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Phenylalanine sensitive K562-D cells for the analysis of the biochemical impact of excess amino acid

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Although it is recognized that the abnormal accumulation of amino acid is a cause of the symptoms in metabolic disease such as phenylketonuria (PKU), the relationship between disease severity and serum amino acid levels is not well understood due to the lack of experimental model. Here, we present a novel *in vitro* cellular model using K562-D cells that proliferate slowly in the presence of excessive amount of phenylalanine within the clinically observed range, but not phenylpyruvate. The increased expression of the L-type amino acid transporter (LAT2) and its adapter protein 4F2 heavy chain appeared to be responsible for the higher sensitivity to phenylalanine in K562-D cells. Supplementation with valine over phenylalanine effectively restored cell proliferation, although other amino acids did not improve K562-D cell proliferation over phenylalanine. Biochemical analysis revealed mammalian target of rapamycin complex (mTORC) as a terminal target of phenylalanine in K562-D cell proliferation, and supplementation of valine restored mTORC1 activity. Our results show that K562-D cell can be a potent tool for the investigation of PKU at the molecular level and to explore new therapeutic approaches to the disease.

Metabolic disorders are often characterized by an imbalance of amino acids in plasma. Although it has been recognized that the accumulation of a particular amino acid or associated toxic metabolite(s), or else the deficiency of an essential amino acid, are causes of these diseases, the biochemical linkage between amino acid and pathophysiological changes often remain to be clarified.

Phenylketonuria (PKU) is an autosomal recessive disorder caused by a deficiency in hepatic phenylalanine hydroxylase (PAH; EC 1.14.16.1)^{1,2}. Since disease severity correlates with levels of serum phenylalanine, dietary restriction of phenylalanine in combination with the supplemental use of glycomacropeptide or neutral amino acids is the central component of PKU treatment. In a subset of PKU patients, supplementation with the PAH activator sapropterin dihydrochloride (BH₄) is sufficient to beneficially reduce plasma phenylalanine levels³.

Amino acids cross the plasma membrane through amino acid transporters and serve as building blocks for protein synthesis, energy-generating metabolites, substrates for enzymes such as nitric oxide synthase (NOS), or carriers for signaling molecule such as nitric oxide⁴. Recent studies have shown that amino acids regulate cell proliferation and protein synthesis through mammalian target of rapamycin complex (mTORC)^{5,6}. The majority of these studies have focused on amino acid starvation, and a little attention has been paid to the effect of excess accumulation of amino acids^{7,8}.

Hyperalimentation with balanced amino acids has been advocated in metabolic diseases, but this intervention cannot always correct the severe symptoms in congenital metabolic disorders. An elucidation of the mechanisms underlying the pathophysiological effects of amino acid imbalance would contribute to the better understanding of inherited metabolic diseases and to the development of novel therapeutic strategies. Due to the lack of the experimental model *in vitro* to analyze the biochemical impact of excess phenylalanine, the molecular mechanism(s) of phenylalanine toxicity remain poorly understood. Here, we have developed a cellular model (K562-D cells), which possesses higher sensitivity in cell proliferation to the content of phenylalanine in the culture medium within the clinically observed range in PKU patients. This system enabled us to investigate the molecular mechanism of phenylalanine toxicity.

Results

Differentiated K562-D cells are prone to the excess phenylalanine. It has been reported that oxidative stress status in the blood from PKU patient is closely linked to serum phenylalanine levels⁹, and nutritional anemias are



prevalent in patients with inborn errors of metabolism¹⁰. We have found that K562 cells¹¹ acquire phenylalanine sensitivity in cell proliferation once they differentiated and the phenotype could be used as an *in vitro* model to assess the effect of excess phenylalanine. In the case of severe PKU patients without dietary restriction of phenylalanine, the serum phenylalanine level may increase more than 2 mM^{1,12}. Thus cell proliferation rate of K562-D cells was used as a read-out to evaluate the cellular effects of phenylalanine up to 5 mM added to the culture medium.

Cell proliferation was monitored by measuring cell density every 24 h for 5 d following the addition of phenylalanine. K562-D cells, differentiated by hemin and Am80, showed significant sensitivity to phenylalanine at 3 mM compared to the parental K562 cells (Figure 1a, b). Although there was no significant difference between 0–3 mM of phenylalanine in parental K562 cells (Figure 1a), K562-D cells exhibit slow proliferation in concentration dependent fashion after 120 h of inoculation with 3 mM or greater phenylalanine (Figure 1b). However, at 10 mM phenylalanine, parental K562 cells showed slow proliferation that was comparable to K562-D cells treated with 5 mM phenylalanine. Since cell-counting method cannot distinguish the effects of cell death from slow proliferation, LDH activity measurement in cell culture media and counting of pyknotic

nuclei were chosen to evaluate cell viability. There was no significant increase on LDH activity or any evidence of changes in nuclear morphology at 5 mM of phenylalanine until 5 d after inoculation (Figure 1c). It suggests that counts of the cell number represent mainly cell proliferation, and has less contribution by cell viability and cell death. In order to increase assay throughput, resazurin method was employed to estimate cell numbers, which quantifies the amount of resazurin metabolite, resorufin (see materials and methods) as a surrogate of cell counts¹³. This resazurin method was highly comparable to standard hemocytometer-based counting and the results were highly reproducible. Since K562 and K562-D cells reach confluence by 120 h after inoculation in the control setup, proliferation rate was compared at 96 h after the addition of phenylalanine (Figure 1d). Although parental K562 cells did not show growth retardation by phenylalanine concentrations less than 3 mM, the K562-D cells were significantly sensitive to phenylalanine at concentrations greater than or equal to 1 mM (Figure 1d). At 3 or 5 mM phenylalanine, the degree of inhibition was 3.4, and 4.8 times higher in K562-D cells than K562 cells, respectively. Of note, increased osmotic pressure by the addition of phenylalanine did not cause similar effect since the control cells with sodium chloride (5 mM, equivalent to 10 mM phenylalanine) added to the culture

