An Antibody-GAA Fusion as a Novel Therapeutic for Lafora Disease

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Abstract

Introduction

Lafora Disease (LD, OMIM #254780) is a severe, invariably fatal form of neurodegeneration manifesting as childhood dementia with progressive myoclonic epilepsy (Gentry *et al*, 2018; Turnbull *et al*, 2016). LD is caused by autosomal recessive mutations in *EPM2A* that encodes the glycogen phosphatase laforin; or *EPM2B/NHLRC1* that encodes the E3 ubiquitin ligase malin (Minassian *et al*, 1998; Serratosa *et al*, 1999; Chan *et al*, 2003; Gentry *et al*, 2005; Worby *et al*, 2006; Tagliabracci *et al*, 2007).

LD patients develop normally for the first decade of life and then exhibit a generalized seizure and/or myoclonic jerks (Minassian, 2001; Turnbull *et al*, 2016). Epileptic episodes increase both quantitatively and qualitatively for the next decade and the patient exhibits dramatic and rapid cognitive decline (Shahwan *et al*, 2005; Minassian, 2001). Palliative care with antiepileptic therapies is useful during the early stages of LD, but these become ineffective as the seizures become more intractable. Approximately 10 years after disease onset, patients descend into severe childhood dementia with ataxia and aphasia, enter a vegetative state, and die from status epilepticus or aspiration pneumonia (Minassian, 2001; Serratosa, 1999; Turnbull *et al*, 2016). Age of onset, severity, and rate of progression through disease symptoms are impacted by the specific patient mutation in EPM2A or EPM2B (Brewer *et al*, 2021).

The deficiency in laforin or malin results in cytosolic accumulation of aberrant glycogen-like aggregates, called Lafora Bodies (LBs). LBs are Periodic Acid-Schiff staining positive (PAS+) aggregates in the cytoplasm of cells from nearly all tissues, including both neurons and astrocytes (Sullivan *et al*, 2017; Tagliabracci *et al*, 2007, 2008; Duran *et al*, 2020; Augé *et al*, 2018; Duran *et al*, 2021; Rubio-Villena *et al*, 2018). In the brain of LD mouse models, >90% of astrocytes and ~10% of neurons contain LBs (Duran *et al*, 2021; Lahuerta *et al*, 2020; Rubio-Villena *et al*, 2018). Astrocytic LBs are hypothesized to be important contributors to neurodegenerative pathophysiology including increased inflammation and autophagy, while neuronal LBs are more directly linked to epileptic activity (Duran *et al*, 2021). Further, LD mice also exhibit perturbed brain glucose metabolism and aberrant N-linked protein glycosylation (Brewer *et al*, 2019b; Markussen *et al*, 2021; Sun *et al*, 2021). Importantly, multiple labs, using several LD mouse models, have demonstrated LBs are the etiological agent driving the disease (Turnbull *et al*, 2011, 2014; Duran *et al*, 2014; DePaoli-Roach *et al*, 2010; Valles-Ortega *et al*, 2011; Ganesh *et al*, 2002; Pederson *et al*, 2013). Many labs are therefore developing therapies targeting LBs to treat LD (Gumusgoz *et al*, 2021; Nitschke *et al*, 2021; Markussen *et al*, 2021; Austin *et al*, 2019; Brewer *et al*, 2019b; Tang *et al*, 2020).

Antibody based therapies are used to treat numerous disorders from immunodeficiency, autoimmune diseases, cancer, and glycogen storage diseases (GSDs) (Zhou *et al*, 2019). These therapies exhibit a range of complexities and functions from simplistic intravenous immunoglobulin to complex bi-

and tri-specific antibodies, antibody-enzyme fusions (AEFs), and antibody-directed enzyme prodrug therapy (Zhou *et al*, 2019; Elgundi *et al*, 2017). Delivery of therapies into cells is a substantial hurdle for biologic therapeutics (Rehman *et al*, 2016). The naturally occurring, anti-DNA 3E10 auto-antibody identified in a mouse model of systemic lupus erythematosus, is capable of delivering cargo into cells (Hansen *et al*, 2005; Weisbart *et al*, 1998). The entire 3E10 antibody, its antigen binding fragment (Fab), and its single chain variable fragment have been utilized as efficient cargo carriers for the delivery of therapeutic proteins in multiple disease models without generating pathogenic side effects (Hansen *et al*, 2006, 2007a; Heinze *et al*, 2009; Weisbart *et al*, 2005; Lawlor *et al*, 2013). The 3E10 antibody and its derivatives penetrate cells via the equilibrative nucleotide transporter 2 (ENT2, SLC29A2), a key receptor in the nucleoside salvage pathway, and gain direct access to the cytoplasm and nucleus (Zack *et al*, 1996; Weisbart *et al*, 2005; Hansen *et al*, 2007b) A humanized Fab (hFab) fragment of 3E10 was fused to the 50 kDa human pancreatic alpha-amylase (AMY2A) to generate the AEF VAL-0417. We previously demonstrated that VAL-0417 degrades LBs *in vitro*, ablates brain LBs in LD mouse models when delivered into brain ventricles, and normalizes brain metabolism in *Epm2a-/-* LD mice (Austin *et al*, 2019; Brewer *et al*, 2019b).

The same 3E10 hFab was also fused to the 110 kDa human acid, alpha-glucosidase (GAA) protein to create the AEF, VAL-1221 (Yi *et al*, 2017; Kishnani *et al*, 2019). GAA is the lysosomal enzyme responsible for cleaving the α-1,4- and α-1,6- glycosidic bonds of lysosomal glycogen. GAA gains access to lysosomes via the mannose-6-phosphate (M6P) receptor. Pompe disease (glycogen storage disease type II) is caused by mutations in the *GAA* gene and is driven by aberrant lysosomal and cytoplasmic glycogen accumulations in multiple organ systems, primarily the cardiac and skeletal muscle (van der Ploeg & Reuser, 2008). Pompe disease patients have been treated by enzyme replacement therapy of recombinant human acid alpha-glucosidase (rhGAA, Alglucosidase alfa) since 2006 (REF). A Pompe disease mouse model treated systemically with VAL-1221 exhibited reduced lysosomal and cytoplasmic glycogen inclusions in multiple tissues (Yi *et al*, 2017). Importantly VAL-1221 treatment of late stage Pompe disease patients displayed promising clinical outcomes in a phase I/II clinical trial with no report of major adverse indications (Clinical trials.gov NCT02898753) (Kishnani *et al*, 2019).

Given the success of VAL-1221 to degrade glycogen inclusions in Pompe disease, we investigated VAL-1221 as a method to degrade LBs in LD models. VAL-1221 displayed robust *in vitro* ability to degrade LBs purified from two LD mouse models and to gain cytoplasmic access in cell culture. An LD mouse model treated intravenously with VAL-1221 exhibited significantly decreased LB loads in multiple tissues. Excitingly, intracerebroventricular administration of VAL-1221 dramatically decreased LBs and normalized both brain metabolism and N-linked protein glycosylation. Thus, VAL-1221 is a novel and effective pre-clinical therapeutic for LD.

