leucine (Leu), isoleucine (Ile) and valine (Val)), with MAIB displaying the mildest and most selective effects. We extended those studies to evaluate additional analogues of MAIB, including 2-methyl-2-(methylamino)butanoic and 3-methyl-2-

* Corresponding author at: Experimental and Systems Pharmacology (ESP), WSU College of Pharmacy, PBS Building Room 347, 412 E. Spokane Falls Blvd, Spokane, WA 99202-2131, United States.
E-mail address: mike.gibson@wsu.edu (K.M. Gibson).
[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.ymgmr.2015.03.009

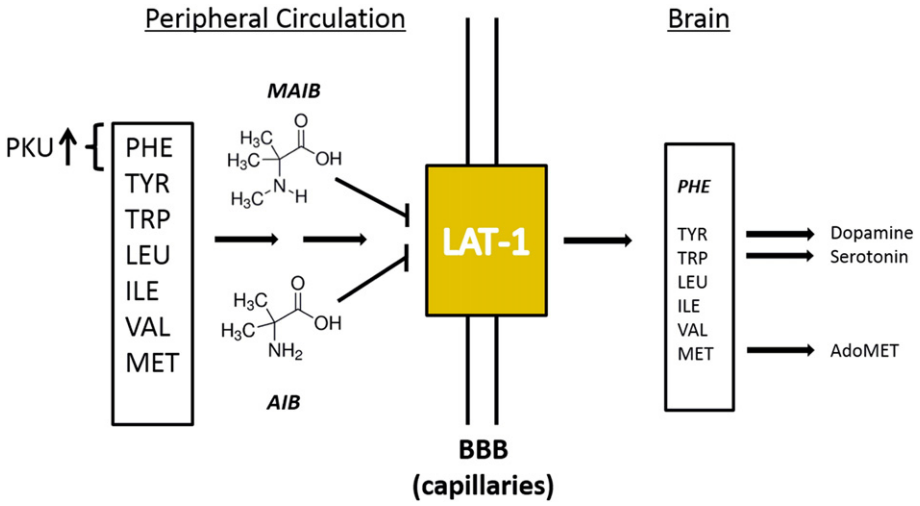

Fig. 1. Schematic rationale for the therapeutic utility of MAIB and AIB in phenylketonuria. MAIB (*N*-methyl-2-aminoisobutyrate) and AIB (2-aminoisobutyrate) are employed to selectively retard the accretion of Phe in brain. Abbreviations: Phe, phenylalanine; Tyr, tyrosine; Trp, tryptophan; Leu, leucine; Ile, isoleucine; Val, valine; BBB, blood–brain barrier; LAT-1, large neutral amino acid transporter; AdoMET, *S*-adenosylmethionine.

(methylamino)pentanoic, and the closely related derivative isovaline [7]. Those studies further highlighted that AIB and MAIB appeared to be our most promising leads capable of restricting Phe accretion in the brain of *Pah*$^{enu2\,-/-}$ mice.

Based on these pilot studies, we have now obtained pilot pharmacokinetic analyses of AIB and MAIB in the mouse. Here, we report on initial pharmacokinetic evaluation of both AIB and MAIB in *Pah*$^{enu2\,-/-}$ mice, data that are needed for the design of additional preclinical safety, toxicity and tolerability studies in animals.

2. Materials and methods

2.1. Reagents

Aminoisobutyric and methylaminoisobutyric acids were purchased from Sigma-Aldrich (St. Louis, MO, USA), and stock solutions were prepared in phosphate buffered saline.

2.2. Animal subjects

Heterozygous *Pah*$^{enu2\,+/-}$ mice were bred monogamously and maintained under a 14:10 light to dark cycle [7]. As previously demonstrated [6] there were no significant metabolic differences between *Pah*$^{enu2\,+/+}$ and *Pah*$^{enu2\,+/-}$ mice; accordingly, data from these two groups were pooled as control for *Pah*$^{enu2\,-/-}$ mice. Diet consisted of Harlan Global Teklad 2019 (19% protein) pelleted rodent chow with ad libitum access to food and water. Animals were genotyped by two primer PCR analysis [6]. Mice were 4–8 weeks of age at the time of study; they were injected once daily for either 1 day or 7 days consecutively following an intraperitoneal (i.p.) injection protocol [7].

Mice of both genders were employed, with n = 3–10 for each set of measurements. Occasionally, only 2 subjects were available for selected parameters. For both compounds, studies were conducted at three dose levels, including 150, 300 and 500 mg/kg once daily, along with PBS vehicle as control. Initial statistical analyses for these dosages with respect to key amino acid measurements (Phe, Tyr) were performed with one-way ANOVA. If no statistical differences were observed, in most cases drug dosing data for both AIB and MAIB were combined to increase n values. At sacrifice (24 h post final dose), blood was collected by cardiac puncture. The brain was rapidly excised, divided sagittally, snap frozen and stored at −80 °C. The liver was excised and processed similarly. Serum was harvested from blood following a 10 minute low-speed centrifugation at 4 °C and stored at −80 °C. All animal work was approved by the Washington State University IACUC (AFS 4232; 4276).