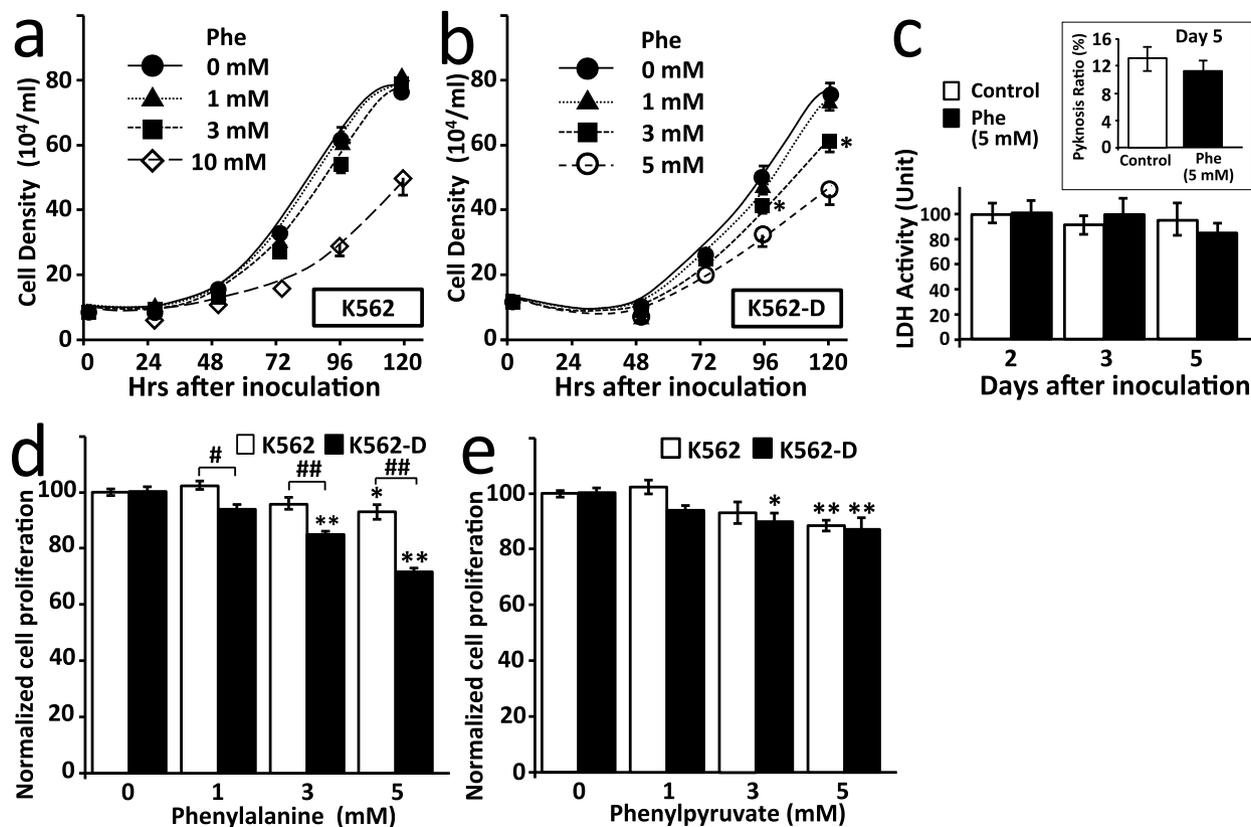


Figure 1 | Phenylalanine-sensitive slow proliferation phenotype in K562-D cells. (a–b) Cell growth was monitored every 24 h for 5 d. Cells were cultured with various amount of phenylalanine in the culture medium and cell density was calculated by using a counting grid under the microscope ($n = 4$ for each condition). The parental K562 cells (a) and the differentiated K562-D cells (b) showed marked differences in cell proliferation. K562-D cells were sensitive to more than or equal to 3 mM of phenylalanine; however, the parental K562 cells showed growth retardation at 10 mM of phenylalanine. Data are presented as mean \pm S.E. (Two-way ANOVA, * $p < 0.01$ vs. K562) (c) LDH activity (Wróblewski unit) was measured in culture medium of K562-D cells for 5 d. There was no significant difference between control and phenylalanine (5 mM) treated cells within 5 d, indicating less contribution of cell death in the slow growth induced by phenylalanine ($n = 4$). Inset: Nuclear morphology was observed on day 5 and pyknotic nuclei were counted. There was no increase in ratio of pyknotic nuclei by phenylalanine overload ($n = 4$). (d) Cell proliferation was assayed on the fourth day of incubation with phenylalanine by resazurin method as described in Materials and Methods. Value was normalized to the control cells. Cell proliferation showed significant difference between K562-D and K562 cells at >1 mM phenylalanine. Phenylalanine significantly inhibited the cell proliferation in K562-D cells at ≥ 3 mM ($n = 12–24$). (Two-way ANOVA, # $p < 0.05$, ## $p < 0.01$ K562 vs. K562-D) (e) Phenylpyruvate did not show marked differences on proliferation of K562-D and K562 cells. Further, growth inhibition was far less than with phenylalanine in (b) ($n = 6–16$). (Two-way ANOVA, * $p < 0.05$, ** $p < 0.01$ vs. 0 mM).



medium did not induce slow proliferation as observed in phenylalanine treated cells (data not shown).

