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Responsiveness of sphingosine phosphate lyase insufficiency syndrome to vitamin B6 cofactor supplementation

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AUTHOR CONTRIBUTIONS

Piming Zhao generated and analyzed data, helped write the manuscript, and reviewed the manuscript. Peter I. Benke, Jeremy Selva, Federico Torta, Markus R. Wenk, Olivia West, Weixing Ou, Emily Tang generated and analyzed data and reviewed the manuscript. JAE performed molecular modeling, helped to write the manuscript and reviewed the manuscript. Denise L.M. Goh, Stacey K. H. Tay conceived of the idea and reviewed the manuscript. Alwin Loh and Jeffrey B. Hodgin performed pathological analysis of kidney biopsies and helped to write the manuscript. Megan A. Cooper reviewed the immunological data, helped to write and review the manuscript. Isaac D. Liu, Hui-Kim Yap, Evren Gumus, Karin Chen, Martin Zenker, Friedhelm Hildebrandt, Suha Salim, Abdullah A. Alangari, Khalid Alhasan, Austin Larson, Nicole Weaver, Bonnie Sullivan contributed patient data, performed data analysis, and reviewed the manuscript. JDS (corresponding author) conceived the idea, designed the study, analyzed data, wrote the manuscript.

CONFLICT OF INTEREST

Piming Zhao, Isaac Liu, Jeffrey Hodgin, Peter Benke, Jeremy Selva, Federico Torta, Markus Wenk, James Endrizzi, Olivia West, Weixing Ou, Emily Tang, Denise Goh, Stacey Tay, Hui-Kim Yap, Alwin Loh, Nicole Weaver, Bonnie Sullivan, Austin Larson, Megan Cooper, Khalid Alhasan, Abdullah Alangari, Suha Salim, Evren Gumus, Karin Chen, Martin Zenker, Friedhelm Hildebrandt and Julie Saba declare that they have no conflict of interest.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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Abstract

Sphingosine-1-phosphate (S1P) lyase is a vitamin B6-dependent enzyme that degrades sphingosine-1-phosphate in the final step of sphingolipid metabolism. In 2017, a new inherited disorder was described caused by mutations in *SGPL1*, which encodes sphingosine phosphate lyase (SPL). This condition is referred to as SPL insufficiency syndrome (SPLIS) or alternatively as nephrotic syndrome type 14 (NPHS14). Patients with SPLIS exhibit lymphopenia, nephrosis, adrenal insufficiency, and/or neurological defects. No targeted therapy for SPLIS has been reported. Vitamin B6 supplementation has therapeutic activity in some genetic diseases involving B6-dependent enzymes, a finding ascribed largely to the vitamin's chaperone function. We investigated whether B6 supplementation might have activity in SPLIS patients. We retrospectively monitored responses of disease biomarkers in patients supplemented with B6 and measured SPL activity and sphingolipids in B6-treated patient-derived fibroblasts. In two patients, disease biomarkers responded to B6 supplementation. S1P abundance and activity levels increased and sphingolipids decreased in response to B6. One responsive patient is homozygous for an SPL R222Q variant present in almost 30% of SPLIS patients. Molecular modeling suggests the variant distorts the dimer interface which could be overcome by cofactor supplementation. We demonstrate the first potential targeted therapy for SPLIS and suggest that 30% of SPLIS patients might respond to cofactor supplementation.

Keywords

pyridoxal 5′-phosphate; *SGPL1*; sphingolipidosis; sphingosine phosphate lyase; sphingosine-1-phosphate; SPL insufficiency syndrome; vitamin B6

1 | INTRODUCTION

Sphingolipidoses are lysosomal storage disorders caused by inactivating mutations in genes involved in the degradation of complex sphingolipids.[1] Sphingolipids are metabolized through a common degradative pathway, which results in the production of bioactive intermediates, such as ceramide, sphingosine, and sphingosine-1-phosphate (S1P). These intermediates play important roles in cell signaling, programmed cell death, vascular biology, and immune cell trafficking.[2] Recently, inborn errors of metabolism affecting enzymes involved in degradation of ceramides and sphingoid bases have been discovered.[3,4] These conditions represent non-lysosomal, atypical sphingolipidoses that exhibit distinct pathophysiology and require unique therapeutic strategies.[5]

Sphingosine phosphate lyase (SPL) is a pyridoxal-5′-phosphate (PLP)-dependent enzyme that catalyzes the irreversible catabolism of S1P and other phosphorylated sphingoid in bases in the final metabolic step of sphingolipid degradation.[6,7] In 2017, a novel syndrome was described, caused by inactivating bi-allelic pathogenic variants in *SGPL1*, which encodes SPL.[8–12] This condition, which is referred to as nephrotic syndrome type 14 (NPHS14) or SPL insufficiency syndrome (SPLIS), is associated with steroid-resistant nephrotic syndrome, adrenal insufficiency, ichthyosis, lymphopenia, and neurological defects.[13] The number of reported cases is now over 40.[14–16] The age range and clinical presentations of SPLIS are broad, and the natural history remains poorly understood. Many SPLIS patients are diagnosed after irreversible damage to kidneys, adrenal gland or nervous system have already occurred. Clinical management has largely focused on treating complications.

Some *SGPL1* missense variants affect SPL residues not directly involved in the enzyme's catalytic activity. As in many other inborn errors of metabolism, there is evidence that these non-catalytic site variants may lead to structural variations that can induce SPL protein misfolding, aggregation and/or proteasomal clearance.[11] Reducing SPL misfolding with pharmacological chaperones could represent a potential therapeutic approach for SPLIS.[13] Interestingly, the SPL cofactor PLP—the active form of vitamin B6—has been shown to function as a chaperone in inborn errors of metabolism involving PLP-dependent enzymes.[17,18]

We now provide biochemical and immunological evidence that two SPLIS patients responded to B6 supplementation, supported by findings in patient-derived fibroblasts.

2 | METHODS

2.1 | Study participants

Following informed consent, we obtained clinical data, blood samples, skin biopsies, or archived skin fibroblast samples from individuals with SPLIS from worldwide sources. Approval for human subject research was obtained from the UCSF Benioff Children's Hospital Oakland Institutional Review Board, the National University Health System, Washington University Medical Center, and from the local human subjects Review Boards (Harran University Medical Center and King Saud University Medical Center). Control skin fibroblast lines used in this study were generated as previously reported.[11]

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000. Informed consent was obtained from all patients for being included in the study. Additional informed consent was obtained from all patients for which identifying information is included in this article.

