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Characterization of 2-(methylamino)alkanoic acid capacity to restrict blood–brain phenylalanine transport in *Pah^{enu2}* mice: Preliminary findings[☆]

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Abstract

Background—Our laboratory seeks a pharmacotherapeutic intervention for PKU that utilizes non-physiological amino acids (NPAAs) to block the accumulation of phenylalanine (Phe) in the brain. In previous studies (Vogel et al. 2013), methylation of the amino group of 2-aminoisobutyrate (AIB) provided an enhanced degree of selectivity for Phe restriction into the brain of *Pah^{enu2}* mice in comparison to unmethylated AIB, leading to the hypothesis that 2-(methylamino)alkanoic acid analogs of AIB might represent targeted inhibitors of Phe accretion into the brain.

Methods—*Pah^{enu2}* and control mice were intraperitoneally administered (500–750 mg/kg body weight, once daily; standard 19% protein diet) AIB, methyl AIB (MAIB), isovaline, and two MAIB analogs, 2-methyl-2-(methylamino)butanoic (MeVal) and 3-methyl-2-(methylamino)pentanoic (MePent) acids for one week, followed by brain and blood isolation for amino acid analyses using UPLC.

Results—In the brain, AIB significantly reduced Phe accretion in *Pah^{enu2}* mice, while MeVal significantly improved glutamine and aspartic acids. Four of five test compounds improved brain threonine and arginine levels. AIB, MAIB and IsoVal significantly reduced blood Phe, with no effect of any drug intervention on other sera amino acids.

Conclusions—Further evaluation of AIB and the 2-(methylamino)alkanoic acids as inhibitors of brain Phe accumulation in *Pah^{enu2}* mice is warranted, with more detailed evaluations of route of administration, combinatorial intervention, and detailed toxicity studies.

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Conflict of interest statement

There is no conflict of interest.

Keywords

Phenylketonuria (PKU); *Pah^{enu2}* mice; 2-(Methylamino)alkanoic acids; Large neutral amino acids (LNAA); Large neutral amino acid transporter (LAT-1); Non-physiological amino acids (NPAA)

1. Introduction

Contemporary therapy for phenylketonuria (PKU) requires restriction of dietary phenylalanine (Phe) intake and supplementation with special medical foods, which prevents the developmental delays associated with long-term, untreated hyperphenylalaninemia [1]. Nonetheless, rigid dietary intervention presents challenges, including institution and maintenance of diet, stigmatization of patients, and emerging evidence of mild neurocognitive deficits even with good metabolic control [2–4]. Moreover, overrestriction of dietary amino acid intake may have untoward consequences. In the last decade, a number of novel therapeutic approaches to PKU have emerged, including cofactor intervention (Kuvan[®]; sapropterin, Biomarin), enzyme therapy (phenylalanine ammonia lyase; PEG-PAL), which degrades circulating Phe into non-toxic *trans*-cinnamic acid, and glycomacropeptide intervention (a cheese byproduct devoid of Phe), as well as gene therapy and hepatocyte transfer to the liver (www.clinicaltrials.gov). Despite these advances, a targeted pharmacological approach to PKU treatment remains undeveloped.

More than 60 years ago, the demonstration of competition between large neutral amino acids (LNAAs) for uptake across the blood brain barrier (BBB) led to the hypothesis that LNAA supplements could decrease Phe levels and replenish depleted LNAAs in PKU [5,6]. Those studies suggested that therapeutic competition for amino acid transport into the brain of individuals with PKU might be feasible [7,8]. At least four mammalian amino acid transporters have been identified that are responsible for movement of LNAAs (Phe, Tyr, Trp, Leu, Ile, Val, Met, as well as His and Thr) across the BBB and intestinal mucosa [9], and these LNAA transporters (so-called LNAA transporters, or LATs 1–4) constitute the system L transport system [10,11]. Despite the specificity of the LATs for LNAAs, considerable evidence indicates that most brain amino acid transport systems (including system L (the LATs), small neutral amino acids (transported on the A (alanine) and ASC (alanine-serine-cysteine) transport systems)), and even the acidic and basic amino acid transporters share considerable overlap in amino acid trafficking [12]. Moreover, as demonstrated by Pardridge and Oldendorf [13] nearly 50 years ago, the K_m values for the system L transporter vary considerably, with the highest affinity for Phe and Trp (~30–50 μ M), somewhat lower affinity for Met, Tyr and Leu (~80–90 μ M) and the lowest affinity for Ile, Val and His (~140–170 μ M). The preceding data suggests that selected therapeutic agents may have treatment relevance in competing against LNAAs (e.g., Phe in PKU) from uptake into the gut and brain of patients with PKU.