Since phenylpyruvate is a disease-related characteristic metabolite of phenylalanine in PKU, K562 and K562-D cells were examined the effects of phenylpyruvate up to 3 mM. There was neither significant growth inhibition nor a difference between K562 and K562-D cells at these concentrations of phenylpyruvate (Figure 1e). Therefore, the extent of slow proliferation in K562-D cells made it possible to evaluate the effect of phenylalanine within the pathophysiological concentration range in PKU.

LAT2 and 4F2hc expressions are elevated in K562-D cells. Among the biochemical constituents of PKU pathogenesis, significant attention has paid to the L-type amino acid transporter, LAT. Since large neutral amino acids (LNAA) including phenylalanine share LAT as a common transporter through the plasma membrane, excess phenylalanine competes with other neutral amino acids at the transporter level, resulting in the decreased uptake of other amino acids in the cell¹⁴. Since differentiated K562-D cells have higher phenylalanine sensitivity than their parental K562 cells, the expression levels of the gene involved in the large neutral amino acid transport were examined by semi-quantitative real-time PCR method (qPCR). Although there was no significant change on LAT1 (SLC7A5) expressions before and after the differentiation induced by hemin and Am80, the expression level of LAT2 (SLC7A8) and 4F2hc (SLC3A2) genes were increased by 2.36 and 4.87 times higher than parental cells, respectively (Figure 2a). 4F2hc (4F2 heavy chain antigen, also known as CD98) is a type II transmembrane protein that is covalently associated with a number of transport proteins, such as LAT1 and LAT2, to localize these transporter proteins on the plasma membrane and allow amino-acid transport¹⁵. Phenylalanine is selectively transported through LAT1 and LAT2 among other LNAAs, the augmentation of phenylalanine sensitivity is induced in conjunction with the functional complex formation of LAT2 and 4F2hc through increases in gene expression. LAT is a bilateral transporter which imports LNAA in exchange with glutamine; thus, glutamine transporter, ASCT2 (SLC1A5), and intracellular glutamine synthase, GLUL, functionally couples with LAT.

Expression levels of ASCT2 and GLUL mRNA were further analyzed, but no significant changes were observed (data not shown).

LAT1 has been suggested as a potential amino acid transporter at the blood-brain barrier (BBB) that is responsible for high phenylalanine uptake into the brain of PKU patients^{16,17}. Although there was no induction of LAT1 gene post-differentiation, there is substantial amount of basal expression of LAT1 in K562 and K562-D cells. In order to determine the contribution of LAT1 on phenylalanine sensitivity, a human bladder transitional-cell carcinoma cell line, T24, was chosen to the assay since LAT1 has been shown to be the dominant transporter of neutral amino acids including phenylalanine in T24 cells¹⁸. As expected, the expression of LAT2 in T24 cells was negligible compared to LAT1 (Figure 2a). T24 cells showed a similar negligible response to excess phenylalanine as K562 cells (Figure 2b). Collectively, the data suggest that K562-D cells acquired higher phenylalanine sensitivity through the induction of LAT2 and 4F2hc.

Supplementation of amino acids does not compensate for the cell proliferation inhibited by phenylalanine.

Phenylalanine toxicity may be mediated by the competitive inhibition of the uptake of other essential amino acids by LAT¹⁴. Leucine was chosen as a representative LAT substrate to investigate whether supplementation of LNAAs might restore this imbalance in amino acids transport, since leucine has low Km value for LATs^{19–21}. Since the culture medium (RPMI1640) contains >90 μ M phenylalanine, the possible beneficial effects of added leucine in K562-D cells were assayed from 100 to 1000 μ M leucine in the presence of 3 mM added phenylalanine; however, there was no significant recovery of the cell proliferation (Figure 3a). Other amino acids were then investigated the rescue of growth inhibition in cells treated with 5 mM phenylalanine. Various amino acids (e.g. Asn, Ala, Gly, Lys, Pro, Arg, His, Ser, Met, etc.) were supplemented at 1 mM in the culture medium over phenylalanine (5 mM), and cell proliferation was analyzed after four days. No significant recovery of cell proliferation was observed in this group of assay except for methionine, which further decreased the growth rate by 1.5 times (Figure 3b). These results suggested that competition of other essential amino acid transporter by excess phenylalanine are not be critical for toxicity in this model.