2.2 | Genetic testing methods

For cases 1 to 6, whole-exome or -genome sequencing was performed by next-generation sequencing on blood samples using accredited commercial or academic laboratories (Cases 1 and 2, no parental testing done, Centogene, Rostock, Germany; Case 3, trio Cincinnati

Children's Hospital Medical Center Division of Human Genetics Diagnostic Laboratory; Case 4, trio, Centogene; Case 5, trio Rady Children's Institute for Genomic Medicine, San Diego, California; Case 6 Centogene, parental alleles confirmed by Sanger sequencing). In each case, *SGPL1* was the only gene in which pathogenic variants were reported. In case 7, diagnosis was made with a nephrotic syndrome gene panel showing positivity for *SGPL1* mutations in both alleles (Prevention Genetics). The diagnosis of SPLIS was assigned when patients presented with one or more of the main disease features (neurological/developmental defect, nephrotic syndrome, primary adrenal insufficiency, lymphopenia, acanthosis/ichthyosis) and genetic testing identified *SGPL1* pathogenic variants. In some cases, genetic testing of the parents was confirmatory, but this was not carried out in all cases.

2.3 | Primary fibroblast lines

Archived primary fibroblast lines were provided when available. In other cases, primary fibroblasts were isolated from fresh skin punch biopsy using a previously reported protocol with some modifications.[19] Briefly, using a sterile blade skin biopsy was cut into 10 to 15 evenly sized pieces, which were placed in 1% collagen type I (Millipore Sigma, 3867–1VL)-coated six-well plates containing 800 μL Dulbecco's Modified Eagle's Medium (DMEM) plus 20% fetal bovine serum (FBS). Plates were incubated at 37°C, and 200 μL DMEM plus FBS was added every 2 days. Once the fibroblasts reached confluence, the cells were sub-cultured and maintained in DMEM plus 10% FBS.

2.4 | Generation of immortalized fibroblasts

Primary fibroblasts were transduced with hTERT using the lentivirus vector plv-hTERT-IRES-hygro (Addgene plasmid # 85140). Two days after transduction, 100 μg/mL hygromycin was added for selection. After stable cells were selected, the immortalized fibroblast lines were amplified and sub-cultured for further experiments.

2.5 | SPL activity assay

SPL activity was quantified in whole-cell extracts of primary skin fibroblasts from SPLIS patients and control fibroblasts by measuring the formation of the (2E)-hexadecenal product. This was performed by quantification of a hydrazine derivative of the product by liquid chromatography–tandem mass spectrometry (LC-MS/MS) separation with multiple reaction monitoring essentially as described.[20] Briefly, 90% confluent fibroblasts propagated in 100 mm tissue culture dishes were washed twice with ice-cold PBS, scraped in 1 mL PBS, and cell pellets were harvested after centrifugation for use in SPL activity assays and lipidomic analysis. For SPL activity assay, 40 μg fibroblast protein extract was incubated with 50 μM S1P substrate in the reaction buffer (50 mM potassium phosphate buffer pH 7.4, 1 mM EDTA, 5 mM DTT, 0.4 mM pyridoxal 5′-phosphate,100 mM sucrose, 0.1% Triton X −100) for 1 hour. The reaction was stopped and product derivatized by addition of derivatization buffer (5 mM 2-hydrazinoquinoline, 5% perchloric acid in acetonitrile) for 1 hour. (2E)-hexadecenal(d5) (Avanti Polar Lipids, Inc. # 857476) was added as internal standard, and product was quantified by LC–MS/MS using an Agilent 6490 triple quadrupole mass spectrometer coupled with a 1290 high-performance liquid chromatography instrument.

2.6 | Sphingolipid quantitation

Anticoagulated blood samples obtained over time from patient 4 were placed on ice until plasma isolation by centrifugation the same day, after which plasma was maintained at −20°C until use. Lipid extraction and S1P quantitation were performed locally by LC-MS/MS after derivatization with TMS-diazomethane as previously described.[21] For all other blood samples, plasma was isolated by centrifugation locally, maintained at −20°C, and shipped overnight on dry ice to the corresponding author's laboratory where lipid extraction and S1P quantitation by LC-MS/MS were performed as we described.[22] Sphingolipid quantitation of cell pellets was performed in similar manner.

2.7 | Western blotting

Western blotting was performed on fibroblast whole-cell extracts as we described.[23] Human SPL was detected using a polyclonal anti-human SPL (R&D Systems) at 1:2000. GAPDH loading control, was detected using anti-GAPDH (Santa Cruz Biotechnology) at 1:10 000. Signals were visualized using the SuperSignal West-Pico kit (Fisher Scientific, Rockford, Illinois) and quantified by densitometry using ImageJ software (NIH).

2.8 | Pyridoxal 5′-phosphate and B6 vitamer treatment

SPLIS patient fibroblasts were grown in custom RPMI medium lacking all B6 vitamers (GE Healthcare—Hyclone) and 10% FBS for 1 week. B6 vitamers pyridoxal (PL), pyridoxine (PN), pyridoxamine (PM) or pyridoxal 5′-phosphate (PLP) were delivered to fibroblast cultures at a dose of 50 μM unless otherwise stated in polyethylenimine (1 mg/mL stock in water). Cells were incubated for an additional 5 to 7 days. Cells were harvested, and whole-cell extracts were prepared for western blotting, SPL assay or sphingolipid profiling.

2.8.1 | Molecular modeling of SPL proteins—Wild type and R222Q mutant SPL proteins were analyzed using the PyMOL Molecular Graphics System, Version 2.1. (Schrodinger, LLC, 2015).[24]

2.8.2 | Statistical methods—Two-tailed Student's *t* test was used to establish significant differences between treatment groups of N = 3 or more, with $P < .05$ considered the cutoff for significance.