Results

VAL-1221 degrades LBs *in vitro*

VAL-1221 has exhibited preclinical and clinical efficacy in Pompe disease, but its use in other GSDs has not been defined. Importantly, the glycogen-like aggregates in GSD patients and mouse models differ between diseases by both composition and location (Ellingwood & Cheng, 2018). Many GSDs are driven by aberrant glycogen-like aggregates, but these aggregates differ with respect to glucose chain length, branching frequency, and levels of glucose phosphorylation. Additionally, these aggregates can be in the lysosome or cytoplasm, and many are tissue specific. Therefore, an enzyme-based therapy

must gain access into the appropriate subcellular compartment of the correct tissue and be capable of degrading the aggregate.

VAL-1221 is an AEF comprised of the 3E10 IgG hFab heavy chain fragment fused to human GAA and co-expressed with the corresponding 3E10 IgG hFab light chain (Figure 1A). VAL-1221 was purified from HEK293-6E culture media by affinity chromatography and the purity was assessed by reducing and non-reducing SDS-PAGE (Figure 1B). The heavy chain-GAA fusion peptide dissociated from the light chain peptide with the addition of β-mercaptoethanol (BME) on a reducing gel yielding two distinct bands of 135 and 25 kDa. In the absence of BME, there is a prominent single band at 160 kDa that corresponds to the intact hFab-GAA fusion. The specific activity of VAL-1221 was compared to rhGAA using a kinetic assay with 4-methylumbelliferyl-α-D-glucopyranoside (4-MU-GP) as a substrate, which is converted to glucopyranoside and a fluorescent product, 4-methylumbelliferone. The activity of VAL-1221 was identical to rhGAA throughout the duration of the assay (Figure 1C).

Given robust VAL-1221 activity against the artificial 4-MU-GP substrate, we tested the activity of VAL-1221 against LBs purified from laforin KO and malin KO mouse models. VAL-1221 displayed robust activity against LBs from both LD mouse models (Figure 1D). The products released by this degradation were determined by high-performance anion-exchange chromatography coupled with pulsed amperometric detection (HPAEC-PAD) from aliquots taken from the digestions at 24-, 48-, 72-, and 168-hours. The major product of LB digestion with VAL-1221 was glucose and the next most abundant product was maltose (Figure 1F, Supplemental Figure 1, Supplemental Table 1). Additionally, LBs were digested *in vitro* with VAL-1221 or PBS for 72-hours and visualized by light microscopy with using 10X Lugol's iodine (Figure 1G). Cumulatively, these data demonstrate that VAL-1221 robustly digests LBs *in vitro* primarily releasing glucose.

VAL-1221 Uptake *in vitro* and *in vivo*

To degrade LBs, VAL-1221 must penetrate the cell membrane and access the cytoplasm. VAL-1221 was engineered to enter cells via both the M6P receptor and the ENT2 transporter. The M6P receptor is located on the cell surface and shuttles proteins to the lysosomes via endosomes. ENT2 is a bidirectional nucleoside transporter found on most cell types. To assess the entry of VAL-1221 into cells, C2C12 mouse myoblasts were treated with 50 µg/mL VAL-1221 or PBS for 4 or 24 hours, and then VAL-1221 was imaged via immunofluorescence. After treatment, cells were fixed, VAL-1221 was detected using an anti-Fab nanobody, lysosomes were visualized using an anti-LAMP2 antibody, and nuclei were stained with DAPI. Notably, VAL-1221 was observed largely in the cytoplasm at both timepoints with increased lysosomal colocalization after 24 hours (Figure 2A).

To assess the biodistribution of VAL-1221 *in vivo*, VAL-1221 and rhGAA were radiolabeled by chemical conjugation with ^{89}Zr. rhGAA enters cells via the M6P receptor but lacks the 3E10 hFab and therefore does not engage the ENT2 transporter to enter cells. WT mice were administered radiolabeled VAL-1221 or rhGAA by tail vein injection (i.v.). 120-hours post administration the mice were euthanized, and the heart, brain, quadriceps, liver, and spleen were harvested. The radioactivity of the collected tissues was quantified and the percent injected dose per gram of each tissue was calculated. As expected, the majority of rhGAA and VAL-1221 was detected in the liver and spleen (Supplemental Figure 2). Strikingly, the percent injected dose delivered to the heart, muscle, and brain tissues was significantly higher for VAL-1221 than rhGAA (Figure 2B). These data demonstrate that VAL-1221 penetrates cells and gains access to the cytoplasm both *in vitro* and *in vivo*.

VAL-1221 Degrades Systemic LBs in LKO Mice after i.v. Administration

High LB loads have been reported in heart and skeletal muscle tissues from LD mouse models and LD patients (Villalba-Orero *et al*, 2017; Wick & Byard, 2006). To assess the viability and efficacy of VAL-1221 at degrading LBs *in vivo*, we administered VAL-1221 to (I don't know the ages) mice via i.v. WT and LKO mice received four 0.2 mL injections of 10 mg/mL VAL-1221 or PBS on days 1, 4, 8 and 12 (Figure 3A). The mice were euthanized on day 13 and the heart and quadriceps tissues were rapidly dissected and washed with PBS. Tissues were divided with half flash frozen in liquid nitrogen for biochemical analysis and the other half formalin fixed PAS staining. Val-1221 dramatically decreased total polysaccharide levels in heart and quadriceps (Figure 3B-C). Conversely, total polysaccharide levels were similar for WT mice regardless of treatment and these levels were consistent with previous reports for C57BL/6 mice (Figure 3B-C) (Tagliabracci *et al*, 2008). Formalin fixed PAS-stained heart sections were consistent with the biochemical polysaccharide quantification with fixed tissue exhibiting marked decreases in PAS+ LBs in LKO heart tissue treated with VAL-1221 (Figure 3D). These data demonstrate that after i.v. injection, VAL-1221 penetrates cells, gains access into the cytoplasm, is active, and degrades LBs *in vivo*.

Intracerebroventricular Administration of VAL-1221 Ablates Brain LBs

Pre-clinical data from multiple laboratories utilizing several independent LD mouse models have demonstrated that cerebral LBs cause LD epilepsy and neurodegeneration (Valles-Ortega *et al*, 2011; Duran *et al*, 2012, 2014, 2021; García-Cabrero *et al*, 2012; Brewer *et al*, 2019b). Since most antibody-based drugs do not cross the blood-brain barrier (Zuchero *et al*, 2016), subdural delivery of VAL-1221 into the brain or spinal cord is likely necessary for it to efficiently penetrate the brain parenchyma and degrade cerebral LBs (Zhou *et al*, 2019; Kumar *et al*, 2018). Intracerebroventricular (i.c.v.) administration has proven safe in pediatric and adult populations for the administration of Cerliponase Alfa for Late-Infantile Neuronal Ceroid Lipofuscinosis Type 2, antibiotics for meningitis, and chemotherapy for various cancers (Cohen-pfeffer *et al*, 2017; Slavc *et al*, 2018; Lewis *et al*, 2020).