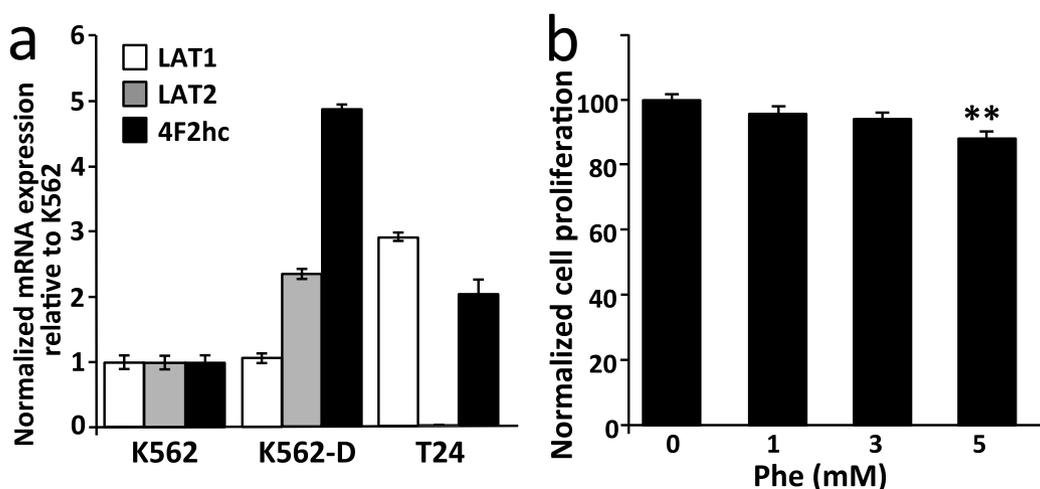


Figure 2 | Differentiation induced higher expressions of LAT2 and 4F2hc genes in K562-D cells. (a) Gene expression levels were analyzed by semi-quantitative qPCR with RPL4 as internal standard. ($n = 2-3$). The expression levels of LAT2 and 4F2hc genes were markedly higher in K562-D cells compared to K562 cells; whereas little difference was found on LAT1. There is little or no expression of LAT2 gene in T24 cells as reported previously¹⁸. (b) Contribution of LAT1 to phenylalanine-dependent slow proliferation was not significant in T24 cells. Following a 4-day incubation with different amount of phenylalanine, cell proliferation was assayed by resazurin method. There was a little difference at 5 mM phenylalanine on cell proliferation; however, the normalized cell proliferation was 71% in K562-D cells (Figure 1b) vs. 88% in T24 cells at 5 mM phenylalanine. ($n = 12-16$ for each group). (** $p < 0.01$ vs. Phe 0 mM) Data are presented as mean \pm S.E.

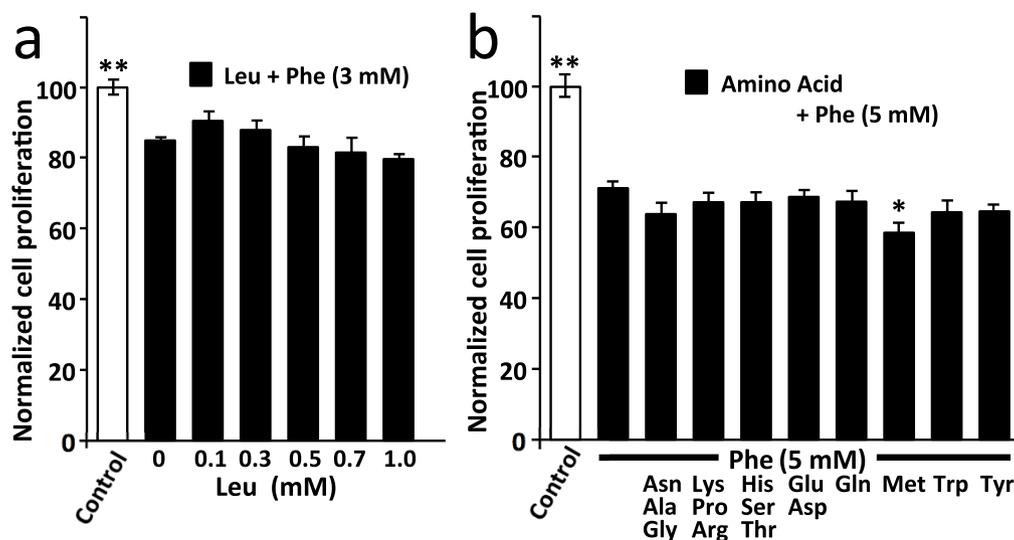


Figure 3 | Supplementation of amino acids over phenylalanine did not restore the slow proliferation induced by phenylalanine. K562-D cells were cultured with phenylalanine (a: 3 mM, b: 5 mM) and other amino acids for 4 d to analyze the effect of supplementation of amino acids over phenylalanine by the resazurin method. (a) Addition of leucine from 0 to 1 mM to the culture medium containing phenylalanine (3 mM) did not cause significant changes on cell growth. There was slight increase at 0.1 mM of leucine, which was not significant to the cells without leucine ($n = 4-16$ for each group, one-way ANOVA, $**p < 0.01$ vs. phenylalanine without leucine). (b): Addition of amino acids (1 mM for each, with exception for Gln (5 mM), Trp (0.5 mM), and Tyr (0.03 mM)) to the culture medium containing phenylalanine (5 mM) did not improve the K562-D cell growth inhibited by phenylalanine ($n = 4-16$ for each group, one-way ANOVA, $**p < 0.01$ vs. phenylalanine alone). Methionine showed further cell toxicity over phenylalanine. Data are presented as mean \pm S.E.