3 | RESULTS

3.1 | Identification of eight novel SGPL1 variants in seven patients with SPLIS

Seven unrelated cases from diverse geographic locations in the Middle East, Asia, and the United States were diagnosed with SPLIS based on the identification of bi-allelic pathogenic *SGPL1* variants (Table S1). An eighth case can be inferred from the history of a sibling of patient 7 who died of a similar condition. Three of the patients were homozygous for one pathogenic variant, and four patients were compound heterozygotes. Patients 1 and 4 harbored *SGPL1* amino acid substitutions that have been previously reported.[11,12,16] The remaining five patients harbored novel *SGPL1* pathogenic variants resulting in amino acid substitutions (patients 2–7), truncation (patient 3), splicing defect (patient 5), and frameshift (patient 7) of the SPL open reading frame. Pathogenic variants affecting the highly

conserved PLP cofactor binding domain (exons 10–12) appear most frequently. One novel pathogenic variant resulted in a substitution at residue 353, replacing the cofactor binding lysine with an arginine, a change that is known to destroy enzyme activity.[25] SPLIS-associated pathogenic variants identified to date including those in the present cohort are represented schematically in Figure S1. All patients in the current cohort presented with at least one feature of SPLIS within the first 5 years of life (Table S2). Similar to other reports, glomerular kidney disease, and/or proteinuria was a prominent feature in six of the seven SPLIS patients in our cohort. In one case, renal pathology was consistent with collapsing variant focal segmental glomerulosclerosis, an entity that is associated with rapid progression to end-stage renal disease (Figure S2 and Table S3).[26] In a retrospective analysis, we found that two additional patients with SPLIS exhibited collapsing variant focal segmental glomerulosclerosis (Figure S2 and Table S3). Retinopathy and other novel phenotypes observed in our cohort that have not previously been reported in SPLIS patients are described in Supplementary Material and shown in Figure S3. Three out of four SPLIS patients exhibited high circulating levels of the SPL substrate S1P in comparison to healthy age-matched controls (data not shown).

3.2 | SPLIS patients uniformly exhibit T-cell lymphopenia

All patients in our cohort exhibited lymphopenia, consistent with the known role of SPL in regulating S1P chemotactic gradients needed for lymphocyte egress (Table S4).[27,28] For the four patients in which T, B, and natural killer (NK) cell populations were tested (patients 3–7), three had pan-lymphopenia (patients 3–5), while one had isolated T-cell deficiency. Antibody responses to vaccination were tested in four patients and found to be protective (patients 2–5), although two of those had low total immunoglobulin G (IgG) levels. Newborn screening for severe combined immunodeficiency (SCID) detects the presence of naïve T-cells in the peripheral blood using a quantitative PCR assay to detect T cell receptor excision circle (TRECs), DNA excised during T-cell receptor recombination.[29] Two of the three patients born in the United States at the time of SCID newborn screening had abnormal TRECs detected (Table S4).

3.3 | SPL biomarkers respond to vitamin B6 supplementation in some SPLIS patients

Given that vitamin B6 is a known cofactor for SPL and that other metabolic disorders with B6 dependent enzyme deficiencies can be B6 responsive, several patients were placed on B6 supplementation by the treating physician. All four of these patients were already in kidney failure at the time of B6 treatment. There was no uniformity in the method of administration, dosage or form of B6 supplementation. We then retrospectively collected lab values from before and after B6 was instituted in these patients. Considering the variable clinical features in our cohort as well as the unlikely event that end-organ damage would be reversed by B6 therapy, we gathered information on the absolute lymphocyte count and percent lymphocytes before and after the intervention as a biomarker of SPL activity and B6-responsiveness in the cohort. In patient 1 (homozygous R222Q), oral pyridoxine was initiated at 5 mg/kg/day. As shown in Figure 1A and Table S4, the patient’s absolute lymphocyte counts increased significantly in response to pyridoxine. Similarly, the percentage of lymphocytes increased in response to therapy with B6 (Figure 1B). Neither patient 2 (homozygous G360V) nor patient 3 (heterozygous for F290L and Y331*)

responded to vitamin B6 supplementation (Table S4), and both died within months of starting supplementation (Figures S4,S5). In patient 4 (heterozygous S202L and Y416C), oral PLP was initiated at 30 mg/kg/day. At baseline, the patient was persistently lymphopenic (Figure 1C,D) and exhibited low CD3, CD4, and CD8 counts with a decreased CD4/CD8 ratio (Figures 1E–F and Table S4). There was also low-mitogen response to phytohemagglutinin and concanavalin A (data not shown). Serum immunoglobulin G (IgG) levels ranged from 1.77 to 3.89 g/L, and monthly intravenous immunoglobulin was instituted for a period of 2 years. PLP therapy was initiated, following which IgG increased to 7.5 g/L in 4 months at which time the intravenous immunoglobulin was discontinued. Beginning at 4 months after therapy, CD3, CD4, CD8, and CD4/CD8 ratio improved (Figures 1E,F, and Table S4). At that time, pyridoxine given at 8 mg/kg/day substituted for PLP due to persistently elevated liver enzymes. Lymphocyte counts remained in the normal range, and the liver enzymes improved. Clinically, there was marked improvement in the patient's hair growth and resolution of bald patches (data not shown). Prior to instituting therapy, sphingoid base phosphate SPL substrates were measured by LC-MS/MS in patient 4 plasma on five occasions in comparison to healthy age-matched controls. Baseline plasma levels of the major SPL substrate S1P were higher than age-matched controls, whereas dihydro-S1P levels were lower (Figure 1G). Retrospective analysis revealed that the high plasma S1P levels in patient 4 declined subsequent to the initiation of vitamin B6 supplementation (Figure 1H). Both patients 1 and 4 remain alive and are awaiting kidney transplantation 2 years after starting vitamin B6 supplementation.

3.4 | SPL insufficiency is confirmed using patient-derived fibroblasts

To confirm the biochemical impact of *SGPL1* variants identified in our cohort, primary fibroblast cell lines were established from skin biopsy samples of patients 1 to 4. Cell lines were immortalized by stable expression of hTERT. A previously reported healthy control primary fibroblast line was also immortalized with hTERT.[11] Both primary and transformed lines were used for analysis of SPL expression and activity levels and yielded similar results. As shown in Figure 2A and Figure S6, SPL protein levels in primary SPLIS fibroblasts were variable, with cells from patients 1 to 3 exhibiting slightly lower SPL expression levels than control, whereas patient 4 fibroblasts showed a profound reduction in SPL expression. Despite variability in SPL protein levels, all primary SPLIS fibroblast lines exhibited significantly lower SPL enzyme activity than controls (data not shown). Similarly, transformed SPLIS fibroblast lines exhibited SPL activity levels less than 10% of control levels (Figure 2B). When sphingolipid profiling was performed, significantly higher S1P levels were observed in all SPLIS fibroblast lines compared to controls (Figure 3A). Three out of the four SPLIS fibroblast lines also exhibited higher sphingosine levels and lower dihydro-S1P levels than controls (Figure 3B,C). Only patient 4 fibroblasts exhibited higher dihydrosphingosine levels compared to control fibroblasts (Figure 3D).