Zinnanti and colleagues [14] were the first to examine this type of competition study in a murine model of a large neutral aminoacidopathy. These investigators demonstrated that feeding of 5% D,L-norleucine (NL; a non-physiological amino acid (NPAA)) to mice genetically engineered to recapitulate MSUD (a large neutral amino aciduria, like PKU) resulted in significant behavioral and neurometabolic improvements with concomitant

lowering of brain Leu levels. Our laboratory extended these studies by piloting a study of selected NPAA in a murine model of PKU. The NPAA evaluated included D,L-norleucine; 2-aminoisobutyric acid (AIB); 2-methyl-2-(methylamino)propionic acid (also referred to as methyl-aminoisobutyrate (MAIB); and 2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid (BCH)), compounds possessing differential affinities for various brain amino acid transporters [15]. This study, with MAIB and BCH (the prototypical LAT-1 competitive inhibitor) was the first exploration of these compounds in a mammalian species. Using the genetically-engineered *Pah^{enu2}* mouse model as experimental platform [16], we found that all species employed (except AIB), at dietary intake levels of 3–5% w/w mouse chow, resulted in significant reduction of brain Phe, yet also resulted in reductions of other LNAAs as well. Only MAIB, fed to *Pah^{enu2}* mice at a dietary concentration of 3%, led to reasonably specific Phe reduction (accompanied by a modest reduction in brain Tyr). This finding was of interest, since MAIB is purported to be specific for the system A transporter [15], which has overlap with the L system transporter [12]. The absence of an effect with AIB in our initial feeding studies, with a more specific and significant effect from the methylated species (MAIB), prompted us to hypothesize that 2-(methylamino) alkanolic acids might represent potentially valuable competitive inhibitors of the L system (LAT-1). In the current report, we have examined this hypothesis in the *Pah^{enu2}* mouse model.

2. Materials and methods

2.1. Design and rationale

In previous studies, unsaturated D,L-norleucine species was effective at inhibiting accretion of Phe into the brain of *Pah^{enu2}* subjects [15]. This finding, coupled with results for AIB and MAIB feeding (see above) suggested that longer-chain species whose 2-amino group was methylated might be selective inhibitors of brain Phe transport. Accordingly, along with MAIB (2-methyl-2-(methylamino)propionic acid), we examined 3-methyl-2-(methylamino)butanoic acid (methyl valine, or MeVal) and 3-methyl-2-(methylamino)pentanoic acid (MePent), alkanolic acid species one and two methylene groups longer than MAIB, respectively (Fig. 1). As control for the methylated nitrogen, we employed 2-amino-2-methylbutanoic acid (isovaline, or IsoVal). The expense of these compounds precluded their use in mouse chow, as previously employed [15], and accordingly we opted for once-daily i.p. administration (see below). Moreover, i.p. administration ensured uptake and the potential to explore blood and brain uptake of dietary amino acids. Since our design included i.p. administration of drug, we repeated studies with AIB and MAIB as well, which had previously been examined using dietary intervention. All drugs (except AIB; Sigma-Aldrich, St. Louis, MO) were obtained from MolPort (SIA MolPort, Riga, Latvia) by custom synthesis, and purity exceeded 99% in all cases.

2.2. Animal husbandry and drug administration

Heterozygous *Pah^{enu2}* mice were bred monogamously and maintained under a 14:10 light to dark cycle. In an earlier study [15], we directly compared metabolite levels between homozygous normal (*Pah^{enu2+/+}*) and heterozygous (*Pah^{enu2+/-}*) mice, and observed no statistically significant differences. Accordingly, to increase our control data range in this pilot evaluation, metabolic findings from both homozygous unaffected and heterozygous

mice were pooled as controls. 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 amplification, restriction enzyme digestion (BbsI and BsmAI) and 4% agarose gel electrophoresis, as previously reported [15]. Mice were injected for one week from age 3 to 10 days, and animals of both genders were included. Entire litters were injected with 50 μ L total volume of PBS (vehicle control), AIB (500 mg/kg), MAIB (500 mg/kg), MeVal (500 or 750 mg/kg), IsoVal (500 mg/kg) or MePent (500 mg/kg). These dosages were chosen based upon previous feeding and dietary consumption characteristics for this strain [15]. Higher dosages and studies of dose response are in progress. Our calculations indicated that daily consumption of diet (powdered) containing approximately 2–3% w/w of drug equated to an approximate injection dosage of 500–750 mg/kg drug, and accordingly we chose this dose range for preliminary evaluation. Drugs were dissolved in PBS at 100 mg/mL and stored at -20°C . Drug administration commenced on the third day of life and continued once daily for 7 days based upon body weight. Because we had no information concerning potential drug toxicity, we limited our evaluations of subjects to a one week duration of study. On DOL 10 animals were sacrificed, and following cardiac puncture to obtain blood for sera collection, the brain was rapidly excised, divided sagittally, snap frozen and stored at -80°C . Serum was collected 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. Amino acid determinations

Amino acids were quantified by UPLC and tandem mass spectrometry as previously described [15].