To deliver VAL-1221 to the brain, i.c.v. cannulas were implanted in the lateral ventricle of 7-month-old LKO mice and attached to a subdermal osmotic pump implanted in the posterior neck. The i.c.v. system delivered a continuous infusion of VAL-1221 (0.03 mg/day) or PBS. Mice received continuous administration of VAL-1221 or PBS for 48- or 168-hours (2 or 7 days) and then were euthanized (Figure 4A). Following euthanasia, the brains were rapidly removed and hemisected along the medial longitudinal fissure. The left hemisphere was fixed in neutral buffered formalin for PAS staining and the right hemisphere was flash frozen and pulverized in liquid nitrogen for biochemical analysis.

To assess if VAL-1221 was successfully delivered to the brain after i.c.v. administration, brain homogenates were assayed using an immunocapture enzyme activity assay using 4-MU-GP as a substrate (Supplemental Figure 3). Robust levels of VAL-1221 were detected from brain samples that were administered the drug for 168 hours and low detection after 48 hours. Negligible levels were detected in PBS-treated brain samples (Figure 4B). Brain homogenates were then assessed for total polysaccharide levels via a recently established method utilizing gas chromatography mass spectrometry (GCMS) (Young *et al*, 2020). VAL-1221-treated mice exhibited a slight reduction in total polysaccharide levels after 48 hours and significantly reduced levels after 168 hours of treatment (Figure 4C). The polysaccharide levels in the PBS-treated animals remained constant at both timepoints (Figure 4C).

Formalin-fixed, PAS-stained LKO brains treated with PBS exhibited an abundance of LBs at the 48-hour and 168-hour timepoints in all regions of the brain, including the cortex, thalamus, cerebellum, and brain stem (Figure 4D & 5). The HALO digital pathology platform was utilized to quantify LB loads in brain regions and the entire brain. In PBS-treated animals, LBs were most abundant in the cerebellum and brainstem with the frontal cortex and thalamus also displaying high LB loads (Figure 5 and Supplemental Figure 4). LB loads were dramatically diminished in all regions of the brain after 168 hours of VAL-1221 treatment (Figure 5 and Supplemental Figure 4).

i.c.v Administration of VAL-1221 Normalizes Metabolic and N-linked Glycosylation in LKO Mice

Glycogen is catabolized to glucose-1-phosphate, converted to glucose-6-phosphate (G6P), and G6P is a substrate for myriad metabolic pathways such as glycolysis, the pentose phosphate pathway, and amino acid synthesis, either directly or indirectly. Disruption of glucose metabolism contributes to multiple physiologic disruptions including dysregulation of neurotransmitters (Duran *et al*, 2021; Markussen *et al*, 2020).

Targeted metabolomics is being utilized for biomarker discovery, defining network perturbations, and establishing molecular mechanisms that drive metabolic diseases (Chen *et al*, 2019; Liu *et al*, 2018; Tasdogan *et al*, 2020; Dang *et al*, 2009; Ward *et al*, 2010; Kind *et al*, 2007; Claudino *et al*, 2007; Sellers *et al*, 2015). We previously demonstrated that LKO mice exhibit perturbations in central carbon metabolism that generate a unique metabolic signature (Brewer *et al*, 2019b). To assess the treatment efficacy of VAL-1221, we employed GCMS to assess central carbon metabolites of glycolysis, the pentose phosphate pathway, the tricarboxylic acid (TCA) cycle, and amino acid metabolism (Fiehn *et al*, 2000; Fiehn, 2016; Kind *et al*, 2009). Polar metabolites were extracted from the pulverized brain tissue of the LKO mice treated with PBS or VAL-1221 via i.c.v. for 48 and 168 hours. We identified >100 metabolites from all the groups that were used in supervised clustering analysis to assess the overall metabolic profiles. The WT untreated and LKO untreated brain metabolomes were distinct, consistent with prior results (Figure 6A) (Brewer *et al*, 2019b). The metabolic profile of LKO mice treated with VAL-1221 shifted more closely to WT untreated animals (Figure 6A). Conversely, the LKO PBS-treated mice exhibited a profile distinct from all the other groups, likely reflecting metabolic changes caused by the i.c.v. surgery(Duran *et al*, 2021). The heatmap clustering further confirmed the sPLSDA results. The metabolic profile of the VAL-1221-treated LKO mice were primarily interspersed with WT profiles while the metabolic profile of the PBS-treated LKO mice was more like LKO untreated (Figure 6B).

Recently, it was demonstrated that brain glycogen is comprised of ~20% glucosamine and disruption in the ability of the glucosamine to be released from glycogen causes critical dysregulation of N-linked glycosylation (Sun *et al*, 2021). It was also reported that glucosamine is sequestered in LBs and that LKO mice exhibit abnormal glycosylation patterns in the brain. We assessed brain N-linked glycosylation patterns using matrix-assisted laser desorption/ionization imaging mass spectrometry (MALDI-IMS) to image sections of LKO mouse brains i.c.v. administered with VAL-1221 or PBS for 168-hour compared to untreated WT and LKO brains (Figure 7 and Supplemental Figure 5). sPLSDA plots show distinct glycan profiles in the WT and LKO mice in five different brain regions (brain stem, cerebellum, thalamus, hippocampus, and cortex). The sPLSDA plots further show that when treated with VAL-1221, the N-linked glycan profile of LKO mice in each region evaluated, becomes indistinguishable from the WT profile, however, the PBS treatment does not return the N-linked glycosylation profile of the LKO mice to WT. From the data making up the total N-linked glycosylation profile seen in the sPLSDA plot, we were able create VIP score plots to parse out the glycans that were most changed within each region.

Interestingly, different glycans made up the most significantly changed group in the various regions, suggesting that the different regions of the brain have specific N-linked glycosylation profiles. However, core fucose glycans seemed to make up the majority of the significantly modified glycans, possibly indicating some core brain specific glycan processing pathways that are not region specific. Indeed, representative MALDI-IMS images of the most changed glycans from the VIP score plot, demonstrate VAL-1221 treatment restores region specific N-linked glycosylation. Together the GCMS metabolomics data and the MALDI-IMS glycan distribution data show that VAL-1221 restores the brains of LKO mice to a WT state after seven days of ICV administration.

Discussion

LD is a GSD with patients exhibiting intractable myoclonic epilepsy and childhood dementia that results from the buildup of aberrant glycogen-like LBs. Because it is well established that LBs drive disease progression, LBs are a key target for LD therapies that are being developed (Brewer *et al*, 2019a, 2019b; Gentry *et al*, 2020; Markussen *et al*, 2021). In this study, we demonstrate the pre-clinical efficacy of a novel AEF therapy for LD. Using multiple established and novel techniques, we establish that VAL-1221 degrades LBs via the GAA domain and penetrates cells in multiple tissues. Upon i.v. administration, VAL-1221 degrades muscle and heart tissue LBs in LD mice. Importantly, i.c.v. administration of VAL-1221 resulted in ablated LBs in multiple brain regions LD mice. Finally, we demonstrate that VAL-1221 i.c.v. administration normalizes the metabolic profile of LD mouse brains and restores WT-like N-linked glycosylation.