Valine supplementation over phenylalanine partly restored K562-D cell proliferation. Pyruvate kinase (PK) has been identified as a target of phenylalanine *in vivo*²². Phenylalanine binding maintains PK in an inactive T-state by the allosteric effects. Decreased activity of PK by phenylalanine may contribute to decreased energy metabolism in PKU patients²³. Thus the PK pathway has drawn attention in K562-D cells. If the inhibition of PK by phenylalanine is key to the reduction in cell growth, enhancement of the TCA cycle by supplying the metabolic intermediates, such as acetyl-CoA or succinyl-CoA, might rescue ATP synthesis, resulting in the recovery of cell proliferation. These intermediates are the metabolic products of branched chain amino acids (BCAA), such as leucine, isoleucine, and valine. Thus, each BCAA at 1 mM was added to the culture medium over 5 mM phenylalanine. As shown previously, leucine had no effect on the recovery of cell growth; however, valine partly restored the cell growth (Figure 4a). Interestingly 0.2 molar equivalents of valine to phenylalanine successfully restored the cell growth by around 50%. At a fixed concentration of valine at 1 mM, cell growth was evaluated over the different concentrations of phenylalanine (Figure 4b). At 3 mM of phenylalanine, 71% of recovery was achieved by 1 mM valine. Collectively, valine at 1 mM can restore the cell growth to normal levels at concentrations of up to 2.2 mM phenylalanine. Of note, addition of valine to the medium did not reduce the proliferation up to 10 mM (Data not shown). Although D-valine showed no effect against phenylalanine (5 mM), even low concentrations of L-valine (0.1–1.0 mM) effectively competed for phenylalanine-induced slow proliferation (Figure 4c). There was no significant change with an addition of phenylalanine in the expression level of LAT1/2 and 4F2hc mRNA analyzed by qPCR method (data not shown).

As a result of the metabolic process of BCAA in the peripheral tissue, valine and leucine supply succinyl-CoA and acetyl-CoA, respectively. Both metabolic intermediates are necessary for the TCA cycle. As shown in figure 4a, valine significantly restored cell growth inhibited by phenylalanine. This result may indicate the possibility that only succinyl-CoA, but not acetyl-CoA, appears to

counteract the effects of phenylalanine. To estimate the involvement of leucine/acetyl-CoA in this process, higher concentrations of leucine (3 or 10 mM) were applied to K562-D cells. Although leucine at 1 mM did not show an inhibitory effect on the K562-D cell growth over phenylalanine (Figure 4a), higher concentrations of leucine actually significantly inhibited the cell growth (Figure 4d). Interestingly, valine successfully restored the growth inhibition by leucine.

Tyrosine deficiency due to the inactivation of PAH could be a cause of PKU symptom. Since PAH expression is rather limited in the liver and kidney, K562-D cells does not have PAH expression in significant level. Since rescue effect of tyrosine in sepiapterin reductase deficient mice has been reported⁷, tyrosine (0.3 or 1.0 mM) was supplemented over phenylalanine to see whether the cell proliferation can be enhanced. However, at least within the concentrations tested, there was no effect by tyrosine on cell proliferation, suggesting that sensitivity to phenylalanine in K562-D cells is not due to tyrosine deficiency.

Excess phenylalanine inhibits mTOR activity in K562-D cells. It has been reported that amino acid deficiency has an impact on cell proliferation through the regulation of mTORC (mammalian target of rapamycin complex) activity^{24,25}. Since mTORC is one of the major regulators of cell proliferation, the activity of mTORC1 was analyzed by measuring phosphorylation at Thr389 of the p70 S6 kinase (p70S6K). Phosphorylation of p70S6K at this site was significantly lower when K562-D cells were cultured with phenylalanine (5 mM) for three days; however, the addition of valine (1 mM) to phenylalanine successfully restored the phosphorylation level of p70S6K back to control levels (Figure 4e).

Discussion

Here, we have presented a new *in vitro* culture system, K562-D cells, as a tool to investigate biochemical processes induced by phenylalanine overload, and to screen compound which can modify cell proliferation rate against phenylalanine. Our findings demonstrated that the elevated expressions of LAT2 and 4F2hc in K562-D cells allow