2.4. Statistical analyses

Data is presented in column analysis format, with mean and SEM (error bars). Statistical evaluations were comprised of a two-tailed t test between genotypes (grouped wild-type and heterozygous subjects compared to mutant (Pah^{enu2}) subjects). Previous metabolic studies had verified that it was acceptable to group wild-type and heterozygous subjects, and this greatly improved animal numbers (n value) in the current study [15]. When there was a significant difference between control and Pah^{enu2} subjects, we applied ANOVA and Tukey post-hoc [15] in the Pah^{enu2} group to determine if drug intervention led to significant alteration in metabolite levels as compared to untreated (PBS vehicle) Pah^{enu2} subjects. To indicate when ANOVA was applied in the Pah^{enu2} group, the letter “A” appears above the vehicle-treated Pah^{enu2} subjects (in the brain, this value was $p = 0.052$ for aspartate, and the ANOVA evaluation was still applied in only this instance). In the absence of a significant difference between vehicle-treated control and Pah^{enu2} subjects, a two-tailed t test was performed between control and mutant only within the respective treatment group. The n values for blood measurements were smaller than those for brain evaluations since the volume of sera obtained was below the limit required (0.02–0.03 mL) for comprehensive amino acid studies in some animals.

3. Theory

Pilot studies [15] revealed that non-physiological amino acids (NPAAs) could both selectively and non-selectively lower Phe accretion in the brain of *Pah^{enu2}* mice, a murine model of PKU. In those studies, methylation of the amino group of 2-aminoisobutyrate (AIB) provided an enhanced degree of selectivity for Phe restriction into the brain using a dietary feeding intervention as compared to the unmethylated parent compound. This observation resulted in the hypothesis that 2-(methylamino)alkanoic acid analogs of AIB might represent highly specific inhibitors of Phe accretion in the brain of this mouse model, potentially providing additional evidence for a pharmacotherapeutic approach to PKU treatment.

4. Results

Findings for quantitative amino acid analysis in brain extracts are depicted in Figs. 2 and 3. The large neutral amino acids (LNAA) are shown in Fig. 2, and for the brain this includes both His and Thr. Met was omitted as there were no significant differences by genotype across the treatment groups. In Fig. 3, additional amino acid data is depicted, notably including the amino acid neurotransmitters (GABA, Glu, Asp, Gly and the Glu precursor Gln), as well as other amino acids where significant differences were observed, including Arg and Ala. The abnormalities observed with these brain amino acids were generally consistent with our previous studies on the brain extracts of untreated *Pah^{enu2}* mice [17]. The data of Figs. 4 and 5 depict results from quantitative sera amino acid analyses corresponding to the same subjects from whom brain data had been obtained. Fig. 4 depicts all large neutral amino acids, with the exception of His and Thr, which are shown with blood Gln levels in Fig. 5.

5. Discussion and conclusions

5.1. Brain amino acid characterization

Under normal protein intake (19% diet), our studies revealed that AIB significantly lowered brain Phe using an i.p. administration approach. This was at variance with earlier findings (dietary feeding) in which AIB administration was ineffective at lowering brain Phe [15]. For MAIB, i.p. administration revealed a tendency toward lower brain Phe which failed to achieve significance ($p = 0.114$), consistent with earlier data in which MAIB feeding significantly lowered brain Phe [15]. There was no effect on brain Phe levels with administration of IsoVal, MeVal nor MePent (Fig. 2). Intraperitoneal administration of MAIB, IsoVal, MeVal and MePent significantly increased Thr in *Pah^{enu2}* mice as compared to PBS-treated subjects, while MAIB intervention resulted in a further lowering of Tyr (which showed the most dramatic reductions of all the LNAAs in *Pah^{enu2}* mice), consistent with our previous studies using MAIB applied via dietary administration [15].