Developing a traditional enzyme replacement therapy (ERT) for LD is not an optimal choice since it can be caused by loss of either laforin or malin. Preclinical LD gene therapy strategies are being developed and early results are promising (Vemana *et al*, 2021), however, achieving widespread brain distribution has only recently been shown to be feasible in humans (Thomsen *et al*, 2021). An antisense oligonucleotide (ASO) therapy for LD targeting glycogen synthase (Gys1) has shown promising results at inhibiting LD progression in pre-clinical mouse models (Gumusgoz *et al*, 2021; Ahonen *et al*, 2021). The ASO prevents LBs from developing, but LBs that are present prior to treatment remain and are not degraded. Thus, ASO therapy would likely halt disease progression, but do little to reverse the disease manifestations already present in patients (Nitschke *et al*, 2021; Gumusgoz *et al*, 2021; Ahonen *et al*, 2021). This result of halting disease progression was observed with the ASO drug for spinal muscular atrophy (SMA), nusinersen, which has led to the implementation of neonatal screening for SMA so that patients can be treated as early as possible (Finkel *et al*, 2017; Mercuri *et al*, 2018; Kemper *et al*, 2018; Ojala *et al*, 2021).

Bioavailability is an important factor in the development of any therapy including VAL-1221. VAL-1221 penetrated cells *in vitro*, which is consistent with previous IF experiments (Yi *et al*, 2017). Furthermore, we show that when delivered systemically, VAL-1221 gains access into multiple tissue types and is active within those tissues. Importantly for LD, VAL-1221 achieves broad biodistribution through all regions of the brain and penetrates deep into the parenchyma when administered via i.c.v. Region specific PAS staining and N-linked glycan imaging demonstrate that VAL-1221 is active in multiple brain regions because of the reduction of LBs and correction of glycan profiles.

Like many mouse models of human disease, LD mouse models do not completely mirror the human condition (Perlman, 2016; Ganesh *et al*, 2002; García-Cabrero *et al*, 2012; Sánchez-Elexpuru *et al*, 2017). While LD mice develop LBs and they are more sensitive to seizure inducing drugs, they do not exhibit frequent spontaneous seizures, nor do they exhibit a reduced lifespan. Alternatively, there is an

emerging trend that mouse models share similar metabolic signatures with their respective human disease. Metabolic similarities between humans and mice have been reported for Alzheimer's disease, Huntington's disease and XXX. LKO mice have a unique metabolic signature with respect to central carbon metabolites, which is not surprising given that glycogen metabolism impacts multiple metabolic pathways (Katy's cell met 2019, our 2021 trends review, something else linking glycogen to metabolism). In addition to a metabolic signature, LKO mice exhibit dramatic hypo-glycosylation of brain proteins (Ramon 2021 call met). To define the efficacy of VAL-1221, we assessed the metabolomic profiles and brain glycosylation patterns in LD mice with and without VAL-1221 treatment. VAL-1221 normalized the metabolic profile and brain glycosylation of the LKO mice. It should be noticed that PBS treatment also impacted both profiles, indicating an effect caused by the surgery itself. This effect is likely due to neuroinflammatory markers being elevated after i.c.v. surgery. The surgical size and impact of the surgery on a mouse is proportionally much larger than i.c.v. surgery on a human and it is well reported that i.c.v. is well tolerated in the clinic (Cohen-pfeffer *et al*, 2017; Slavc *et al*, 2018; Lewis *et al*, 2020). Therefore, the effects of surgery alone may not be as significant as observed in this study.

While VAL-1221 ablated brain LBs and normalized brain physiology in LD mice, there are unanswered questions regarding VAL-1221 and LD treatment that need to be defined. First, the rate of LB accumulation in humans and in LD models is not established. This information is crucial to the establishment of a lifelong therapy regimen of VAL-1221 for LD. Furthermore, comparisons between continuous VAL-1221 delivery with a repeated single injection schedule should be defined. Once LBs are ablated, the re-accumulation rate also needs to be defined. Defining these parameters will aid in determining the ideal dosing regimen and whether LD patients will require injections through a port like CLN2 disease or a wearable pump similar to an insulin pump.

Since VAL-1221 is one of multiple treatment strategies being evaluated simultaneously a combination therapy may prove most optimal to control LD progression and relieve symptoms. VAL-1221 ablated LBs and the ASO therapy targeting Gys1 inhibits LB formation. Current ASO therapies involve intrathecal injections every six to twelve months. Thus, one could envision a combination whereby VAL-1221 is administered to the CNS to remove LBs in a single treatment and then an ASO therapy is provided every six to twelve months. Currently, physicians focus on CNS related symptoms given LD patient seizures and dementia. GSDs where patients exhibit glycogen aggregates in non-CNS organs typically display pathology in the organ that contains the aggregates, i.e. liver, skeletal muscle, and/or heart. If LD LBs are ablated and CNS symptoms are controlled, then it is possible that LD patients could develop cardiac manifestations similar to familial Wolff-Parkinson-White patients and/or muscle wasting similar to Pompe disease patients. Indeed, there is one report of cardiac hypertrophy in a LD mouse model (Villalba-Orero *et al*, 2017). VAL-1221 should be well positioned to degrade systemic LBs given the pre-clinical data in this study and the recently completed successful Pompe disease safety clinical trial (NCT02898753).

This study provides ample pre-clinical evidence that VAL-1221 is a viable therapeutic option for the treatment of LD. VAL-1221 degrades the LBs that are the pathognomonic cause of LD and normalizes brain metabolism and glycosylation of LD mice. The tools we have highlighted and developed in this study will be crucial moving forward as VAL-1221 enters studies in other organisms and eventually enters clinical trials. Furthermore, VAL-1221 has shown efficacy in preclinical models of two different GSDs: LD in the present study, and previously in a Pompe disease mouse model (Yi *et al*, 2017)). These data, along with evidence that rhGAA alone (current ERT for Pompe disease) is useful in relieving

symptoms in PRKAG2 syndrome (Austin *et al*, 2017) demonstrates that therapies degrading glycogen accumulations like VAL-1221 have a future in treating multiple GSDs, which impact 1:20,000 patients.