2.3. Quantitation of AIB and MAIB in tissues and serum

Twenty milligrams of tissue was homogenized with 0.75 ml of buffer (40:60 0.1% formic acid in water:acetonitrile) using a mechanical homogenizer. The homogenizer was rinsed 2× with 0.5 ml of buffer and 0.2 ml of methanol. The rinses were added to the homogenate and well-mixed. Fifty microliters of the pooled homogenate was transferred to a microcentrifuge tube, and 20 μl of internal standard solution (tranexamic

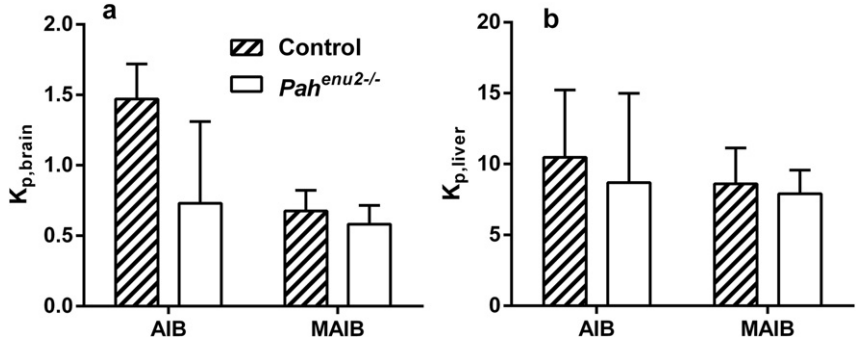

Fig. 2. NPAA partitioning into brain (a) and liver (b) in control and *Pah*$^{enu2\,-/-}$ mice. NPAAs were administered at either 150 or 300 mg/kg i.p. (one day study). Each bar graph incorporates pooled data for each respective genotype. Bar graphs represent mean ± SEM.

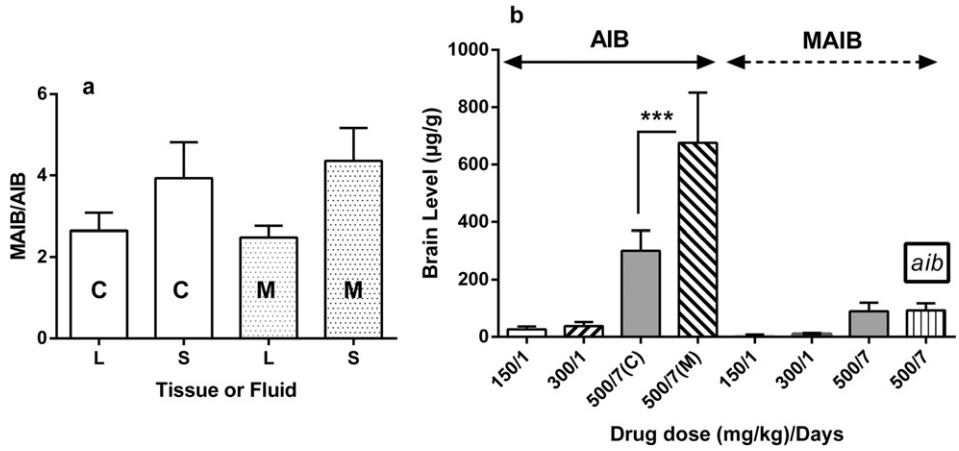

Fig. 3. (a) MAIB to AIB ratios measured in mice treated with MAIB for one day i.p. and (b) the measured brain drug concentration across various treatments. AIB or MAIB was administered at 150 mg/kg for one day (150/1), 300 mg/kg for one day (300/1) or 500 mg/kg for seven days (500/7). The specific agent administered is shown above the bar graphs, which is also the agent determined in brain, with the exception of the final bar graph labeled *aib*. For that graph, MAIB was administered (500 mg/kg for seven days) and AIB quantified in brain. There was no difference in accumulated level of drug in brain across genotypes, except for AIB administered at 500 mg/kg for seven days; the latter bar graphs are the only ones presented by genotype (C, control; M, mutant). Abbreviations: M, mutant ($Pah^{enu2\,-/-}$ mice); L, liver; S, sera.

acid, 5 ng/µl in methanol) and 0.6 ml of methanol were added to the microcentrifuge tube. The sample was vortexed for 30 s and ultra centrifuged at 20,000 ×*g* for 10 min at 4 °C. The supernatant was decanted to a glass culture tube and evaporated to dryness in air at 40 °C. The residue was dissolved in 0.1 ml of 3 M HCl in 1-butanol and heated to 100 °C in a dry-block for 45 min. After cooling briefly on ice, the solvent was evaporated. The residue was reconstituted in 0.2 ml of methanol and 1 µl was injected on the LC–MS system. Standard curves were prepared using blank tissue from non-dosed animals. Serum samples (10 µl) were prepared by protein precipitation and worked-up as for tissues.

For LC–MS analysis, the instrumentation consisted of an Agilent Technologies (Santa Clara, CA) 1100 series HPLC consisting of binary pump, chilled auto-sampler, and thermostatted column compartment. Separation was achieved on an ACE (Mac-Mod, Chadds Ford, PA) C8 2.1 mm × 150 mm × 3 µm column maintained at 30 °C. The mobile phase consisted of a binary mixture of 10 mM ammonium formate and methanol at a flow rate of 0.3 ml/min with the following gradient (time, % methanol): 2 min, 10%; 17 min, 85%; 19 min, 85%; 19.1 min, 10%. Total run time was 22 min. The HPLC was coupled to an Agilent G1946D quadrupole mass spectrometer which was operated in the