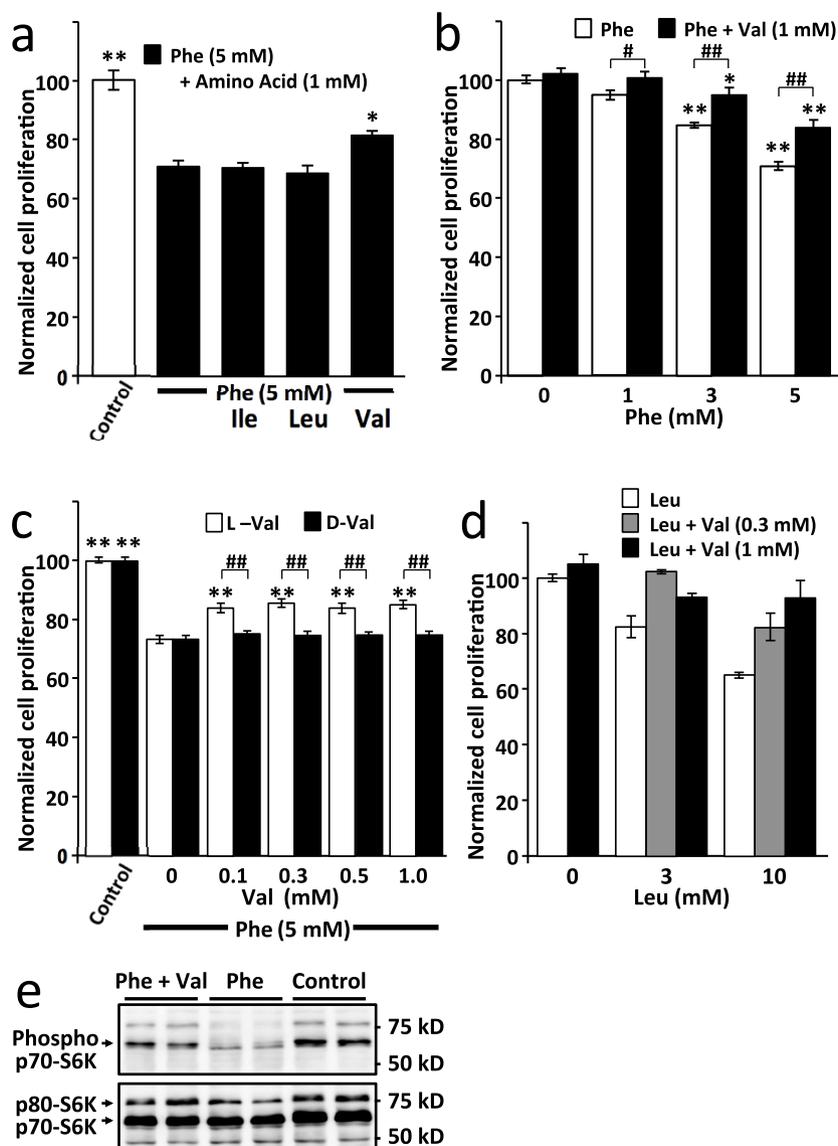


Figure 4 | Synergistic effects of branched chain amino acids (BCAA) over phenylalanine-induced slow growth in K562-D cells. (a) Addition of valine to the culture medium with phenylalanine partially restored K562-D cell proliferation. K562-D cells were cultured for 4 days with phenylalanine (5 mM) and a BCAA at 1 mM ($n = 4-16$ for each group). Only valine improved cell proliferation (one-way ANOVA: $*p < 0.05$, $**p < 0.01$ vs. phenylalanine (5 mM)). (b) Valine reduced the inhibitory effect of phenylalanine on K562-D cell proliferation. The effect of valine (1 mM) on the cell proliferation was compared against different concentration of phenylalanine (0, 1, 3 and 5 mM) after 4 days of culture. Although valine did not facilitate cell proliferation by itself, valine successfully reduced phenylalanine-dependent growth inhibition in all range examined. ($n = 12-20$ for each group, two-way ANOVA, $*p < 0.05$, $**p < 0.01$ vs. phenylalanine 0 mM; $\#p < 0.05$, $\#\#p < 0.01$ Phe vs. Phe + Val (1 mM)). (c) L-valine, but not D-valine, reduced the inhibitory effect of phenylalanine on cell proliferation. K562-D cells were cultured for 4 days with phenylalanine (5 mM) and L-valine or D-valine. L-valine markedly improved the cell proliferation from 0.1 mM and higher (empty bar); however, D-valine had no effect on the cell proliferation in all range examined (filled bar) ($n = 7-21$ for each group, two-way ANOVA, $**p < 0.01$ vs. Phe (0 mM); $\#\#p < 0.01$ L-Val vs. D-Val). (d) Leucine at high concentration inhibited cell growth and valine exhibited protective effect against leucine. K562-D cells were cultured for 4 days with leucine (empty bar), and valine at 0.3 mM (gray filled) or 1 mM (black filled). Leucine slowed the cell growth in concentration dependent manner. Valine canceled the effect of leucine both at 0.3 and 1 mM. ($n = 2-14$ for each group). Data are presented as mean \pm S.E. (e) A treatment with phenylalanine reduced mTOR activity in K562-D cells and valine restored the activity. K562-D cells were cultured for 3 days with phenylalanine (5 mM) alone or in combination with valine (1 mM). Thirty μ g of total cell lysate was separated on 10% SDS-PAGE followed by western blotting with a pThr389-specific p70S6 kinase antibody (upper panel) followed by reprobing with p70S6 kinase antibody (middle panel) and GAPDH as loading control (lower panel). A treatment with phenylalanine reduced phosphorylation of p70S6K at Thr3889, which suggests the inhibition of mTORC1 activity; however, addition of valine to phenylalanine markedly restored the phosphorylation level of p70S6K. The image is the representative of three independent experiments. The full-length blots used to crop images shown in panel e are presented in Supplementary Figure.

phenylalanine influx into the cell, leading to impaired cell proliferation through the inhibition of the mTORC1. The lack of an *in vitro* model system for the study of amino acid overload has been a significant limitation for the detailed molecular analyses of metabolic

diseases including PKU. Animal models and the material obtained from the patients have not so far allowed us to sufficiently test hypotheses or to screen compounds for the potential treatment. This *in vitro* model system with K562-D cells allowed us to uncover the



compensative effects of valine, which may have important clinical significance.

It has been implicated that elevated levels of phenylalanine will overwhelm LAT homeostasis and increase its uptake to the detriment of other LNAs¹⁷. In this study, however, supplementation of amino acids except valine did not reduce inhibitory effect by phenylalanine. This evidence indicated an alternative pathway which might be responsible for effects induced by excess phenylalanine. It has been reported that glucose metabolism²⁶ and pyruvate kinase activity²⁷ in the brain are significantly low in PKU patients due to the elevated phenylalanine in the cytoplasm. Since pyruvate uptake into the mitochondria is the rate-limiting step for ATP synthesis²⁸, excess phenylalanine could cause insufficient production of ATP through the TCA cycle, leading to the inhibition of mTORC1 resulting from the activation of AMPK²⁴. Since AMP kinase (AMPK) is a negative regulator of mTORC^{24,29}, insufficient synthesis of ATP decreases ATP/AMP ratio, which in turn activates AMPK. It may suggest the existence of regulatory process originated from the inhibition of pyruvate kinase and attenuated ATP synthesis and AMPK activation, resulting in the inhibition of mTORC activity, which directly suppresses proliferation of K562-D cells. Even when pyruvate kinase activity is suppressed by phenylalanine, glucose supplementation could increase the substrate availability for pyruvate kinase such as phosphoenolpyruvate (PEP) as a product of glycolysis; however, K562-D cells are highly sensitive to phenylalanine even in normal media, which contains relatively high concentration of glucose (about 11.1 mM in RPMI1640). This evidence suggests that further biochemical evaluation is necessary to clarify relation between glucose metabolism and pyruvate level in K562-D cells.