3.5 | SPLIS patient-derived fibroblasts demonstrate responsiveness to treatment with exogenous vitamin B6

Absolute lymphocyte counts and plasma S1P serve as useful biomarkers of tissue SPL activity. We investigated the impact of exogenous B6 on SPL abundance, activity and S1P levels in patient 4 fibroblasts, as these cells had the lowest SPL abundance and corresponded

to a patient who exhibited a clinical response to B6. Patient 4 fibroblasts were propagated for 1 week in medium lacking all B6 vitamers, followed by an additional week in B6-deficient medium (control) or medium supplemented with B6 vitamers at 50 μM concentration, a dose chosen based on previous literature describing B6 chaperone effects.[30] Comparison of SPL expression in fibroblasts receiving vehicle, PLP, pyridoxal (PL) or pyridoxamine (PM) revealed an increase in SPL abundance after treatment with PLP and PM, as shown by western blotting in Figures 4A and S7. Sphingolipid profiling in fibroblasts treated with different B6 vitamers showed that the S1P and sphingosine levels were significantly reduced after treatment with PLP, PL, PM or PN—with PN and PM showing greatest effect (Figures 4B–C). In contrast, no significant changes were observed in dihydrosphingosine or dihydroS1P levels regardless of treatment (data not shown). Patient 4 fibroblasts were then propagated for 1 week in medium containing a range of PN concentrations. A dose response to PN, as shown by a reduction in cellular S1P, was detectable starting at 0.3 μM, with greatest effect at 50 μM (Figure 4D). SPL activity was augmented in patient 4 fibroblasts by treatment with 50 μM PN (Figure 4E).

3.6 | Response of SPLIS patient-derived fibroblasts to PN treatment correlates with in vivo response

Finally, all four SPLIS patient-derived fibroblast lines were compared for their responsiveness to 50 μM PN based on reduction in cellular sphingolipids. Fibroblasts derived from patients 1 and 4 demonstrated PN responsiveness, as shown by the reduction in total cellular S1P levels (Figure 5A) and sphingosine levels (Figure 5B). In contrast, fibroblasts from patients 2 and 3 were unresponsive to PN based on lack of significant changes in cellular S1P and sphingosine (Figure 5A,B). No consistent differences in cellular dihydrosphingosine or dihydro S1P levels were observed between treated and untreated fibroblasts from any of the four cases (Figure 5C,D).

3.7 | Molecular modeling of the B6 responsive R222Q variant of SPL

Using the crystal structure of human SPL with an inhibitor molecule in the active site as a guide,[31] we interrogated the predicted impact of the SPL^{R222Q} substitution. Within SPL AB dimers, the arginine 222 side chain of monomer A donated by helix 210 to 227, stabilizes the C-terminus of helix 243 to 252 of monomer B and maintains the register of the helices through direct interactions with both helices in a symmetrical arrangement, as shown in Figure 6A. Substituting glutamine for arginine would abrogate charge-dipole interactions at the C-terminus of helix 243 to 252, compromising helix stability due to electrostatic repulsion, represented in Figure 6B. Helix misalignment would likely perturb PLP binding by repositioning the N-terminus of helix 243 to 252, a key determinant for PLP recognition. A reduction in binding affinity for PLP could be compensated for by increasing the concentration of PLP through vitamin B6 supplementation. Using Boltzmann's equation,[32] we calculated that increasing the concentration of PLP by 100 to 200-fold (equivalent to pharmacological PN dosing) should compensate for the reduction in dimer stability and rescue the minor energetic defects introduced via the glutamine substitution. Destabilization of the intact, active, dimeric SPL structure due to the glutamine substitution could be compensated for by PLP binding through multiple direct hydrogen bonds with residues of both monomers and a water molecule and extensive van Der Waals interactions. PLP could

additionally act as a chaperone if covalently bound to SPL, regardless of the K_m for PLP in solution.

4 | DISCUSSION

In this report, we describe seven new SPLIS cases with one additional presumed case. Thus, we contribute 18% of the world's diagnosed cases of SPLIS, bringing the total known patients close to 50. SPL insufficiency causes accumulation of the enzyme's main substrate S1P, consistent with our finding of high S1P levels in patient plasma and skin fibroblast lines. The low plasma dihydro-S1P levels observed in patient 4 could be explained by the fact that dihydro-S1P is generated via the de novo pathway, which may be inhibited by ORM proteins in the presence of high sphingolipid concentrations.[33] This differential effect also suggests that the plasma S1P/dihydroS1P ratio could be helpful in confirming the diagnosis in patients with typical disease features and missense *SGPL1* variants of unknown significance. We observed several unusual features, including two cases of retinopathy and three cases of collapsing variant focal segmental glomerulosclerosis (one in our series and two in previously reported cases). Further analysis will be required to establish the frequency of these conditions in the context of SPLIS. The wide range in severity and heterogeneity of SPLIS presentations could be explained by the degree of SPL inactivation. However, long-term follow up will be required to characterize the natural history of SPLIS and confirm genotype to phenotype correlations.

All patients in our cohort exhibited lymphopenia, which highlights the importance of the known role of SPL in generating S1P gradients that control lymphocyte migration.[27,28] Two patients exhibited pan lymphopenia and two exhibited T-cell-specific lymphopenia. S1P receptors are present on all major lymphocyte populations, and S1P gradients are thought to be important signals for lymphocyte migration.[27,28] It is uncertain why there is variability in B- and NK-cell lymphopenia, but it could be related to the presence of other receptors that promote migration of these lymphocytes. The positive response of SPLIS patients to vaccine challenge suggests that while circulating lymphocytes are low, functional responses may be intact. However, some patients with SPLIS develop recurrent infections, suggesting that SPLIS could be considered a monogenic cause of immune deficiency.