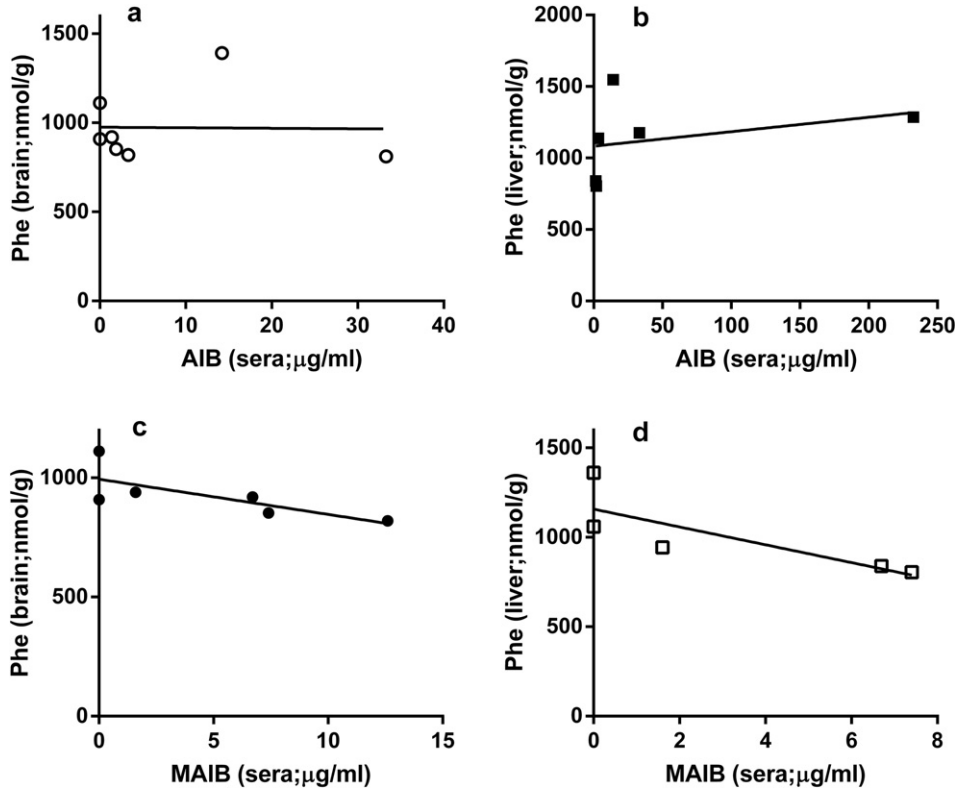

Fig. 4. Brain (a, c) and liver (b, d) phenylalanine concentration versus serum NPAA concentration. Solid lines denote a fitted linear regression. The data presented depict all data obtained from a one day i.p. administration study of either AIB or MAIB (intraperitoneal administration; 150 or 300 mg/kg) for $Pah^{enu2\,-/-}$ mice.

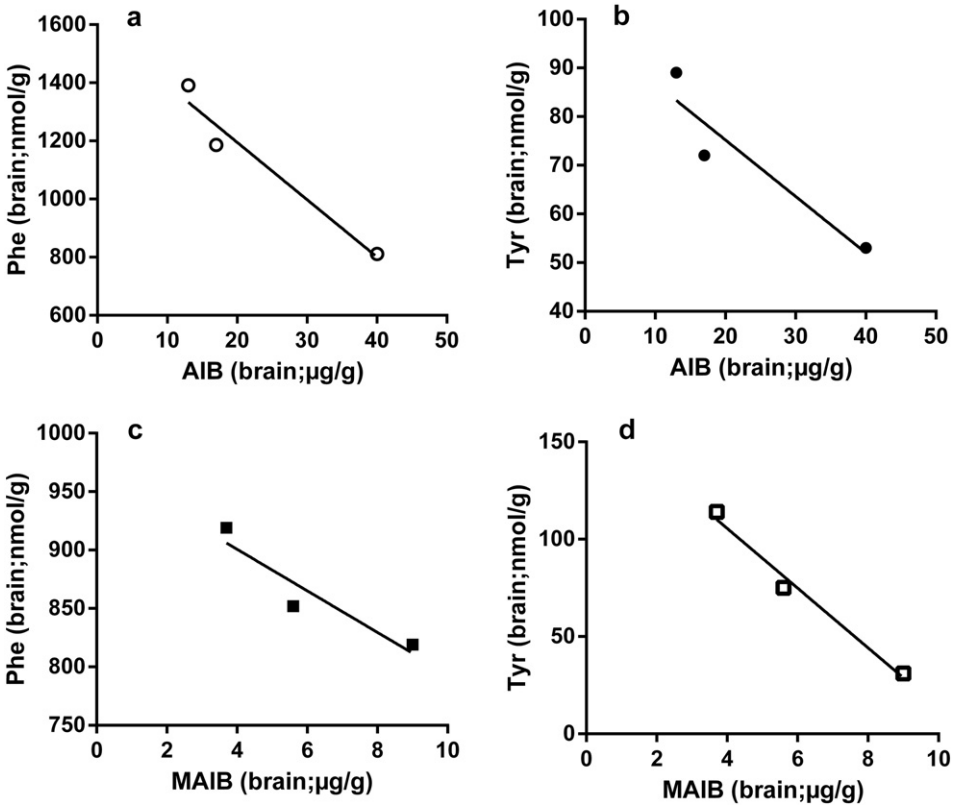

Fig. 5. Effect of brain AIB (a, b) and MAIB (c, d) concentration on brain phenylalanine and tyrosine in *Pah*$^{enu2\,-/-}$ mice. The concentration of drug in the brain correlated with the amino acid level, although the *n* value was insufficient to derive significance. All data shown is derived from a seven day study, with injection of AIB or MAIB (500 mg/kg).

ESI + mode with unit resolution. Nebulizer gas was nitrogen at 35 psi. Drying gas was nitrogen at 10 l/min and 350 °C. The capillary voltage and dwell time were set to 3000 V and 144 ms, respectively. Analytes were detected using the M + H ions of 174.1 (MAIB), 160.1 (AIB) and 214.2 m/z (tranexamic acid, internal standard) with corresponding fragmentor voltages of 80 (MAIB), 80 (AIB) and 110 V (tranexamic acid). Calibration curves over the dynamic range of 20–1970 μg/g (AIB) and 2–197 μg/g (MAIB) for tissue and 0.2–40 μg/ml (AIB) and 0.02–4 μg/ml (MAIB) for serum were constructed using a quadratic fit forced through the origin without weighting.

2.4. Quantitation of amino acids

Amino acids were quantified by UPLC and tandem mass spectrometry [6,7].