The therapeutic strategy for PKU is the reduction of blood phenylalanine level. Although dietary restriction is the basic strategy to reduce phenylalanine intake, it is challenging particularly for adults with PKU since the diet is bland and not affordable for all PKU patients. Thus, poor dietary adherence continues to be a major problem¹. Supplementation with sapropterin³ or large neutral amino acids (LNAA)³⁰ partially fulfill the dietary desire by increasing the allowable intake of phenylalanine, but the therapeutic cost of sapropterin is more than that for phenylalanine-free foods. There could be a possibility that enrichment of valine may bring a better outcome even under the situation where the phenylalanine level has not been controlled well. It would be interesting to monitor the blood phenylalanine level in combination with valine for better understanding of pathophysiology of PKU patients.

Based on our screening, valine was the only amino acid effectively restored mTORC1 activity and cell proliferation in phenylalanine-treated K562-D cells. Phenylalanine inhibits both glucose metabolism²⁶ and pyruvate kinase²², so it is necessary to circumvent these problems to maintain the ATP synthesis by supplying metabolic intermediates into the TCA cycle, such as acetyl-CoA from leucine or succinyl-CoA from valine. Since K562 cells are peripheral leukemia cells derived from the pleural fluid, they do not have cellular machinery to process various amino acids but BCAA. Leucine is required for mTORC activation⁵, and it is a source of acetyl-CoA, so it was expected to compete with phenylalanine on K562-D cell proliferation. However, even at high concentrations, leucine did not reverse phenylalanine toxicity, but rather inhibited cell proliferation (Figure 3a, 4d); these effects are likely due to the occupation of LAT resulting in the prevention of other amino acid influx. Although BCAA have been utilized for large neutral amino acid therapy^{31,32}, contribution of individual amino acids (leucine, isoleucine, and valine) on the effectiveness of the therapy has left to be elucidated. Thus, K562-D cells would be useful to clarify the effect of each amino acid over phenylalanine, and to lead an improvement in the current therapeutics.

Depression is one of the common symptoms in adult off-diet PKU patients^{33,34}; however, biochemical linkage of the blood phenylala-

nine level to the depression state has not been resolved. Recent studies in depression have demonstrated that the reduced cerebral protein synthesis, which is also reported in PKU patients with elevated plasma phenylalanine³⁵, is due to low mTORC activity in neuronal cells³⁶. In analogous to K562-D cells, phenylalaninemia might be linked to decreased mTORC1 activity in PKU patients, which subsequently becomes a cause of depression.

Although further biochemical evaluation of K562-D cells is necessary to further substantiate these cells as a tool for investigating the molecular basis for PKU pathology, they have enormous potential for *in vitro* modeling of PKU pathobiology and therapeutic interventions.

Methods

Materials and Reagents. All materials were from Sigma-Aldrich unless otherwise indicated. Hemin (51280) and Am80 (Tamibarotene, T3205) were dissolved in DMSO and stored at -20°C until use. Amino acid stock solutions were made in RPMI1640 (R8758) medium and filter sterilized before freezing them at -20°C in small aliquots to avoid repeated freeze-thaw cycles. Amino acids used in this report are L-type unless otherwise noted.

Cell culture. The human cell lines, K562 derived from chronic myelogenous leukemia (RCB1897) and T24, derived from bladder transitional-cell carcinoma (RCB0431), were provided by the RIKEN BRC through the National Bio-Resource Project of the MEXT, Japan. All of the cells were cultured at 37°C in a 5% CO_2 , humidified atmosphere. Cells were grown in RPMI1640 with 10% heat-inactivated fetal bovine serum (SH30396, HyClone), 100 units/ml penicillin, and 100 $\mu\text{g}/\text{ml}$ streptomycin (Nacalai Tesque, Japan).

Differentiation of K562 cells to K562-D cells. K562 cells were cultured with hemin (10 μM) and Am80 (1 μM) for 4 days to induce differentiation³⁷. The resulting cells were termed as K562-D cells, and used for the further experiments. K562-D cells were prepared fresh for each set of experiments. Erythroid differentiation was assayed by benzidine staining of hemoglobin accumulated in the cells³⁸. Following the staining, at least 500 cells were examined by light microscopy to count the number of benzidine-positive cells. Under typical conditions, at least 96% of total cells were benzidine-positive at the time of amino acid addition. The parental K562 cells under the regular culture condition did not show stained cells.

Amino acid treatments and growth assay. Cells were seeded at the density of 1.7×10^5 cells/well in a 24-well plate. For K562-D cells, hemin (10 μM) and Am80 (1 μM) were kept in the culture medium. Amino acid stock solutions (100 mM in serum-free RPMI1640, with the exception of tyrosine at 3 mM, and tryptophan at 50 mM) were added to each well to increase amino acid levels in the culture medium. The resulting amino acid concentration was annotated in this study as the amount of added to the standard culture medium (RPMI1640) from the stock solution, since the amino acid concentration in RPMI1640 medium is relatively low (e.g. Phenylalanine 91 μM , Leucine 381 μM , Valine 171 μM). Serum-free RPMI1640 was added to each well to adjust the total volume of culture medium. Cells were then cultured for 4 days in a CO_2 incubator at 37°C . Cell counts in each well were estimated by the amount of resorufin formed from resazurin (7-Hydroxy-3H-phenoxazin-3-one 10-oxide, R7017)¹³. On the last day of assay, resazurin was added to each well to a final concentration of 10 $\mu\text{g}/\text{ml}$, and incubated for 1.5 to 2 h. The resorufin was detected in the culture medium by fluorescence measurement (Ex. 570 nm, Em. 582 nm, Shimadzu RF5300PC) following separation of cells by a centrifugation. Relative proliferation rate was expressed as fluorescence counts in the presence of test compound (amino acid) as a percentage of that in the vehicle control.