The greatest significance of our study lies in the responsiveness of two SPLIS patients to cofactor supplementation as measured by its impact on two biomarkers of SPL activity, namely plasma S1P levels and lymphocyte counts. The rise in lymphocyte counts in response to B6 was mirrored by increased SPL protein and activity and reduced sphingolipids in patient-derived fibroblasts. PLP serves as a cofactor for more than 140 different enzyme reactions.[34] In addition to its catalytic functions, PLP can serve as a chaperone, facilitating the folding of nascent polypeptides of some PLP-dependent enzymes to attain their optimally active three-dimensional structures.[17] This feature of PLP has been leveraged therapeutically in primary hyperoxaluria and other metabolic diseases.[35,36]

No targeted therapy is currently available for patients with SPLIS. Our results suggest that some manifestations of SPLIS may be preventable by cofactor supplementation in patients with B6-responsive *SGPL1* alleles. We speculate that other chaperone-type drugs could also

potentially be useful in the treatment of SPLIS. Studies comparing murine “SPL knockout” mice which die at weaning to “humanized SPL knock-in” mice which have 10% to 20% of normal SPL activity and are healthy demonstrate that a small amount of SPL activity may suffice to prevent serious sequelae of SPL insufficiency.[37] Thus, modest increases in SPL activity afforded by B6 supplementation may be clinically consequential. Lymphocyte trafficking is highly sensitive to disturbances of S1P gradients, as shown by the persistent lymphopenia in otherwise healthy humanized SPL knock-in mice. Thus, the rise in lymphocyte counts we observed may be a harbinger of a more global responsiveness of SPLIS pathology to cofactor supplementation. Our results demonstrating correlation between in vivo and in vitro responsiveness to B6 suggest that skin fibroblasts may be useful for theratyping, that is, establishing genotype-specific responsiveness to B6 and/or other therapies.

Limitations of our study include the retrospective design, lack of uniformity in the treatment regimen and timing of intervention, lack of information on baseline vitamin B6 status, and differences in patient genotypes, age, medications, and medical history. Like many other micronutrients, B6 contributes to optimal immune function with particular impact on lymphocytes, and supplementation can alleviate neutropenia and lymphopenia especially in individuals with B6 deficiency.[38] In our study, we used the lymphocyte count as a biomarker of SPL function rather than as an indicator of overall immune status, infection risk or chance of survival. Although the two B6 responsive patients survived and the two B6 non-responsive patients did not, the differences in outcome could have been due to many factors that differed between patients including concurrent infections in patients 2 and 3, medications (particularly anticonvulsants which may affect B6 bioavailability) and *SGPL1* pathogenic variant. Testing a standardized vitamin B6 regimen for efficacy in well-designed prospective longitudinal studies—ideally coupled with cell-based testing—will be required before firm conclusions can be drawn regarding the benefit of vitamin B6 in the context of SPLIS.

The two B6-responsive SPLIS patients harbor previously reported *SGPL1* pathogenic variants. Patient 1 is homozygous for R222Q. Substitution of glutamine for arginine disrupts the alignment of helices, distorting the active site and reducing cofactor binding affinity. Importantly, the R222 residue is substituted in nearly 30% of reported SPLIS cases. Thus, a significant proportion of SPLIS patients might benefit from cofactor supplementation. Patient 4 is compound heterozygous for S202L and Y416C. It is not possible to distinguish which allele is responsive to B6 without further analysis. in vitro studies measuring wild type and mutant SPL interactions and mutant SPL protein folding in the presence of PLP will be important next steps.

The greatest potential of cofactor supplementation would be to prevent the devastating neurological sequelae and end-organ damage to kidneys and endocrine glands associated with SPLIS. The development of methods for early diagnosis could provide a window of opportunity for intervention with vitamin B6 supplementation or other targeted therapies as they become available.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

DMEM	Dulbecco's modified Eagle's medium
FBS	fetal bovine serum
FSGS	focal segmental glomerulosclerosis
LC-MS/MS	liquid chromatography-tandem mass spectrometry
NPHS14	nephrotic syndrome type 14
PL	pyridoxal
PLP	pyridoxal 5′-phosphate
PM	pyridoxamine
PN	pyridoxine
S1P	sphingosine-1-phosphate
SCID	severe combined immunodeficiency
SPL	sphingosine phosphate lyase
SPLIS	sphingosine phosphate lyase insufficiency syndrome
TREC	T-cell receptor excision circles

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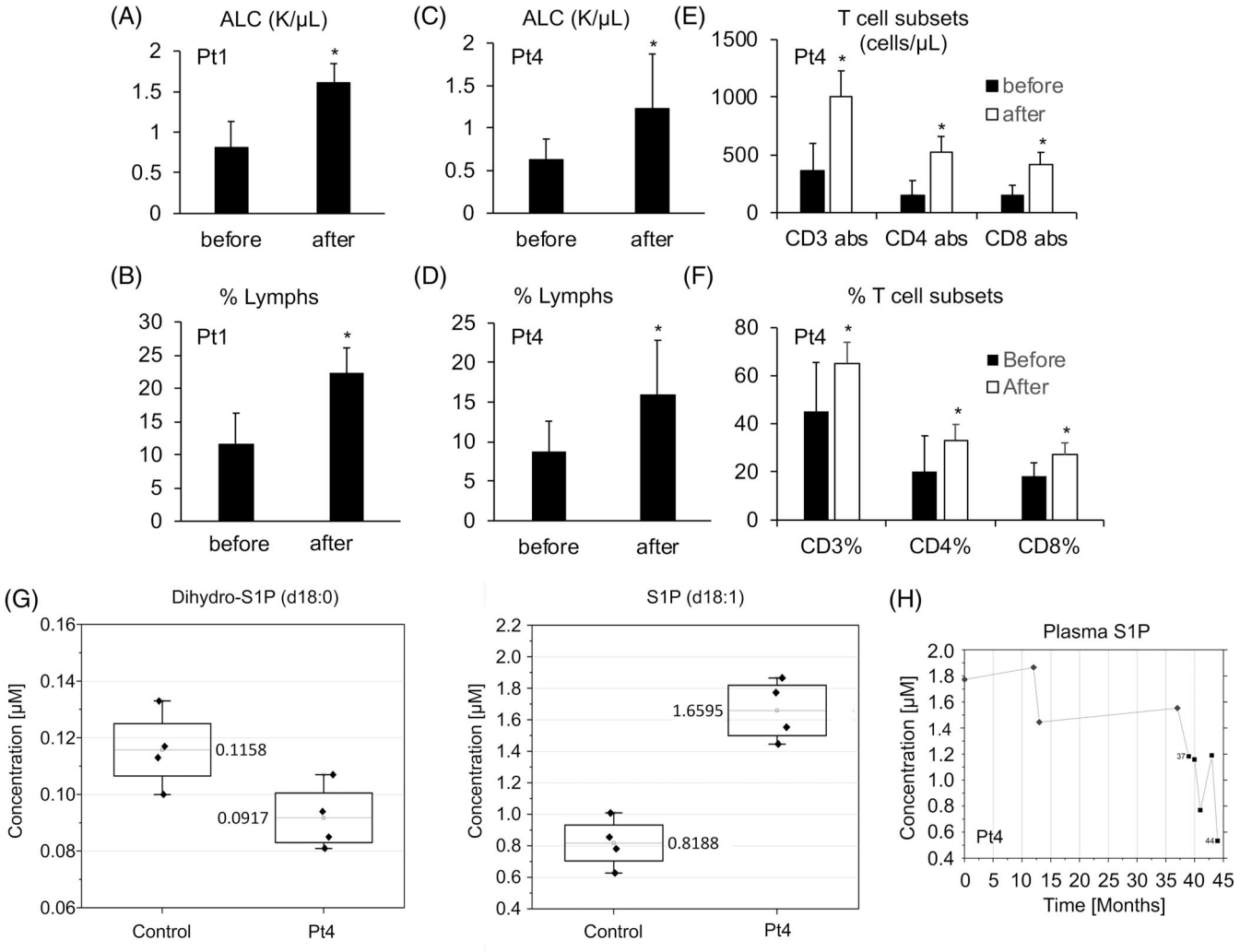