2.5. Kinetic and statistical analyses

Cohort–control designs were employed that evaluated single-dose tissue distribution, as well as multiple drug dosage applications (7 days, for both AIB and MAIB). Partitioning coefficients for the brain ($K_{p,brain}$) and liver ($K_{p,liver}$) were determined as the ratio of the tissue-to-serum concentration with Excel. Descriptive statistics and estimates of intra- and intersubject variability and correlations were obtained using GraphPad Prism 6. Between-group analyses were achieved using one-way ANOVA with post-hoc Bonferroni *t* tests, and/or Tukey multiple comparison analyses.

3. Results

The partitioning of MAIB and AIB from serum to the liver and brain was examined as a function of genotype (Fig. 2). AIB showed a higher partitioning into the brain of control mice compared to *Pah*$^{enu2\,-/-}$ mice, although there was no statistical significance. Overall, both AIB and MAIB partitioned similarly, with an approximate 10-fold higher accumulation in the liver compared to the brain. After one day of MAIB administration to both control and *Pah*$^{enu2\,-/-}$ mice, quantifiable amounts of AIB in the liver and serum (combined doses, 150 and 300 mg/kg) were detected (Fig. 3a). For the MAIB groups, the parent (MAIB) to metabolite (AIB) ratios were examined (sera and liver), revealing a consistent ~ 2:1 ratio between genotypes. Despite the lower ratio in the liver, this was not significant as determined by one-way ANOVA.

The brain concentration of each drug (AIB, MAIB) was then examined across dosages for both mutant and *Pah*$^{enu2\,-/-}$ mice (Fig. 3b). In this analysis, we found that AIB accumulated in the brain over 7 days depending upon genotype. The brain-to-serum partition ratios did not vary with dose in a 1 day study for either MAIB or AIB (150 versus 300 mg/kg; Fig. 3a). AIB was detected in the brain after 7 days of MAIB treatment (Fig. 3b). In comparison to MAIB, AIB accumulated to approximately 3 to 7-fold higher concentration in the brain.

We next investigated the correlation between brain Phe concentration and serum NPAA concentration (Fig. 4). The data of Fig. 4 (only *Pah*$^{enu2\,-/-}$ animals) were generated incorporating AIB data derived from AIB-, MAIB-, and vehicle-treated mice (baseline); the MAIB data were derived from MAIB- and vehicle-treated mice. The data indicated a slight (but non-significant) decrease of Phe with increasing MAIB serum concentration in both the brain and liver in a one day dosing study. This was not observed with AIB. These data suggest that the serum concentration of MAIB might be driving the Phe lowering effect in the brain, although we have not yet completed a parallel set of correlations with brain MAIB. No AIB was detected in the brain of MAIB-treated mice (150 and 300 mg/kg dosage, 1 day study; data not shown), consistent with the results of Fig. 3b.

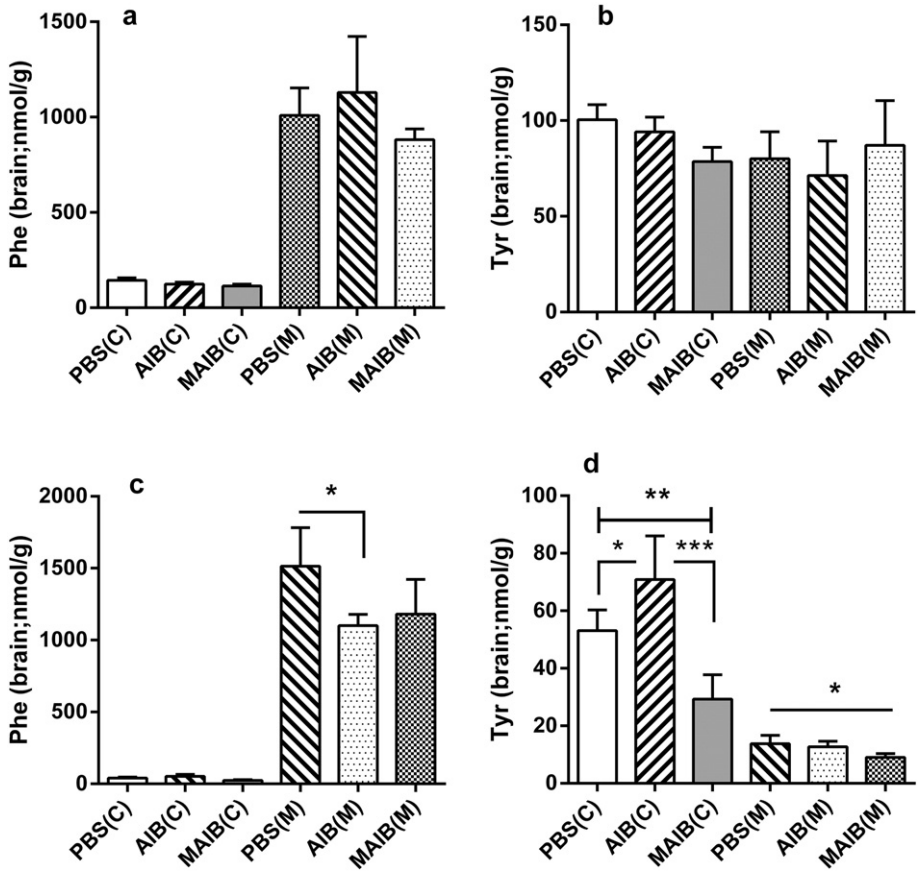

Fig. 6. Brain phenylalanine (Phe; a, c) and tyrosine (Tyr; b, d) concentration across treatment. Results shown are from a one day i.p. study (a, b) and a seven day i.p. study (c, d). Dosage levels were 150–300 mg/kg for a and b, and 500 mg/kg for c and d. In the one day study, treatments did not significantly impact Phe or Tyr levels in the brain. AIB decreased brain Phe (c), but not Tyr in a seven day dosing paradigm (d). The effect of MAIB on Phe level was a non-significant decrease (c; 1 way ANOVA, multiple comparisons) and on Tyr a significant decrease (d; $P < 0.05$).