With regard to other LNAAs, His was significantly increased in *Pah^{enu2}* subjects in comparison to controls, while the branched chain amino acids (Leu, Ile and Val) and Trp were significantly decreased overall, and there was no effect of drug administration on any of these LNAAs (Fig. 2) [15,17]. These reductions did not always achieve significance as a

function of treatment, particularly when a selected drug administration led to lowering of the control level of amino acid, or when the SEM was large (as often found with administration of both MeVal and MePent). Met (data not shown) was not significantly different by genotype, but there was a significant decrease in its concentration in control subjects treated with AIB intervention, consistent again with previous studies employing dietary administration [15].

For the neurotransmitter amino acids (including Asp, Glu, GABA, Gly and the Glu precursor Gln), we observed a significant decrease in Gln as previously observed [17], and this decrease in *Pah^{enu2}* mice was exacerbated by AIB intervention and significantly improved with MeVal intervention (as was also the case for Asp; Fig. 3). For both GABA and Glu, there was a trend toward decreased levels in *Pah^{enu2}* subjects which failed to achieve statistical significance, and there was little effect from drug intervention [17].

We found unusual results for three additional amino acids in brain extracts. As depicted in Fig. 3, Arg was significantly decreased in mutant brain as we had previously observed [17], and its levels were significantly improved with AIB, MAIB, IsoVal and MePent intervention. Additionally, *Pah^{enu2}* mice were hyperglycinemic, as previously observed, and there was no effect with any drug intervention [17], while Ala levels were low which was at variance with the previous finding of its elevation in *Pah^{enu2}* mouse brain. Our results for Asp, Arg, His, Gly and Ala are of interest if we consider the various transporters in the brain and their overlapping specificities [12]. For example, the system L transporter (LAT) has considerable overlap with the A and ASC transporters, and even overlap with the Y transporter (basic amino acids). Conversely, Asp is trafficked only on the X transporter (acidic amino acids), which has no overlap with the L transporter. It is possible that increased phenylalanine levels are altering (competing with) these transporters, even the system X transporter, but it is challenging to explain why His and Gly are increased as opposed to decreased, suggesting an inverse effect (akin to agonism) by elevated Phe on their transport [18,19]. Conversely, there is the potential for unidentified transporters yet to be described for brain amino acid transport.

5.2. Blood amino acid characterization

The results of blood amino acid determinations are shown in Figs. 4 and 5. Overall, the *n* values were lower, which resulted in more data variation. Nonetheless, despite often low *n* values, we pursued blood amino acid analyses to glean insights on drug interventions on the peripheral circulation.

Amino acid analysis revealed that AIB, MAIB and IsoVal all resulted in significant reductions in blood Phe (Fig. 4). For the remainder of LNAAs, the trend was for decreased levels in blood, which was somewhat more pronounced with Tyr and Trp, a finding consistent with results for brain amino acid profiling. More consistent reductions in Gln and His were found in blood, the former consistent with the brain results while the latter was the opposite of the brain results. With the exception of Phe, drug intervention did not correct any blood amino acid. As was the case for brain extracts, there was considerable variation for animals receiving MeVal in addition to IsoVal (Figs. 4, 5), pointing to interindividual

variation and possibly toxicity. Any further studies with these species (IsoVal, MeVal and MePent) will certainly require dosing and toxicity analyses moving forward.

5.3. Large neutral aminoacidopathies, in silico modeling and future considerations

The large neutral aminoacidopathies, PKU and MSUD, share many similarities in metabolic neuropathology, yet some rather distinct features [20,21]. In both disorders, as modeled in knockout mice, disturbances in brain amino acid neurotransmitters follow similar patterns, with elevated Gly and depleted Gln, Glu and Asp. On the other hand, results for Arg, His and Thr are quite different with respect to disease, with depleted Arg in *Pah^{enu2}* mice and elevated brain Arg in mice modeling *msud* [20], elevated His in the brain of *Pah^{enu2}* mice (no demonstrable abnormality in *msud* mice), and depleted Thr in *Pah^{enu2}* mouse brain (elevated in *msud* mice). Another important observation is that the remaining LNAAs (e.g., Tyr, Trp, Met) are not significantly depleted in murine *msud* while quite decreased (at least for Tyr and Trp) in the *Pah^{enu2}* model. This might be explained by the much higher K_m of Phe for the LAT-1 as compared to the K_m values for the BCAAs. Some of these differences in metabolic neuropathology likely relate to accumulation of the BCAA ketoacids in *msud* mice (and patients), especially 2-oxoisocaproic acid, which is expected to gain brain access on the monocarboxylate transporter [22]. The preceding observations suggest that pharmacological intervention in PKU and MSUD, targeting restriction of the offending amino acid from entry into the brain, will require quite different approaches.