FIGURE 1.

Immunological and biochemical responses to vitamin B6 treatment in patients 1 and 4. Repeated blood counts in patient 1 show (A) the absolute lymphocyte count (ALC) and (B) percent lymphocytes increased in response to pyridoxine treatment. Blood counts before treatment, N = 14. Blood counts after initiation of treatment, N = 17. **P*-value for absolute lymphocyte count before and after treatment = .0025; *P*-value for percent lymphocytes before and after treatment = .0013. Repeated blood counts in patient 4 show (C) the absolute lymphocyte counts and (D) percent lymphocytes increased in response to PLP and pyridoxine treatments. Blood counts before treatment, N = 4. Blood counts after initiation of treatment, N = 13. **P*-value for absolute lymphocyte counts = 7.2×10^{-5}; *P*-value for percent lymphocytes = .0002. In patient 4, T-cell CD3, CD4, and CD8 subsets were evaluated over time. Absolute subsets (E) and percentage of each subset (F) increased after B6 was initiated. The *P*-value for percent CD3, CD4, and CD8 before and after treatment = .011, .017, and .007, respectively; *P*-value for absolute CD3, CD4, and CD8 levels before and after treatment = 1.5×10^{-4}, 2×10^{-4}, and 2×10^{-4}, respectively. (G) Concentrations of S1P and dihydro-S1P measured in plasma of five controls matched by age, gender, and ethnicity to patient 4. Data for patient 4 represent four different time points collected before

any treatment was started. For each group, mean and interquartile values are represented. (H) Concentration of patient 4 plasma S1P (d18:1), measured by targeted mass spectrometry before (blue) and after (black) starting treatment with pyridoxal 5′-phosphate. Time is indicated in months after the first plasma sample was taken

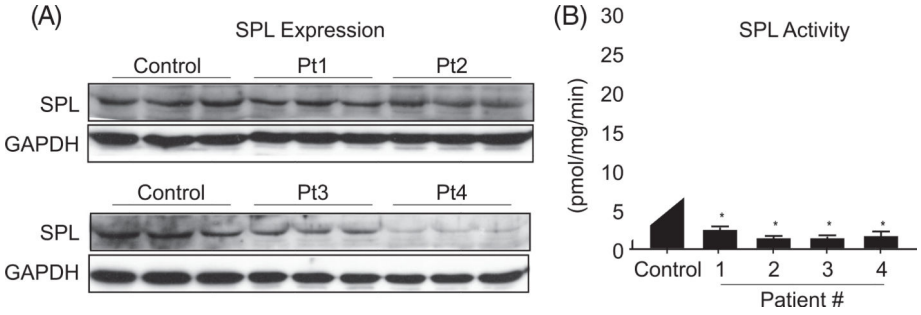

FIGURE 2.

Sphingosine phosphate lyase (SPL) protein expression and enzyme activity in sphingosine phosphate lyase insufficiency syndrome (SPLIS) fibroblasts. Fibroblasts from SPLIS patients 1 to 4 and a healthy control were grown to confluence in B6 replete medium plus 10% FBS, harvested by cell scraping, pelleted, and whole-cell extracts were used for both immunoblotting and SPL activity assays. (A) SPL protein expression in primary fibroblasts from patients 1 to 4; N = 3 per group. For patients 1 to 3 fibroblasts vs control, $P < .05$; For patient 4 fibroblasts vs control, $P < 0.01$. Significance was calculated based on image quantification results shown in Figure S6. (B) SPL activity in primary fibroblasts from patient 1 to 4. N = 4 per group. For each of the four patient-derived fibroblasts vs control, $P < .0003$. These results are representative of three separate experiments, with similar results obtained using either primary or transformed fibroblasts

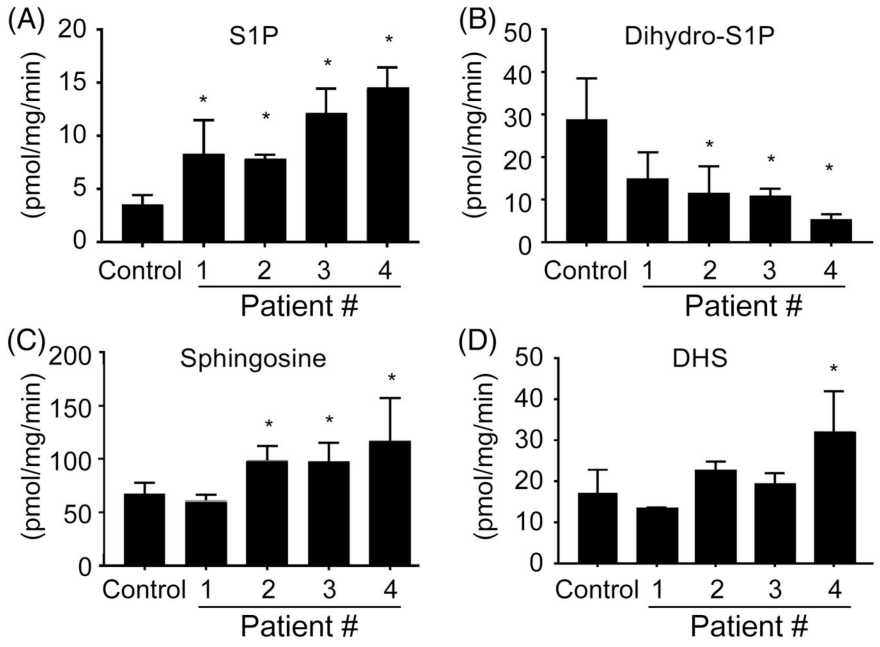

FIGURE 3.