We next examined the effects of AIB and MAIB on brain LNAAs in *Pah*$^{enu2\,-/-}$ mice. There was a reduction of both Phe and Tyr as a function of brain NPAA accumulation in *Pah*$^{enu2\,-/-}$ mice following seven days of treatment (Fig. 5). Phe and Tyr concentration correlated with brain NPAA concentrations, although these relationships did not achieve significance. The more consistent reduction in both Phe and Tyr seen with AIB (~40% for both) revealed a similar magnitude as opposed to the parallel reductions observed with MAIB (~10% for Phe; ~70% for Tyr).

We extended these studies to further examine the brain concentration of Phe and Tyr across treatment durations (Fig. 6a, b = one day study; Fig. 6c, d = seven day study) [7]. In the one day study, there was no effect on Phe or Tyr with either drug. Following a seven day dosing schedule, AIB decreased brain Phe but not Tyr (Fig. 6c, d). In comparison, the effect of MAIB on Phe was a non-significant decrease (Fig. 6c; 1 way ANOVA, multiple comparisons) and on Tyr a significant decrease (Fig. 6d; $p < 0.05$). Interestingly, AIB increased Tyr in control mice, whereas MAIB decreased Tyr in these subjects. Comparable analyses

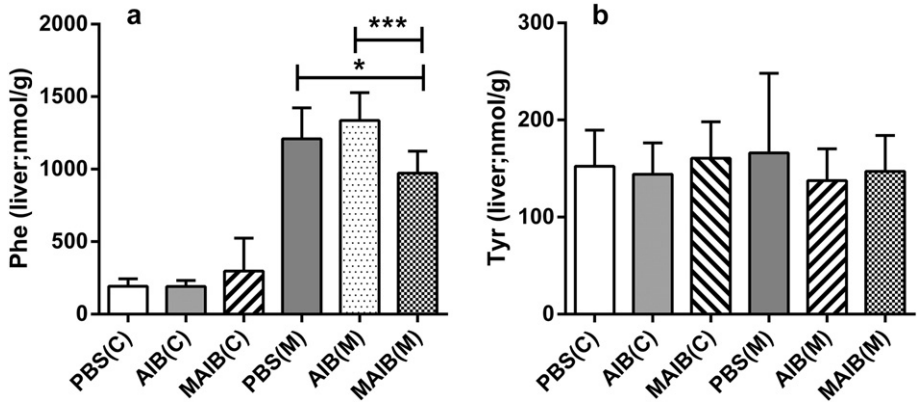

Fig. 7. Liver phenylalanine (Phe; a) and tyrosine (Tyr; b) level across treatment. Both graphs represent a 1 day study. Data were pooled for both AIB and MAIB, with each species administered at 150 mg/kg. MAIB significantly decreased Phe in the liver (a), whereas liver Tyr was unaffected by all treatments (b).

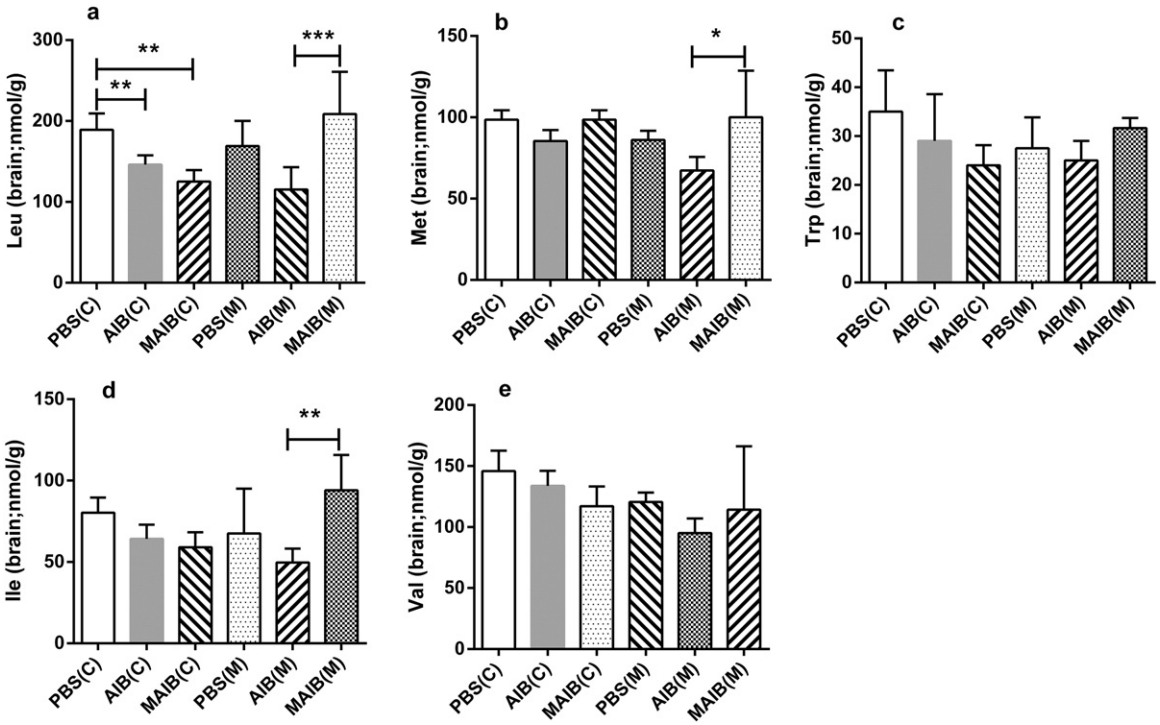

Fig. 8. Large neutral amino acids (LNAAs) in brain across treatments. All data was derived from a one day study (a = leucine (Leu); b = methionine (Met); c = tryptophan (Trp); d = isoleucine (Ile); e = valine (Val)). Data were pooled for both AIB and MAIB, with each species administered at 150 mg/kg. Leu was decreased in control animals with both AIB and MAIB (a). Leucine and isoleucine were both increased in MAIB-treated *Pah*$^{enu2\,-/-}$ mice (a, d). Changes in Met (b), Trp (c) and Val (e) were minimal if present.

were performed in the liver (one day study only; Fig. 7). For the latter, MAIB significantly decreased Phe (Fig. 7a), whereas liver Tyr levels were unaffected by either treatment (Fig. 7b).