Methods

Expression and purification of VAL-1221

VAL-1221 was designed and produced by Enable Therapeutics (Framingham, MA) (Brewer *et al*, 2019b). The cDNA encoding the humanized IgG1 Fab-linker-rhGAA heavy chain and kappa light chain were synthetically produced with codon optimization for mammalian cell expression and cloned into pTT5. HEK293 cells expressing a truncated variant of the Epstein-Barr virus nuclear antigen 1 (HEK293-6E) to increase the volumetric yield of monoclonal antibodies and fragments were used for VAL-1221 expression (Jäger *et al*, 2013). Two 1 L cultures of HEK293-6E cells in 2 L flasks were transfected with 1 mg of total plasmid DNA/L culture in a 1:1 ratio heavy chain:light chain using PolyPlus linear Q-PEI at a 1:1.5 (w/v) DNA:PEI ratio. Culture parameters were monitored for density and cultures were harvested 5 days post transfection via centrifugation for 5 min at 1000 x g. The conditioned culture supernatant was clarified by centrifugation for 30 min at 9300 x g. Pre-packed CaptureSelect IgG-CH1 affinity columns (Fisher) were equilibrated in PBS (pH 7.2). VAL-1221 from 2 L of exhausted supernatant was top-loaded onto affinity columns (2x1 mL columns in tandem) at 4 °C overnight. The column was washed with approximately 15 column volumes (CV) of PBS, 15 CV of buffer B (1 3 PBS with 500 mM NaCl, pH 7.2), and 15 CV of PBS. The resin-bound fusion protein was eluted with 10 CV of Buffer C (30 mM NaOAc, pH 3.5-3.6), collecting the protein in 1 mL fractions, and diluted in 1/10th volume Buffer D (3 M NaAcetate pH ~9.0) to neutralize. To minimize the elution volume, elution was paused for several minutes between each fraction collected. Fractions were analyzed by A_{280} prior to pooling fractions and pools were analyzed by SDS-PAGE. VAL-1221 remained in the non-bound pool from the first affinity chromatography pass. The above procedure was repeated to capture remaining fusion protein. The affinity pools were combined prior to dialysis. The combined CaptureSelect IgG-CH1 affinity pool (18 mL) was dialyzed against 3x1 L of dialysis buffer (20 mM Histidine, 150 mM NaCl, pH 6.5) at 4 °C. The dialyzed pool was concentrated to 1 mg/mL using a VivaSpin 20 (10K MWCO, PES membrane) centrifugal device prior to final analysis and storage at -80 °C. Purified VAL-1221 was analyzed by size exclusion chromatography (Agilent HP1100) showing a single peak before and after a freeze-thaw cycle, indicative of a monodisperse, stable species.

Mouse lines and care

Mouse models used in this study included C57BL/6 WT mice, LKO (*Epm2a-/-*) mice (Ganesh *et al*, 2002), MKO (*Epm2b-/-*) mice (DePaoli-Roach *et al*, 2010), and CD1 IGS WT mice (Charles River Laboratories, Inc Wilmington, MA). C57BL/6 WT, LKO, and MKO mice were housed at University of Kentucky (UK) and CD1 IGS mice were housed at University of California, San Francisco (UCSF). All mice were housed in a 12:12 hr light-dark cycle and were given *ad libitum* access to food and water. Male and female mice were used interchangeably in these studies as there are no sex differences in LD (Gentry *et al*, 2018). All procedures and housing done at UK were approved by the UK Institutional Animal Care and Use Committee (IACUC) as specified by the 1985 revision to the Animal Welfare Act. UCSF is accredited by the American Association for Accreditation of Laboratory Animal Care (AAALAC) and all procedures and housing done at UCSF met or exceeded the requirements by the 1985 revision of the Animal Welfare Act.

VAL-1221 activity assay

The activity of VAL-1221 was performed using a similar protocol to one already established for GAA activity (Khanna *et al*, 2012). VAL-1221 and rhGAA were incubated in a black Costar 3915 untreated 96-well pate at 37°C in a kinetic fluorescent plate reader with the substrate 4-methylumbelliferyl-α-D-glucopyranoside (4-MU-GP). 4-MU-GP was prepared at 4.2 mM in 0.2 M sodium acetate trihydrate solution pH 4.5. The fluorescence was determined using Ex360/Em460 nm wavelengths with reads every 5 minutes for 1 hour starting as soon as the 4-MU-GP substrate was added. Activity was determined using a standard curve of 4-methylumbelliferone.

Brain homogenate degradation assay

LKO and MKO mice were euthanized by CO_2 and decapitation. The brains were dissected, and flash frozen with liquid nitrogen (LN_2). Frozen brain tissue was pulverized over LN_2 using a Freezer/Mill Cryogenic Grinder (SPEX SamplePrep). Powdered tissue was resuspended in 4 volumes 0.2 M Sodium Acetate buffer and split into two aliquots: treatment and control homogenates. VAL-1221 was added to treatment homogenates at a final concentration of 0.05 mg/mL and an equal volume of PBS was added to control homogenates. The treatment and control homogenates were incubated at 37°C for 72 hours on a rotator. Following the incubations, the homogenates were centrifuged for 5 min at 16,000 x g. The supernatant was separated into polar and non-polar fractions using 50% methanol/chloroform (V/V 1:1) and the polar fraction was analyzed for glucose concentration with GCMS as described previously (Young *et al*, 2020). Briefly, the polar fraction was spiked with a known amount of L-norvaline (as an internal control) and then dried using a vacuum centrifuge at 10^{-3} mBar. The dried fraction was derivatized using methoxyamine in pyridine followed by sequential addition of N-methyl-trimethylsilylation using MSTFA. Derivatized samples were quantified on the GCMS.

LB purification

Purification of native LBs was done as previously described (Brewer *et al*, 2019b). LKO mice were euthanized by cervical dislocation and decapitation and the brains were rapidly dissected and flash frozen on LN_2. The frozen tissue was pulverized in LN_2 using a SPEX SamplePrep cryogenic grinder. The pulverized tissue was then homogenized on ice with lysis buffer (100 mM Tris-HCl pH 8.0, 200 mM NaCl, 1 mM $CaCl_2$, 0.5% sodium azide) with a Dounce homogenizer. The homogenate was centrifuged for 10 min at 10,000 x g and 4°C, the supernatant was removed, and the pellet resuspended in lysis buffer with added SDS and Proteinase K at final concentrations of 0.2% and 0.4 mg/mL, respectively. Proteolytic digestion was run overnight in a water bath at 37°C. The digested samples were syringe-filtered sequentially through 140 µm and 60 µm nylon net filters and then centrifuged at 16,000 x g for 5 min and the pellet resuspended in 10% SDS. The LBs were then washed 5 times in LB buffer (10 mM HEPES-KOH pH 8.0, 0.1% sodium azide) by centrifuging at 16,000 x g for 5 min and then resuspending in LB buffer. Final LB pellets were resuspended in LB buffer and stored at -20°C.

***In vitro* LB degradation and Lugol's light microscopy**

Mouse LKO brain purified LBs were digested with 10 µg of VAL-1221 or PBS for 7 days at 37°C in reaction buffer (5 mM sodium acetate pH 5.4, 7.5 mM NaCl, 0.2 mM $CaCl_2$) on a rotator. After digestion, samples were centrifuged at 16,000 x g for 10 min and resuspend in 20 µL of PBS. 5 µl of resuspended LBs were mixed with 5 µl of 20X Lugol's Iodine (1.5 M KI and 100 mM I_2) and put on a glass slide with cover. LBs were visualized with a Nikon Eclipse E600 microscope using Axio vision software.