Sphingolipid profiles in sphingosine phosphate lyase insufficiency syndrome (SPLIS) fibroblasts. Primary and transformed skin fibroblasts from SPLIS patients 1 to 4 and a healthy control were grown to confluence in B6 replete medium plus 10% fetal bovine serum (FBS), harvested by cell scraping, pelleted, and whole-cell extracts were used for analysis of sphingoid bases and sphingoid base phosphates. Results are shown for primary fibroblasts: (A) Sphingosine-1-phosphate (S1P); (B) Dihydrosphingosine-1-phosphate (DihydroS1P); (C) Sphingosine; (D) Dihydrosphingosine (DHS). Each sample was run in triplicate (N = 3); *For S1P levels: patient 1 vs control, $P = .03$; patient 2 vs control, $P < .0006$; patient 3 vs control, $P = .001$; patient 4 vs control, $P < 5.7 \times 10^{-6}$. For DihydroS1P levels: patient 1 vs control, $P = .07$; patient 2 vs control, $P = .04$; patient 3 vs control, $P = .02$; patient 4 vs control, $P < .0003$. For all other results, * $P < .05$. These results are representative of three separate experiments, with similar results obtained using either primary or transformed fibroblasts

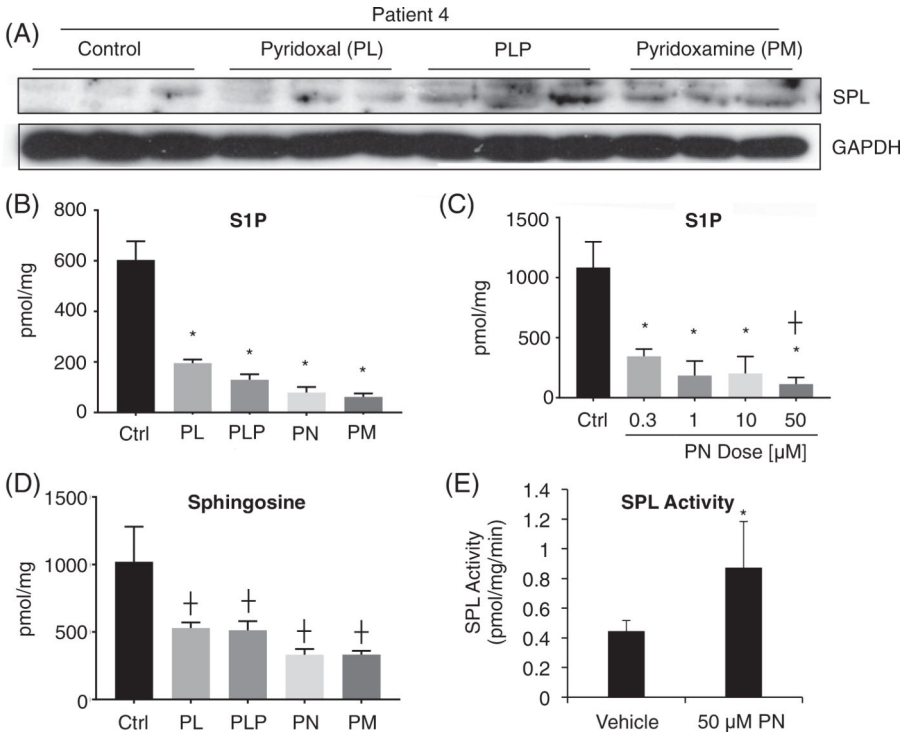

FIGURE 4.

A sphingosine phosphate lyase insufficiency syndrome (SPLIS) patient-derived fibroblast line responds to exogenous PLP and B6 vitamers. Patient 4 fibroblasts were maintained for 1 week in B6-deficient medium and 10% fetal bovine serum (FBS), then switched to B6-deficient medium plus B6 vitamer. Cells were harvested by cell scraping, pelleted, and whole-cell extracts were used for immunoblotting, sphingolipid quantitation or sphingosine phosphate lyase (SPL) assays. (A) SPL protein levels determined by immunoblotting. N = 3 per condition. For pyridoxal (PL) vs vehicle, no significant difference was observed. For PLP vs vehicle control (Ctrl) and pyridoxamine (PM) vs control, $P < .05$. Significance was calculated based on image quantification results shown in Figure S7. (B) Sphingosine-1-phosphate (S1P) and (C) Sphingosine in cells treated with the indicated B6 vitamer; N = 4 per group. *For S1P: $P < .0003$ for all vitamers vs control. + For sphingosine: $P \leq .01$ for all vitamers vs control. These results are representative of three separate experiments. (D) Patient 4 transformed fibroblasts were maintained for 1 week in B6-deficient medium and 10% FBS, then switched to B6-deficient medium-plus vehicle control (Ctrl) or a range of PN doses from 0.3 to 50 μM. Cells were harvested by cell scraping, pelleted, and whole-cell extracts were used for S1P quantitation after 1 week of incubation in the stated conditions. N = 3 per condition. *For 0.3, 1.0, and 10 μM vs vehicle, $P < .005$. + For 50 μM vs vehicle, $P < .002$. (E) Patient 4 transformed fibroblasts were maintained for 1 week in B6-deficient medium and 10% FBS, then switched to B6-deficient medium-plus vehicle control (Ctrl) or plus 50 μM PN. After 1 week of incubation in the stated conditions, whole-cell extracts were harvested and SPL enzyme activity measured. N = 3 per condition; *$P = .035$ for PN vs control