The effect of both AIB and MAIB was less obvious on other LNAAs (one day dosing study; Fig. 8). Both AIB and MAIB decreased Leu in *Pah*^{enu2} control mice (Fig. 8a), whereas Leu and Ile were increased in MAIB-treated *Pah*$^{enu2\,-/-}$ mice (Fig. 8a, d). In the liver, neither AIB nor MAIB had any effect on the concentrations of these amino acids (also a one day dosing study; data not shown). Finally, a comprehensive evaluation of the brain ($K_{p,brain}$) and liver ($K_{p,liver}$) for all amino acids with affinity for the LAT-1 transporter was derived (Tables 1 (brain) and 2 (liver); seven day dosing only). These data revealed no significant differences for partitioning from sera into either organ with regard to either AIB and MAIB.

4. Discussion

The present study represents an extension of our prior studies as we are beginning to develop an in vivo picture of the actions of AIB and MAIB in control and *Pah*$^{enu2\,-/-}$ mice [6,7]. As expected, we found conversion of MAIB to AIB in vivo, most likely carried out by hepatic demethylation systems. Although we have not performed detailed pharmacokinetic evaluations of either AIB or MAIB (particularly in vitro metabolic kinetic parameters, in addition to in vivo area under the curve, clearance, etc.), the demethylation finding is of value, since both AIB and MAIB have effects on Phe accretion in the brain of *Pah*$^{enu2\,-/-}$ mice (Figs. 5, 6). Nonetheless, until we know that the $t_{1/2}$ of AIB is longer than that of its precursor, MAIB, we will not know which agent is optimally employed. Moreover, in the instance of MAIB

Table 1
Brain partitioning ($K_{p,brain}$) of LNAAs in control and *Pah*$^{enu2\,-/-}$ mice.

Amino acid	Control			*Pah*$^{enu2\,-/-}$		
	PBS	AIB	MAIB	PBS	AIB	MAIB
Phe	0.30 ± 0.06	0.36 ± 0.10	0.23 ± 0.03	0.28 ± 0.04	0.35 ± 0.05	0.38 ± 0.08
Leu	0.23 ± 0.03	0.23 ± 0.09	0.22 ± 0.04	0.11 ± 0.04	0.20 ± 0.03	0.24 ± 0.07
Met	0.71 ± 0.23	0.76 ± 0.21	0.70 ± 0.02	0.66 ± 0.12	0.77 ± 0.12	0.87 ± 0.26
Trp	0.19 ± 0.03	0.15 ± 0.04	0.17 ± 0.02	0.11 ± 0.01	0.15 ± 0.03	0.20 ± 0.06
Ile	0.24 ± 0.03	0.22 ± 0.09	0.25 ± 0.03	0.09 ± 0.03	0.18 ± 0.03	0.24 ± 0.08
Tyr	0.31 ± 0.08	0.24 ± 0.13	0.24 ± 0.05	0.12 ± 0.04	0.17 ± 0.06	0.19 ± 0.08
Val	0.28 ± 0.05	0.23 ± 0.09	0.27 ± 0.07	0.10 ± 0.01	0.18 ± 0.03	0.24 ± 0.07
Glu	7.8 ± 4.1	18.6 ± 5.8	23.0 ± 6.6	7.7 ± 5.6	17.2 ± 5.6	27.3 ± 14.3
Asp	5.5 ± 4.4	12.2 ± 5.0	15.4 ± 5.4	6.1 ± 4.5	9.2 ± 2.6	15.6 ± 6.1
Gln	3.9 ± 1.8	3.3 ± 0.8	6.4 ± 4.1	4.5 ± 1.1	3.3 ± 0.7	9.1 ± 2.3
Arg	1.6 ± 2.3	0.60 ± 0.65	1.8 ± 2.6	0.57 ± 0.3	0.74 ± 0.77	1.4 ± 1.0
Gly	1.1 ± 0.5	2.5 ± 0.4	1.1 ± 0.28	1.8 ± 1.3	1.7 ± 0.5	2.3 ± 0.86

Legend: Values are expressed as the mean ± SD for a seven day i.p. administration paradigm. Amino acids are listed in order of their affinity toward the LAT-1 transporter (highest affinity on top).

Table 2
Liver partitioning ($K_{p,\ liver}$) of LNAAs in control and *Pah*$^{enu2\ -/-}$ mice.