Measurement of gene expressions. Gene expression levels were analyzed by semi-quantitative real-time PCR. Cells were pelleted by a centrifugation at 800 g for 3 min at 4°C , and total RNA was extracted with RNAiso (9108 Takara, Japan) according to the manufacturer's instructions. Two μg of total RNA was then used to synthesize first strand cDNA with a SuperScript VILO cDNA Synthesis Kit (11754-050 Invitrogen, USA). mRNA levels were quantified by real-time PCR with SYBR green dye (QPS-201 Thunderbird Sybr qPCR Mix, Toyobo, Japan) with the specific primer sets shown in Table 1, and normalized to ribosomal protein L4 (RPL4) mRNA³⁹.

Assay for cell death. Pyknotic nucleus and dense stained nucleus was used as a marker of cell death⁴⁰. Following a treatment with amino acid for the indicated period, cells were collected in a tube, and cell viability was determined by staining with Hoechst 33258 dye (B2261) for 3 min at room temp, followed by fixation with 4% paraformaldehyde in phosphate buffer (163-20145 Wako, Japan). Nuclei were visualized using a fluorescence microscope (TE300, Nikon, Japan) and digitized. Cells with dense stained nuclei or condensed or discrete fragmented nuclei were counted as dead cells. At least 2500 cells/sample were counted to calculate the ratio of dead cells to total cells.

Lactate dehydrogenase (LDH) activity measurement was also employed as a biochemical indicator of cell death. Culture medium (1 ml) was obtained on day 2, 3, and


Table 1 | Primer sets used for real-time PCR analyses

RPL4 . Human . For	AAGATCCATCGCAGAGTCCT
RPL4 . Human . Rev	TTATCCACCCGGAGCTTGTG
LAT1 . Human . For	GTCCCTGTGTCAGAAGCTCAT
LAT1 . Human . Rev	GTGACTACTTTAACTGGCCTCT
LAT2 . Human . For	GGACACAGAGGAGGCTAATGA
LAT2 . Human . Rev	GCCACGTCCTTGTCCCTTCGT
4F2hc . Human . For	TCTTGATTGCGGGGACTAAC
4F2hc . Human . Rev	GCCTTGCCGAGACAAACTC
ASCT2 . HumanRT . For	CCGTCCTTCAACTCCCTTCAA
ASCT2 . HumanRT . Rev	ACCCACATCCTCCATCTCCA
GLUL . HumanRT . For	GCTGGTGTAGCCAATCGTAGC
GLUL . HumanRT . Rev	GGCTTCTGTCACCGAAAAGG

5 from the dish prepared for the pyknosis assay. Following a centrifugation at 800 g for 3 min at 4°C to separate cells, the LDH assay was conducted according to the manufacturer's instructions (LDH CII, Wako, Japan).

Assays for mTORC1 activity. mTOR complex (mTORC) 1 is essential for the phosphorylation and activation of the 70 kDa ribosomal protein S6 kinase (S6K) 1 and 2⁴¹. Phosphorylation levels were assessed as mTORC1 activity by immunoblotting with a S6K pThr389-specific antibody (#9234 Cell signaling) normalized to total S6K (#2708 Cell Signaling). GAPDH (sc-25778 Santa Cruz) was used as an internal loading control. K562-D cells were cultured in a 10-cm dish for three days with amino acid as indicated. Cells were collected by a centrifugation and stored at -80°C until use. Cells were thawed in hypotonic lysis buffer (20 mM Hepes pH 7.6, 10 mM NaCl, 1.5 mM MgCl₂, 0.1% Triton X-100) supplemented with 1 mM EDTA (pH 8.0) and protease inhibitors (1836170, Roche, USA) and phosphatase inhibitors (07575-51, Nacalai Tesque, Japan). Following incubation on ice for 10 min, cell lysate was cleared by a centrifugation at 20,000 g for 10 min at 4°C. Protein quantity was measured using BCA method with BSA as standard. Total cell lysate (20 µg) reduced in sample buffer was resolved by 10% SDS-PAGE for p70S6K. Proteins were transferred to nitrocellulose membrane, and probed with a specific antibody (1:1000 dilution) overnight at 4°C. Proteins were visualized using anti-rabbit secondary antibody conjugated to HRP and a chemiluminescence detection system (Immobilon Western Chemiluminescent HRP Substrate, Millipore).

Statistical analysis and data managing. Differences between groups were assessed by one-way or two-way analysis of variance (ANOVA). P values of < 0.05 were considered significant unless otherwise stated. Statistical analyses were performed with SPSS 21.0.

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Author contributions

Y.S. and A.M. conception and design of research; Y.S. and A.M. performed experiments; Y.S. and A.M. analyzed data; S.Y., A.M. and H.N. interpreted results of experiments; S.Y. and A.M. prepared figures; S.Y., A.M. and H.N. wrote manuscript; S.Y., A.M., N.S., Y.K. and H.N. approved final version of manuscript.



Additional information

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