To begin to address the latter consideration, we have begun exploring the development of further compounds using an *in silico* approach for ligand docking on the L transporter (LAT-1). To this end, we have used the Schrodinger small molecule drug discovery suite (www.schrodinger.com). As a preliminary step, we have simulated LAT-1 folding. Since the LAT1 crystal structure remains unsolved, the folding must be simulated with the Prime component of Schrodinger. To accomplish this, we have modeled the input sequence from a high homology match of the LAT-1 (*Lat1*: light chain, NM_003486.5), as depicted in Fig. 6. As predicted from this initial modeling, the LAT-1 is composed entirely of α -helical strands which form a perfect pore for LNAA transport. This preliminary modeling data is being refined and will be used to identify potential ligand inhibitors that can be examined in our murine models.

In conclusion, our pilot studies employing the 2-(methylamino) alkanoic acids in the current study provide further supportive evidence for more detailed examinations of AIB and MAIB in *Pah^{enu2}* mice, with a particular focus on routes of administration, toxicity and regional brain characterization. Ongoing studies will work to determine optimal concentrations for these compounds, or those to be identified by in-silico modeling, that will eventually produce a high degree of specificity for restriction of Phe accumulation in the brain. For the longer chain species, namely MeVal and MePent, toxicity may pose a concern and safety considerations will be extensively characterized prior to further *in vivo* studies with these compounds.

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Abbreviations

PKU	phenylketonuria
<i>Pah^{enu2}</i> mice	murine model of PKU developed with ethylnitrosourea mutagenesis
LNAA	large neutral amino acids
A transporter	system A (alanine) transport system (small neutral amino acids)
ASC transporter	system ASC (alanine-serine-cysteine) transport system
X transporter	system X (acidic amino acid) transporter
Y transporter	system Y (basic amino acid) transporter
LAT1-4	LNAA transport system, system L transporter
NPAA	non-physiological amino acid
DOL	day of life
BBB	blood-brain barrier
Phe	phenylalanine
Tyr	tyrosine
Met	methionine
His	histidine
Thr	threonine
Leu	leucine
Ile	isoleucine
Val	valine
Arg	arginine
Ala	alanine
Trp	tryptophan
GABA	gamma-aminobutyric acid
Glu	glutamic acid
Gln	glutamine
Asp	aspartic acid

Gly	glycine
BCAA	branched chain amino acids (Leu, Ile, Val)
AIB	2-aminoisobutyrate (also 2-methyl-2-aminopropionic acid)
MAIB	methylaminoisobutyrate (also 2-methyl-2-(methylamino)propionic acid)
IsoVal	isovaline (also 2-methyl-2-aminobutanoic acid)
MeVal	methylvaline (also 3-methyl-2-(methylamino)butanoic acid)
MePent	3-methyl-2-(methylamino)pentanoic acid
i.p	intraperitoneal drug administration
PBS	phosphate buffered saline

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Fig. 1. Structures of the non-physiological amino acids (NPAAs) evaluated in the current study. A, 2-aminoisobutyric acid (AIB; also 2-methyl-2-aminopropionic acid); B, 2-methyl-2-(methylamino)propionic acid (MAIB); C, isovaline (IsoVal; also 2-methyl-2-aminobutanoic acid); D, 3-methyl-2-(methylamino)butanoic acid (also methylvaline (MeVal)); E, 3-methyl-2-(methylamino)pentanoic acid (MePent).

Fig. 2.

Large neutral amino acids (LNAAs) in brain extracts as a function of diet and genotype.

Amino acid identity is shown in three letter code on the y-axis (units, nmol/g tissue wet weight). Parenthetical values on the x-axis represent the number of animal subjects

evaluated. Abbreviations: C = control, including both wild-type and heterozygous *Pah^{enu2}*

mice; M = *Pah^{enu2}* mice. Statistical analysis as described in the text (*p < 0.05; **p < 0.01;

***p < 0.001).

Fig. 3. Selected brain amino acids as a function of diet and genotype. For abbreviations and other descriptions, see Fig. 1 legend.

Fig. 4.

Large neutral amino acids (LNAAs) in sera as a function of diet and genotype. For abbreviations and other descriptions, see Fig. 1 legend.

Fig. 5.
Selected sera amino acids as a function of diet and genotype. For abbreviations and other descriptions, see Fig. 1 legend.

Fig. 6.

Simulation of LAT1 folding with Prime modeling software (www.schrodinger.com). Comparative modeling of the input sequence (*Lat1*: light chain, NM_003486.5) via fold recognition was performed with the highest identity homolog (3G19C) imported from the default Find Homologs BLAST query of the pre-loaded Prime PDB library. This particular LAT1 structure was built using the knowledge-based (as opposed to energy-based) model-building method within Prime.