Amino acid	Control			*Pah*$^{enu2\ -/-}$		
	PBS	AIB	MAIB	PBS	AIB	MAIB
Phe	0.30 ± 0.06	0.35 ± 0.13	0.28 ± 0.04	0.28 ± 0.04	0.37 ± 0.04	0.39 ± 0.09
Leu	0.23 ± 0.03	0.24 ± 0.11	0.2 ± 0.03	0.11 ± 0.04	0.20 ± 0.04	0.24 ± 0.08
Met	0.71 ± 0.23	0.71 ± 0.26	0.65 ± 0.08	0.66 ± 0.12	0.83 ± 0.06	0.92 ± 0.28
Trp	0.19 ± 0.03	0.15 ± 0.05	0.17 ± 0.02	0.11 ± 0.01	0.16 ± 0.02	0.20 ± 0.07
Ile	0.24 ± 0.03	0.26 ± 0.10	0.24 ± 0.03	0.09 ± 0.03	0.17 ± 0.03	0.24 ± 0.09
Tyr	0.31 ± 0.08	0.31 ± 0.13	0.24 ± 0.04	0.12 ± 0.03	0.14 ± 0.02	0.16 ± 0.05
Val	0.28 ± 0.05	0.28 ± 0.10	0.25 ± 0.03	0.09 ± 0.03	0.17 ± 0.03	0.24 ± 0.09
Glu	8.0 ± 4.1	18.8 ± 7.1	18.5 ± 7.7	7.7 ± 5.6	18.4 ± 3.5	29.8 ± 15.1
Asp	5.5 ± 4.4	14.4 ± 5.6	13.7 ± 5.0	6.1 ± 4.5	9.0 ± 1.3	15.9 ± 7.0
Gln	3.9 ± 1.8	3.5 ± 0.8	5.8 ± 3.5	4.5 ± 1.1	3.1 ± 0.7	9.2 ± 2.6
Arg	0.27 ± 0.1	0.6 ± 0.7	1.7 ± 2.2	0.6 ± 0.3	0.6 ± 0.6	1.4 ± 1.2
Gly	1.0 ± 0.5	1.3 ± 0.4	1.1 ± 0.2	1.8 ± 1.3	1.9 ± 0.4	2.6 ± 0.6

Legend: Mean ± SD, with amino acids again listed in the order of their affinity toward the LAT-1 transporter (highest affinity on top). The liver K_p for Ile and Tyr trended toward normalization with MAIB intervention.

administration, we are in effect looking at a combination of agents administered in sequence. Along these lines, subsequent work should emphasize differentiating the individual contributions by MAIB versus AIB, optimally achieved by conducting a more thorough pharmacokinetic study for each compound (AIB, MAIB) and employing deconvolution modeling for effect differentiation. On the other hand, data supporting the selected use of MAIB is suggested by the finding that the one day i.p. study pointed to a Phe-lowering effect for MAIB (combining both 150 and 300 mg/kg i.p. dosing) in the brain and liver that was not seen with AIB (Fig. 4).

We found that AIB accumulated in the brain over seven days differentially by genotype (Fig. 3b). When comparing the accumulation of MAIB in brain across genotypes, there was no statistical difference between control and *Pah*$^{enu2\ -/-}$ mice, and therefore the data in Fig. 3b for MAIB administration (500 mg/kg, seven days) was pooled without differentiation between genotype. Additionally, AIB was detected in the brain after seven days of MAIB treatment but was not following one day of treatment (Fig. 3b). The preceding data will provide a platform for future studies to further examine the differences in drug response (MAIB vs. AIB effect) to further differentiate whether brain or serum concentrations of AIB or MAIB drive their capacity to lower brain Phe levels. One approach to achieving the latter would be to achieve equimolar concentrations of AIB and MAIB in serum, which will almost certainly require a constant infusion study.

Comparison of Fig. 5 and Table 1 suggests contradictory results. In the former, the data are derived from a seven day administration study of either AIB or MAIB (500 mg/kg). The data of Fig. 5 compares Phe and Tyr concentration in the brain with respect to total brain NPAA (AIB, MAIB) concentration. Increasing NPAA level indicated a reduction both in Phe and Tyr (Fig. 5a–d). Conversely, K_p estimates in Table 1 examine all of the available data (PBS, AIB and MAIB; 0, 150, 300 and 500 mg/kg administration, including one and seven day treatment regimens). In Table 1, the data for brain Phe K_p suggests higher penetration of Phe with MAIB administration, but it should be noted that the results were not significant (the same case for Fig. 5). However, those data were obtained for *Pah*$^{enu2\ -/-}$ mice, while in control animals the trend was in the opposite direction for MAIB. Moreover, the K_p term is a less direct investigation as compared to overall brain concentration. It must also be remembered that K_p is a mathematical calculation, which estimates brain penetration based upon the accumulated error of multiple measurements, whereas the data of Fig. 5 represent single measurements. Overall, we predict that in the face of high serum and brain concentrations of AIB or MAIB, the magnitude of effect would have to be rather high to see a significant change in K_p, but this aspect needs to be scientifically interrogated. Our current working hypothesis is that the hepatic LAT is convoluting our data sets in the intact organism (see below), and thus although the two sets of data (Fig. 5, Table 1) appear to be at odds, they are not directly comparable.

Our data further suggested that MAIB may be acting on the hepatic LAT system, the so-called LAT-3 (Fig. 7). A number of isoforms of the LAT exist in mammalian systems (human, rodent) that demonstrate tissue-selective expression, including LAT-1 in the blood–brain barrier, LAT-2 predominantly in the gastrointestinal tract, LAT-3 predominantly in the liver, and LAT-4 in the kidneys [8–11]. Prior to moving forward with additional preclinical animal studies, the effects of both AIB and MAIB on these transporters will require investigation. This would be especially important for LAT-2 and 3. Future studies for these transporter isoforms will likely employ cultured cell models where the conditions can be more effectively manipulated.

There was no significant change in $K_{p,brain}$ with dose for AIB or MAIB, suggesting that there may be other effects for AIB and MAIB beyond transport-mediated processes (Fig. 1; Table 1). Pertinent processes might include saturation in uptake transport or passive permeability, among many others. Overall, the maintenance of normal $K_{p,brain}$ and $K_{p,liver}$ (Tables 1, 2) for all LAT-1 amino acid substrates indicates that both AIB and MAIB reveal negligible or minimal impact on LNAA central nervous system availability, although quantitative data along these lines are yet to be obtained. The relatively mild effect of both compounds on the accretion of Leu, Ile and Val, and the absence of an effect on both Trp and Met (Fig. 8), are consistent with this hypothesis.

