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Identification of a novel fusion Iduronidase with improved activity in the cardiovascular system

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ABSTRACT

Background: Lysosomal diseases are a group of over 70 rare genetic conditions in which a protein deficiency (most often an enzyme deficiency) leads to multi-system disease. Current therapies for lysosomal diseases are limited in their ability to treat certain tissues that are major contributors to morbidity and mortality, such as the central nervous system (CNS) and cardiac valves. For this study, the lysosomal disease mucopolysaccharidosis type I (MPS I) was selected as the disease model. In MPS I, mutations in the *IDUA* gene cause a deficiency of the α-L-iduronidase (IDUA) enzyme activity, leading to disease pathology in tissues throughout the body, including the CNS and cardiac valves. Current therapies have been unable to prevent neurodevelopmental deficits and cardiac valvular disease in patients with MPS I. This study aimed to evaluate the delivery of IDUA enzyme, via a novel gene therapy construct, to target tissues.

Methods: MPS I mice were hydrodynamically injected through the tail vein with plasmids containing either a codon-optimized cDNA encoding the wild-type IDUA protein or one of four modified IDUAs under the control of the liver-specific human α1-antitrypsin (hAAT) promoter. Two modified IDUAs contained a ligand for the CB1 receptor, which is a highly expressed receptor in the CNS. Iduronidase activity levels were measured in the tissues and plasma using an enzyme activity assay.

Results: The modified IDUAs did not appear to have improved activity levels in the brain compared with the unmodified IDUA. However, one modified IDUA exhibited higher activity levels than the unmodified IDUA in the heart ($p = 0.0211$). This modified iduronidase (LT-IDUA) contained a sequence for a six amino acid peptide termed LT. LT-IDUA was further characterized using a noncompartmental pharmacokinetic approach that directly analyzed enzyme activity levels after gene delivery. LT-IDUA had a 2-fold higher area under the curve (AUC) than the unmodified IDUA ($p = 0.0034$) when AUC was estimated using enzyme activity levels in the plasma.

Conclusion: The addition of a six amino acid peptide improved iduronidase's activity levels in the heart and plasma. The short length of this LT peptide facilitates its use as fusion enzymes encoded as gene therapy or administered as enzyme replacement therapy. More broadly, the LT peptide may aid in developing therapies for numerous lysosomal diseases.

1. Introduction

Lysosomal diseases are a group of over 70 rare, genetic diseases that usually arise from a mutation in a gene coding for an enzyme normally present in the lysosome. This mutation causes a deficiency in the enzyme, accumulation of storage material inside cells, and multi-systemic disorders. Currently, only 12 lysosomal diseases have approved treatments. Therapies for lysosomal diseases include enzyme replacement therapy (ERT), substrate reduction therapy (SRT), protein chaperone therapy, and hematopoietic cell transplant (HCT).

Current therapies for lysosomal diseases have difficulty reaching certain tissues that are sometimes major contributors to morbidity and

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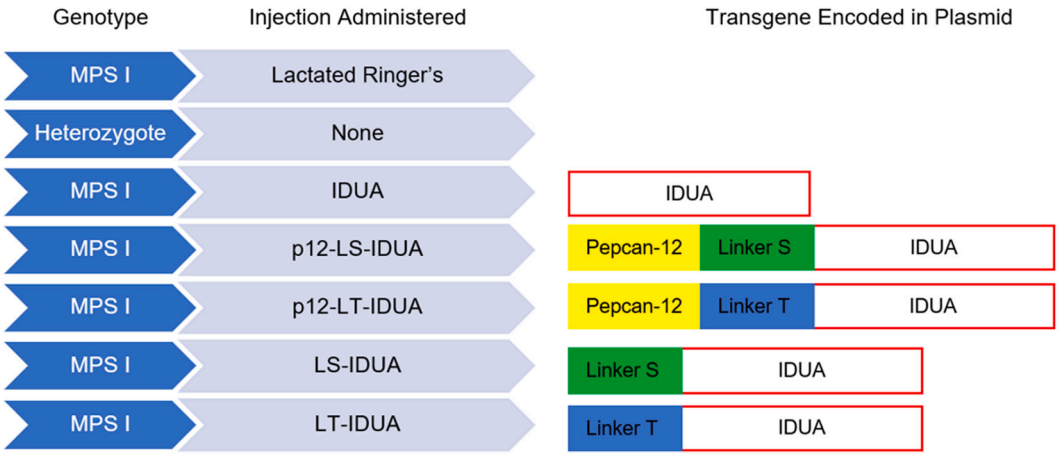

Fig. 1. Experimental Groups in the Identification of Fusion IDUA Candidates.
In the identification of fusion IDUA candidates, seven groups were tested, and each group is represented by a row. Mucopolysaccharidosis type I (MPS I) mice are IDUA$^{-/-}$, and heterozygotes are IDUA$^{+/-}$. Five plasmid constructs were tested, each encoding a different transgene. Plasmids contained the human IDUA cDNA under the control of a human α1-antitrypsin (hAAT) promoter to allow for liver-specific transgene expression. Mice injected with IDUA served as a comparator group. The four modified iduronidases contained inserts for one of two linkers ± pepcan-12. All inserts occurred immediately downstream of IDUA's 57 bp signal sequence. Pepcan-12 is a 12 aa peptide ligand for the CB1 receptor.