HPAEC-PAD determination of degradation products

HPAEC-PAD was performed as previously described (Brewer *et al*, 2019b). Briefly, 80 µg of purified LBs were treated with 2.67 µg of VAL-1221 in degradation buffer (30 mM HEPES-KOH pH 7.5, 5 mM $MgCl_2$, 5 mM $CaCl_2$) to a final concentration of LBs at 1 µg/µL. The digestions were done in triplicate at 37 °C. 20 µL samples were removed at 24-, 48-, and 72-hour intervals followed by addition of 2.67 µg of VAL-1221 after each extraction. The aliquots were stored at -20 °C until they were run. The reactions were continued at 37 °C for 168 hours. Samples were profiled using a CarboPac PA-100 column (Thermo-Dionex, 4 3 250 mm) and detected with PAD detector. 5 µL of samples from each time point were injected on the column. Non-digested LBs in buffer were injected as controls. Glucose and maltose in the degradation reactions were quantified using standards of known amount. Oligosaccharide profiling was standardized using 5 µg Maltrin100.

Cell culture and IF

C2C12 mouse myoblasts (ATCC) were grown DMEM with 10% FBS at 37 °C under 5% CO_2. At 75% confluency, VAL-1221 (final concentration of 50 µg/mL) or PBS was added, cells were incubated for 4- and 24-hours, and then fixed and imaged. Cells were fixed in 4% paraformaldehyde and permeabilized with 0.2% Tween (Bio-Rad) in PBS pH 7.4. A FLAG-tagged proprietary anti-Fab nanobody was incubated for 1 hr at 37 °C at 1:100 dilution in 10% goat serum in PBS. Slides were washed three times and a rabbit anti-FLAG secondary antibody (Invitrogen) and rat anti-LAMP2 primary antibody (Cayman Chemical) were incubated overnight at 4 °C both at a 1:200 dilution. After washing, a goat anti-rat AlexaFluor488 secondary antibody (Invitrogen) and a goat anti-rabbit AlexaFluor546 tertiary antibody (Invitrogen) were incubated for 45 min at 37 °C both at a 1:200 dilution. After final washing, cover slips were mounted using Vectashield with DAPI (Vector Labs). Imaging was performed with an Echo Revolve camera and software (ECHO). All treatment groups were imaged with the same exposure time and equivalent processing.

Radiolabeled VAL-1221 experiment

VAL-1221 and rhGAA were conjugated with the chelator desferoxamine (DFO) by Isotherapeutics Group, LLC (Angleton, TX, USA). ^{89}Zr in 1 M oxalic acid was neutralized with 2 M sodium carbonate. 0.2-1 mg of DFO-conjugated test article, VAL-1221 or rhGAA, was diluted with PBS to ≤ 1 mg/mL. A known volume containing 1 mCi of neutralized ^{89}Zr was then added to the centrifuge tube and left at room temperature for at least 60 minutes depending on the amount of conjugated compound added. After the desired time, an aliquot was spotted onto a filter paper TLC plate and run in 10 mM citric acid. The plate was then developed and read on the TLC scanner to determine labeling efficiency. The solution was then loaded onto a PD-10 column that was conditioned with PBS buffer. Fractions (500 µL) of PBS were loaded onto the column and the elution fractions were counted. In a typical synthesis, 40-60% of the labeled product was recovered in two to three fractions of PBS. ^{89}Zr-VAL-1221 or ^{89}Zr-rhGAA were administered by intravenous tail vein injection (TVI) to CD1 IGS WT mice and mice were euthanized 120 hours after the injection. Brain, lung, spleen, liver, heart, and quadriceps tissue samples were collected. Samples were weighed, loaded into a Perkin Elmer Wallac 3 Gamma counter and the ^{89}Zr radioactivity in each sample was obtained over 1 minute. A 1% injected dose standard was counted along with the samples. The percent injected dose per gram was calculated based on the known dose injected, the 1% standards and tissue weights.

Mouse intravenous (i.v.) experiment

LKO and WT C57BL/6 mice were injected with XXX amount of 1221 or PBS via i.v. 4 injections were performed with 1 injection every 4 days for a treatment schedule of 12 days and the mice were euthanized by cervical dislocation and decapitation on day 13. Hearts and quadriceps muscles were dissected, quickly rinsed in PBS, and hearts bisected with half of each heart fixed in 10% neutral buffered formalin (NBF) for 48 hours and stored in 70% ethanol, and the other half of the heart flash frozen in LN_2. All the quadriceps muscles were flash frozen in LN_2. NBF fixed heart tissues were PAS stained (see "PAS staining and HALO PAS analysis" below) and visualized with XXX. Frozen heart and quadriceps tissues were pulverized using a SPEX SamplePrep cryogenic grinder. Polysaccharide quantification was done using the Pflüger method as described previously (Brewer *et al*, 2019b; Tagliabracci *et al*, 2008). Briefly, pulverized tissue was resuspended and boiled for 2 hours in 10 vol. 30% KOH. After cooling, the samples were mixed with 2 vol 95% cold ethanol with 10 µl 20 mM LiCl and allowed to precipitate overnight at -20°C. The sample was then centrifuged for 10 min at 16,000 x g at 4°C and the pellet washed in ethanol with LiCl and reprecipitated at -20°C for 2 hours. This process was repeated twice, and the final pellet was resuspended in water. The resuspended polysaccharides were digested overnight by amyloglucosidase from *Aspergillus niger* (Sigma), and glucose was determined using a D-glucose kit (Fisher) using the fluorescent readout.

Mouse intracerebroventricular (i.c.v.) administration experiments

Intracerebroventricular (i.c.v.) injections were performed by Northern Biomedical Research (NBR) (Spring Lake, MI). LKO mice were anesthetized using 0.5-1 L/min oxygen with 1-5% isoflurane prior to canula implantation surgery. The i.c.v. canula was attached to an osmotic pump containing VAL-1221 or PBS, the canula was inserted into the cerebral lateral ventricle, and the whole apparatus was enclosed beneath the skin. VAL-1221 (0.03 mg/day) or PBS were administered continuously to the mice for 2 or 7 days with euthanasia by isoflurane/oxygen sedation and perfusion of the left cardiac ventricle by 0.001% sodium nitrite in heparinized saline on days 3 and 8, respectively. Brains were harvested immediately following euthanasia and were bisected into right and left hemispheres. The right hemisphere was rinsed in PBS and then fixed in 10% NBF for 48 hours followed by storage in 70% ethanol. The left hemisphere was flash frozen in LN_2 and then pulverized using a SPEX SamplePrep cryogenic grinder. Fixed tissue was used for PAS staining (see "PAS staining and HALO PAS analysis" below) and pulverized frozen tissue was used for VAL-1221 quantification, polysaccharide quantification, and metabolomics (see "Immunocapture enzyme activity assay," "GCMS polysaccharide quantification," and "PAS staining and HALO PAS analysis" below).