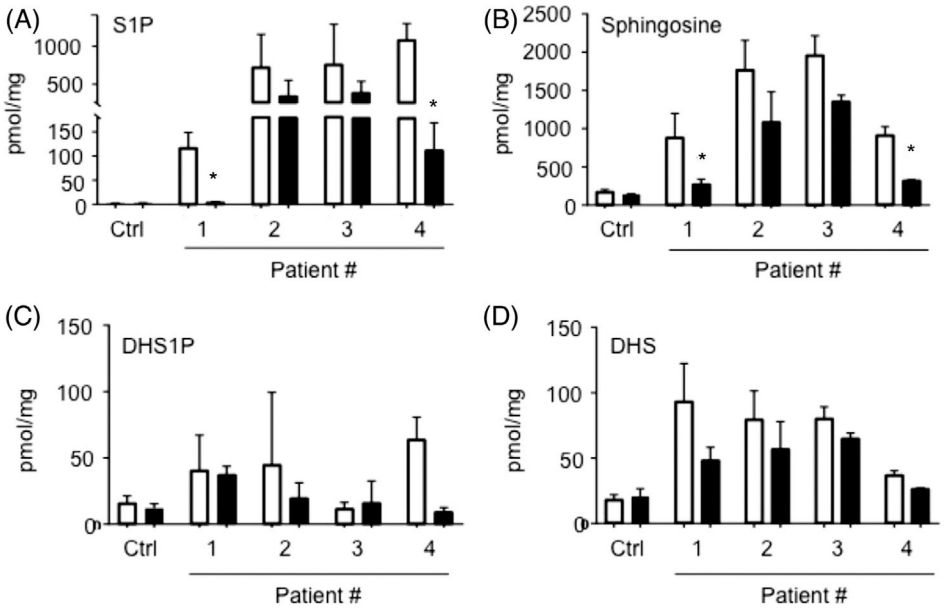

FIGURE 5.

Response of sphingosine phosphate lyase insufficiency syndrome (SPLIS) patient fibroblasts to PN treatment correlates with in vivo response. Fibroblasts from patients 1 to 4 were maintained for 1 week in B6-deficient medium and 10% FBS, then switched to B6-deficient medium plus 50 μM PN. After 1 week of incubation in the stated conditions, whole-cell extracts were harvested and sphingolipid quantitation performed. (A) S1P; (B) Sphingosine; **(C)** dihydroS1P (DHS1P); **(D)** dihydrosphingosine (DHS). N = 3 per group; **P* < .05 or greater significance. For patient 1, PN vs vehicle: S1P, *P* < .0004 and Sphingosine, *P* = .03. For patient 2, PN vs vehicle and patient 3, PN vs vehicle, no significant differences in S1P or sphingosine were observed. For patient 4, PN vs vehicle: S1P, *P* < .002 and sphingosine, *P* < .0007. These results are representative of three separate experiments

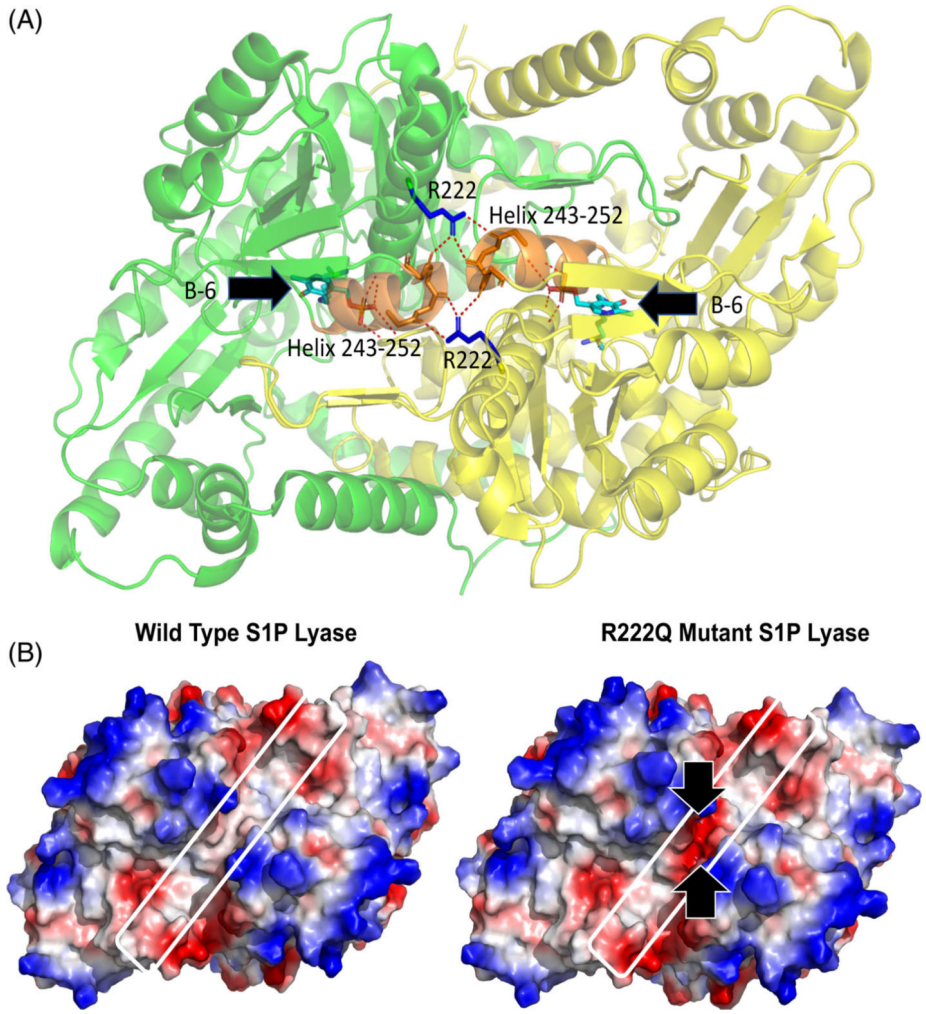

FIGURE 6.

Potential mechanism of B6 interaction with SPL R222Q mutant protein. (A) PyMol-generated illustration of pyridoxal 5′-phosphate (PLP; black arrows) bound to wild type sphingosine phosphate lyase (SPL) monomers at the dimer interface. Monomers within the dimer are designated by green or yellow carbon atoms, while the PLP moiety contains cyan carbon atoms. Helix 243–252 (which would be distorted by substitution of glutamine for arginine) is shown in orange. R222 side chains are shown in deep blue. (B) Electrostatic PyMol surface representations for wild type and R222Q SPL homodimers illustrating electrostatic repulsion (red) at the dimer interface of the R222Q mutant enzyme. The white box represents the dimer interface and the arrows point to the locations of R222 substituted by Q222 in each mutant monomer