mortality, such as the central nervous system (CNS) and cardiac valves. The CNS is affected in two-thirds of all lysosomal diseases [1], and severe neurodevelopmental decline is common in many lysosomal diseases [2]. Treatment of CNS disorders is hindered by the blood-brain barrier (BBB), which restricts the transport of molecules into the CNS, particularly enzymes. One potential strategy to deliver enzymes into the CNS is receptor-mediated transport [3,4]. In receptor-mediated transport, the enzyme of interest is attached to a ligand/antibody that binds to a target receptor expressed on the luminal surface of the BBB. The ligand binding to the receptor triggers endocytosis, allowing enzyme uptake into cells. While there is considerable evidence on targeting the insulin and transferrin receptors [3,4], it is unknown whether targeting other highly expressed receptors can mediate enzyme delivery into the CNS. Furthermore, as most lysosomal diseases are multi-systemic disorders, strategies to deliver enzymes into other affected areas are needed. Complications of mucopolysaccharidoses (MPS) include cardiovascular pathology [5–12]. In particular, cardiac valvular disease is common in mucopolysaccharidosis type I (MPS I), type II (MPS II), and type VI (MPS VI) [5–12]. While HCT and ERT are therapeutic options for some MPS diseases, HCT and ERT do not treat all cardiovascular complications, specifically cardiac valvular disease [5,13–23]. While the exact reasoning is unclear, the limited benefits of HCT and ERT on cardiac valves is thought to be due to the poor vascularization of cardiac valves [5].

Identifying a mechanism that can enhance the delivery of lysosomal enzymes into target tissues could aid in the advancement of therapeutic development for lysosomal diseases. The cannabinoid receptor type 1 (CB1) exhibits several desirable characteristics as a target for receptor-mediated delivery of enzymes. CB1 is highly expressed in the CNS [24], including on the BBB's endothelial cell's luminal surface as well as neurons [25–29]. As such, CB1 is properly localized for transporting enzymes across the BBB and into target cells within the CNS.

The objective of this study was to identify a strategy to facilitate the delivery of therapeutic enzymes into targeted tissues for lysosomal diseases using murine models of MPS I. A secondary objective was to explore new approaches for pharmacokinetic analysis of gene transfer therapies. In this study, MPS I was chosen as the disease model because it is a relatively well-characterized lysosomal disease, which would decrease the likelihood of having an unknown confounding factor. MPS I is an autosomal recessive disease caused by mutations in the *IDUA* gene, which encodes for the enzyme α-L-iduronidase (IDUA; EC 3.2.1.76), resulting in the accumulation of glycosaminoglycans in cells and multi-systemic disorders, including neurocognitive and cardiovascular disorders. It is important to note that, unlike most lysosomal diseases, MPS I has a therapeutic option that can improve CNS outcomes, specifically HCT if it is performed prior to the onset of neurologic damage [30,31].

2. Materials and methods

2.1. Plasmid cloning

A third-generation lentiviral transfer plasmid was used as the backbone. This plasmid contained a human α1-antitrypsin (hAAT) promoter and the hepatic control region of human apolipoprotein E (HCR-apoE), which cause liver-specific expression of transgenes [32,33]. The plasmid contained a codon-optimized sequence of wild-type human *IDUA* that has been used previously [34,35].

The cDNA sequence for pepcan-12 was obtained from the human *HBA1* gene, which encodes for the α chain of hemoglobin [36–38]. In humans, the *HBA2* gene also encodes for the α chain of hemoglobin [37]. In mice, there is only one gene, *Hba-a1*, which encodes for the α chain of hemoglobin [38]. The region of nucleotides that encoded for pepcan-12 was identical among *HBA1*, *HBA2*, and *Hba-a1* [39].

The DNA sequence for pepcan-12 was cgggtggacccggtcaacttcaagctcctaagccac. Linker S was the following 45 bp sequence gggggaggcgggagcgggggaggcgggagcgggggaggcgggagc. Linker T was the following 18 bp sequence ggcggcggcggaacggga. The DNA sequence for linkers was codon-optimized for humans using the Codon Optimization Tool from Integrated DNA Technologies [40].

The plasmid constructs are shown in Fig. 1. The sequences for p12-LS or p12-LT were inserted directly downstream of IDUA's signal sequence. Constructs were cloned by ligation of the polymerase chain reaction (PCR)-synthesized insert using the primers between *Eco*RI/*Blp*I sites. The p12-LS-IDUA and p12-LT-IDUA plasmids used the cloning primers ctcctgacaggttggtgtacagtgaattcatgc and tggggaacagctgccgga tctgctgagcgacg. The LS-IDUA and LT-IDUA plasmids were generated by cloning out the p12 sequence. LS-IDUA plasmid used the cloning primers cagtgaattcatgcggcccctgcggcctagagccgccctgctggc tctcctggcttctctgctggccgggggaggcgggagcgggggaggcggga and tggggaacagctgccggatctgctgagcgacg. LT-IDUA used the cloning primers cagtgaattcatgcggcccctgcggcctagagccgccctgctggctctcctggcttctctgctggccggcg gcggcggaacgggagctcccccctg and tggggaacagctgccggatctgctgagcgacg. The inserts were sequenced by the primers agccctgccctgagactg and attgtactaaccttcttctctttc. Plasmid cloning and sequencing were performed by the