Immunocapture enzyme activity assay

Corning 3925 black high-binding 96-well plates were prepared as previously described (Austin *et al*, 2019) using a proprietary anti-3E10 Fab antibody (Enable Therapeutics). The wells were then blocked with 5% milk in tris-buffered saline (TBS) and washed with TBS before being incubated for 1 hour with pulverized brain tissue samples from VAL-1221 or PBS ICV treated LKO mice that were resuspended in TBS, or purified VAL-1221 in TBS (for standard curve). The wells were washed again with TBS before being run through the GAA activity assay (see "VAL-1221 activity assay" above). By using a standard curve of purified VAL-1221, the concentration of intact VAL-1221 in the tissue sample can be determined. Schematic of this assay shown in Supplemental Figure 3.

GCMS polysaccharide quantification

Relative amounts of polysaccharides from brain tissue following ICV treatment with VAL-1221 or PBS were determined using a modified GCMS procedure to one described by Young et. al. (Young *et al*, 2020). Briefly, purified polysaccharides were hydrolyzed in 200ul of 2N HCl for 2 hours at 95°C. Then, 200ul 100% methanol with 20uM L-norvaline was added for metabolite extraction. Samples were spun at 15,000rpm for 10 minutes at 4°C. 300ul of the supernatant was transferred to a new tube and dried using a vacuum centrifuge at 10^{-3} mBar. Dried samples were derivatized by the addition of 20 mg/mL methoxyamine hydrochloride in pyridine and incubation for 1.5 hr at 30°C. Sequential addition of N-methyl-trimethylsilyl-trifluoroacetamide (MSTFA) followed with an incubation time of 30 min at 37°C with thorough mixing between addition of solvents. The mixture was then transferred to a v-shaped amber glass chromatography vial.

PAS staining and HALO PAS analysis

Heart tissue staining… Combination Periodic Acid-Schiff's Stain (PAS)

Brain tissue staining was performed on 4-micron thick sections cut from formalin fixed paraffin-embedded tissue. Slides were deparaffinized and hydrated stepwise. PAS staining was carried out on 4 mm sections, along with appropriate positive controls per standard protocols (Sheehan & Hrapchak, 1980). After staining, slides were scanned, and images were prepared using the HALO image analysis software (PerkinElmer). Using the HALO software, PAS+ area was quantified both in the brain as a whole and in specific anatomical regions.

Metabolomics with GCMS

Pulverized mouse brain was extracted for polar metabolites using 1 mL of ice cold 50% methanol. The polar fraction was transferred to a V-shaped GCMS glass vial and dried using a vacuum centrifuge at 10^{-3} mBar. Dried polar samples were derivatized by the addition of 20 mg/mL methoxyamine hydrochloride in pyridine and incubation for 1.5 hr at 30°C. Sequential addition of N-methyl-trimethylsilyl-trifluoroacetamide (MSTFA) followed with an incubation time of 30 min at 37°C with thorough mixing between addition of solvents. The mixture was then transferred to a v-shaped amber glass chromatography vial.

Samples were then ran on an Aglient 7800B gas-chromatography (GC) couple to a 5977B single quadrapole mass spectrometry detector. GCMS protocols were similar to those described previously (Young *et al*, 2020; Sun *et al*, 2021), except a modified temperature gradient was used for GC: initial temperature was 130 °C, held for 4 min, risen at 6 °C/min to 243 °C, risen at 60 °C/min to 280 °C, and held for 2 min. The electron ionization (EI) energy was set to 70 eV. Scan (m/z:50-800) and full scan mode were used for mass spectra analysis. For polysaccharide analysis, Metabolite EI fragmentation pattern and retention time were determined by ultrapure standard purchased from sigma. The Ion (m/z) and retention time (min) for Glucose was 160 or 319m/z; 17.4min. Mass spectra were translated to relative metabolite abundance using the Automated Mass Spectral Deconvolution and Identification System (AMDIS) software matched to the FiehnLib metabolomics library (available through Agilent) for retention time and fragmentation pattern matching with a confidence score of > 80 (Fiehn *et al*, 2000; Kind *et al*, 2009; Fiehn, 2016). Values were first normalized to L-norvaline within the sample to account for sample loss, then normalized to DNA-derived thymine to represent input volume.

Glycan distribution with MALDI-imaging

Formalin fixed paraffin embedded brain tissue from the ICV treated LKO mice brains was prepared on Matrix-assisted laser desorption/ionization traveling-wave ion-mobility high-resolution mass spectrometry (MALDI-IMS) imaging compatible glass slides as previously described (Stanback *et al*, 2021). The slides were heated at 60°C for 1 hour and then cooled to room temperature before being deparaffinized using sequential washes in xylene, 100% ethanol, 95% ethanol, 70% ethanol, and water. Following deparaffinization, the slides were placed in a coplin mailer in citraconic anhydride buffer (Thermo, citraconic anhydride and water V/V 1:2, pH 3) and the entire mailer was placed in a vegetable steamer for 25 min. The mailer was allowed to cool before the citraconic buffer was exchanged with water using multiple water washes and the slide was desiccated prior to enzymatic digestion. The desiccated slides were sprayed with PNGase F using an M5 TMSprayer (HTX Technologies LLC) and previously established protocols (Stanback *et al*, 2021; Drake *et al*, 2018; Powers *et al*, 2014). Following PNGase F application, the slides were placed in a 37°C, prewarmed, humidified chamber for 2 hours and then sprayed with α-cyano-4-hydroxycinnamic acid (CHCA) matrix (21 mg CHCA in 3 mL 50% acetonitrile/50% water and 12 µL trifluoroacetic acid). Detection of N-glycans was done using a Waters SynaptG2-Xs high-definition mass spectrometer with traveling wave ion mobility (Waters Corporation) using established settings (Stanback *et al*, 2021; Sun *et al*, 2021). Data acquisition, spectrums were uploaded to High Definition Imaging (HDI) Software (Waters Corporation) for mass range analysis from 750 to 4000m/z.

Figure Legends

Figure 1: VAL-1221 degrades LBs *in vitro*. A. Schematic of the VAL-1221 AEF showing the hFab fragment, made of two peptides linked by disulfide bridges, and the GAA enzyme, fused to the hFab heavy chain fragment peptide. B. Reducing and non-reducing SDS-PAGE gel showing the purity of the VAL-1221 construct. Characteristic banding patterns for VAL-1221 are seen. The non-reducing lane shows the full-size VAL-1221 band as well as faint bands indicating unequal production of the two precursor peptides. The reducing lane shows ablation of the full-size band and shows strong bands at the sizes of the precursor peptides. C. *in vitro* activity assay of VAL-1221 using 4-MU-GP as a substrate with 1-, 3-, and 7-day incubations. D. GCMS glucose quantification assay with VAL-1221 on LKO mouse brain homogenate. E. GCMS glucose quantification assay with VAL-1221 on MKO mouse brain homogenate. F. HPAEC-PAD quantification of the molecules released from purified LKO LBs digested with VAL-1221 as a function of time. G. Light microscopy of Lugol's stained purified LKO LBs digested for 168 hours with PBS or VAL-1221. Data presented as mean with standard deviation (SD) error bars, * $p \leq 0.05$.