In summary, our data supports further investigation of MAIB and AIB as potential therapeutic strategies for PKU. Detailed studies on absorption, distribution and elimination, and especially routes of metabolic transformation, are underway. The challenge from a safety standpoint will be the overlapping effects on Phe and Tyr (Fig. 6), suggesting that future preclinical studies may need to consider the combined use of MAIB or AIB, supplemented with Tyr intake. Studies with Tyr, combined with our optimal NPAA, may provide an even more effective approach toward relaxing the strict dietary protein restriction in PKU patients.

Acknowledgment

This project was supported in part by NIH grant ULI TR000423 from the National Center for Advancing Translational Sciences (NCATS) and UW School of Pharmacy Drug Metabolism, Pharmacokinetics, and Transport Research Program (DMPTR); its contents are solely the responsibility of the authors and do not necessarily represent the official view of NCATS, NIH, the Institute of Translational Health Sciences or the University of Washington School of Pharmacy. Additional support was provided by a grant from the National PKU Alliance (www.npkua.org), and a Pre-Doctoral Fellowship (KRV) from the AFPE.

References

[1] K.M. Camp, M.A. Parisi, P.B. Acosta, G.T. Berry, D.A. Bilder, N. Blau, O.A. Bodamer, J.P. Brosco, C.S. Brown, A.B. Burlina, B.K. Burton, C.S. Chang, P.M. Coates, A.C.

Cunningham, S.F. Dobrowolski, J.H. Ferguson, T.D. Franklin, D.M. Frazier, D.K. Grange, C.L. Greene, S.C. Groft, C.O. Harding, R.R. Howell, K.L. Huntington, H.D. Hyatt-Knorr, I.P. Jevaji, H.L. Levy, U. Lichter-Konecki, M.L. Lindegren, M.A. Lloyd-Puryear, K. Matalon, A. MacDonald, M.L. McPheeters, J.J. Mitchell, S. Mofidi, K.D. Moseley, C.M. Mueller, A.F. Mulberg, L.S. Nerurkar, B.N. Ogata, A.R. Pariser, S. Prasad, G. Pridjian, S.A. Rasmussen, U.M. Reddy, F.J. Rohr, R.H. Singh, S.M. Sirrs, S.E. Stremer, D.A. Tagle, S.M. Thompson, T.K. Urv, J.R. Utz, F. van Spronsen, J. Vockley, S.E. Waisbren, L.S. Weglicki, D.A. White, C.B. Whitley, B.S. Wilfond, S. Yannicelli, J.M. Young, Phenylketonuria Scientific Review Conference: state of the science and future research needs, Mol. Genet. Metab. 112 (2014) 87–122.

[2] F.J. van Spronsen, S.C. Huijbregts, A.M. Bosch, V. Leuzzi, Cognitive, neurophysiological, neurological and psychosocial outcomes in early-treated PKU-patients: a start toward standardized outcome measurement across development, Mol. Genet. Metab. 104 (2011) S45–S51 (Suppl.).

[3] L. Aldámiz-Echevarría, M.A. Bueno, M.L. Couce, S. Lage, J. Dalmau, I. Vitoria, F. Andrade, M. Llarena, J. Blasco, C. Alcalde, D. Gil, M.C. García, D. González-Lamuño, M. Ruiz, M.A. Ruiz, D. González, F. Sánchez-Valverde, Tetrahydrobiopterin therapy vs phenylalanine-restricted diet: impact on growth in PKU, Mol. Genet. Metab. 109 (2013) 331–338.

[4] E.L. Macleod, D.M. Ney, Nutritional management of phenylketonuria, Ann. Nestle Eng. 68 (2010) 58–69.

[5] W.M. Pardridge, W.H. Oldendorf, Kinetic analysis of blood–brain barrier transport of amino acids, Biochim. Biophys. Acta 401 (1975) 128–136.

[6] K.R. Vogel, E. Arning, B.L. Wasek, T. Bottiglieri, K.M. Gibson, Non-physiological amino acid (NPAA) therapy targeting brain phenylalanine reduction: pilot studies in PAHENU2 mice, J. Inherit. Metab. Dis. 36 (2013) 513–523.

[7] K.R. Vogel, E. Arning, B.L. Wasek, T. Bottiglieri, K.M. Gibson, Characterization of 2-(methylamino)alkanoic acid capacity to restrict blood–brain phenylalanine transport in Pah^{enu2} mice: preliminary findings, Mol. Genet. Metab. 110 (2013) S71–S78 (Suppl.).

[8] Y. Kanai, H. Segawa, K. Miyamoto, H. Uchino, E. Takeda, H. Endou, Expression cloning and characterization of a transporter for large neutral amino acids activated by the heavy chain of 4F2 antigen (CD98), J. Biol. Chem. 273 (1998) 23629–23632.

[9] H. Segawa, Y. Fukasawa, K. Miyamoto, E. Takeda, H. Endou, Y. Kanai, Identification and functional characterization of a Na^+-independent neutral amino acid transporter with broad substrate selectivity, J. Biol. Chem. 274 (1999) 19745–19751.

[10] E. Babu, Y. Kanai, A. Chairoungdua, D.K. Kim, Y. Iribe, S. Tangtrongsup, P. Jutabha, Y. Li, N. Ahmed, S. Sakamoto, N. Anzai, S. Nagamori, H. Endou, Identification of a novel system L amino acid transporter structurally distinct from heterodimeric amino acid transporters, J. Biol. Chem. 278 (2003) 43838–43845.

[11] S. Bodoy, L. Martín, A. Zorzano, M. Palacín, R. Estévez, J. Bertran, Identification of LAT4, a novel amino acid transporter with system L activity, J. Biol. Chem. 280 (2005) 12002–12011.