Figure 2: VAL-1221 penetrates cells in culture and distributes to clinically relevant tissues when delivered systemically. A. Immunofluorescence shows C2C12 Mouse myoblasts take up VAL-1221 into the cytoplasm and lysosomes. Blue: DAPI, Green: LAMP2, Red: VAL-1221. B. Radiolabeled VAL-1221 biodistribution in lungs, heart, quadriceps (muscle), and brain in WT mice after TVI compared to radiolabeled rhGAA. Data presented as mean with SD error bars, ** $p \leq 0.01$, **** $p \leq 0.0001$.

Figure 3: VAL-1221 degrades LBs in heart and quadriceps muscle tissue of LKO mice after TVI. A. Schematic of the injection schedule for this experiment. B. Biochemical quantification of polysaccharides in heart tissue in WT and LKO mice with PBS or VAL-1221 TVI treatment. C. Biochemical quantification of polysaccharides in quadriceps tissue in WT and LKO mice with PBS or VAL-1221 TVI treatment. D. Representative PAS staining of LKO mouse heart tissue after TVI treatment with PBS or VAL-1221. Data presented as mean with SD error bars, *** $p \leq 0.001$.

Figure 4: Degradation of LBs in the brains of LKO mice after VAL-1221 ICV treatment. A. Schematic of the treatment schedule for this experiment. B. Quantification of VAL-1221 delivered to the brain after ICV treatment determined by immunocapture enzyme activity assay. C. Quantification, using GCMS, of the degradation of polysaccharides in the brains of LKO mice after PBS or VAL-1221 ICV treatment. D. Quantification, using HALO image analysis software, of the PAS+ area of the brains of LKO mice after PBS or VAL-1221 ICV treatment.

Figure 5: PAS staining of brain regions from LKO mice after PBS or VAL-1221 ICV treatment. Representative PAS staining images of the cortex, thalamus, cerebellum, and brain stem from LKO mice after ICV treatment with PBS or VAL-1221 shown at 40X magnification.

Figure 6: Correction of metabolic to WT in LKO mouse brains after ICV treatment with VAL-1221. PCA (A) and heatmap (B) of the metabolic profiles of WT mouse brains and LKO mouse brains treated via ICV with PBS or VAL-1221 determined using a GCMS approach.

Figure 7: Correction of N-linked glycosylation profiles to WT in LKO mouse brains after ICV treatment with VAL-1221. Cerebellum: sPLSDA (A) and VIP score (B) plots of the N-linked glycan profiles. Representative images (C) of the two most changed cell surface N-linked glycan distributions in WT mouse brains, LKO mouse brains, and LKO mouse brains ICV treated with VAL-1221 or PBS. Hippocampus: sPLSDA (D) and VIP score (E) plots of the N-linked glycan profiles. Representative images (F) of the two most changed cell surface N-linked glycan distributions in WT mouse brains, LKO mouse brains, and LKO mouse brains ICV treated with VAL-1221 or PBS. Schematics of the specific glycans analyzed in these images follow X conventions.

Supplemental Figure 1: HPAEC-PAD chromatograms of LKO mouse brain LB digestions with VAL-1221. Chromatograms showing the quantification of the different molecules released from LKO mouse brain LBs throughout an *in vitro* digestion with VAL-1221. Chromatograms are shown for all timepoints collected during the experiment (0, 24, 48, 72, and 168 hours).

Supplemental Figure 2: Radiolabeled VAL-1221 biodistribution in WT mouse liver and spleen compared to radiolabeled rhGAA. Distribution of radiolabeled VAL-1221 or radiolabeled rhGAA in the liver and spleen in WT mice after TVI. Data presented as mean with SD error bars, ** $p \leq 0.01$.

Supplemental Figure 3: Schematic of the immunocapture enzyme activity assay for VAL-1221. VAL-1221 (red and green) is captured from tissue homogenate with an adsorbed anti-Fab antibody (blue). Then the abundance of VAL-1221 present is measured using the activity of the GAA segment of VAL-1221 against 4-MU-GP

Supplemental Figure 4: PAS+ area of different brain regions in LKO mice after ICV treatment with PBS or VAL-1221. Graphs showing the PAS+ area from the cortex, thalamus, cerebellum, and brain stem, after ICV treatment with PBS or VAL-1221. Quantification was done using HALO image analysis software.

Supplemental Figure 5: Cell surface glycosylation correction in additional brain regions. Brain stem: sPLSDA (A) and VIP score (B) plots of the N-linked glycan profiles. Representative images (C) of the most changed cell surface N-linked glycan distribution in WT mouse brains, LKO mouse brains, and LKO mouse brains ICV treated with VAL-1221 or PBS. Thalamus: sPLSDA (D) and VIP score (E) plots of the N-linked glycan profiles. Representative images (F) of the most changed cell surface N-linked glycan distribution in WT mouse brains, LKO mouse brains, and LKO mouse brains ICV treated with VAL-1221 or PBS. Cortex: sPLSDA (G) and VIP score (H) plots of the N-linked glycan profiles. Representative images (I) of the most changed cell surface N-linked glycan distribution in WT mouse brains, LKO mouse brains, and LKO mouse brains ICV treated with VAL-1221 or PBS. Schematics of the specific glycans analyzed in these images follow X conventions.

Supplemental Table 1: Breakdown product analysis via HPAEC-PAD of purified LBs digested with VAL-1221								
		Glucose	Maltose	DP-3	DP-4	DP-5	DP-6	DP-7
Water Blank	Area (nC*min)	n.a.	2.78	n.a.	n.a.	n.a.	0.93	0.51
	Amount (µg)	n.a.	0.108	n.a.	n.a.	n.a.	n.a.	n.a.
Buffer Blank	Area (nC*min)	1.59	5.93	4.57	2.69	n.a.	0.87	0.51
	Amount (µg)	0.022	0.229	n.a.	n.a.	n.a.	n.a.	n.a.
24hr VAL-1221	Area (nC*min)	0.24	10.32	2.18	2.21	0.77	0.86	0.68
	Amount (µg)	0.003	0.399	n.a.	n.a.	n.a.	n.a.	n.a.
48hr VAL-1221	Area (nC*min)	35.50	9.48	n.a.	n.a.	n.a.	0.90	0.75
	Amount (µg)	0.495	0.367	n.a.	n.a.	n.a.	n.a.	n.a.
72hr VAL-1221	Area (nC*min)	47.58	9.45	n.a.	n.a.	n.a.	0.89	0.65
	Amount (µg)	0.663	0.366	n.a.	n.a.	n.a.	n.a.	n.a.
168hr VAL-1221	Area (nC*min)	64.44	9.61	n.a.	n.a.	n.a.	0.84	0.80
	Amount (µg)	0.898	0.372	n.a.	n.a.	n.a.	n.a.	n.a.

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