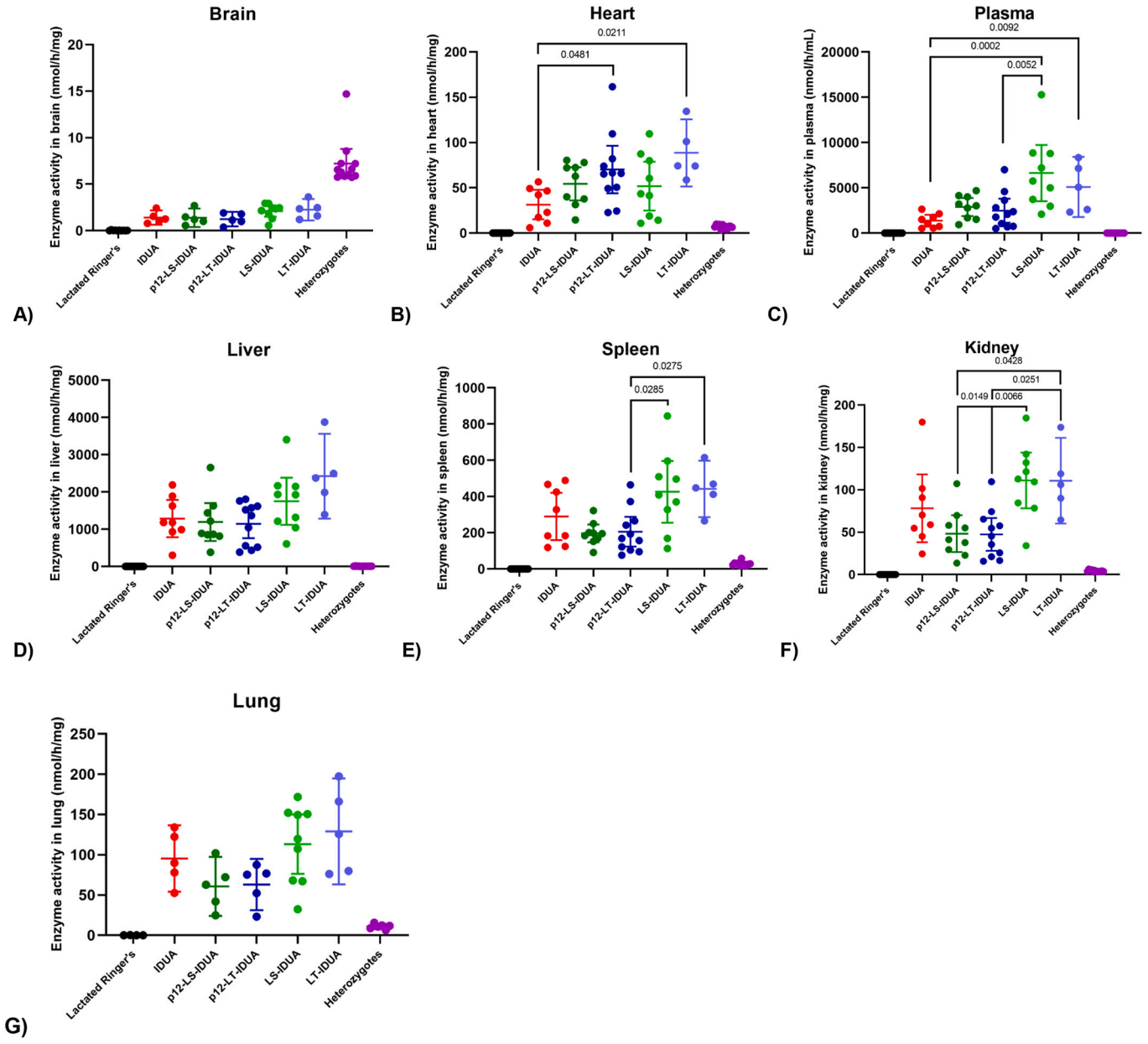

Fig. 2. Enzyme activity levels in the tissues and plasma.
Mice were hydrodynamically injected into the tail vein with 50 μg of plasmids encoding different iduronidase enzymes (IDUA, p12-LS-IDUA, p12-LT-IDUA, LS-IDUA, and LT-IDUA). The IDUA group served as a comparator group for the four modified IDUAs. After 48 h post-injection, iduronidase levels were measured in the tissues and plasma using an enzyme activity assay. Each data point represents one mouse, and the mean ± 95% confidence interval for each group is shown. A one-way ANOVA with multiple comparisons adjustment was performed, and statistically significant differences between plasmid-treated groups are shown.

Custom Cloning Division within the Emory Integrated Genomics Core.

2.2. Hydrodynamic injection

All protocols involving animals were approved by the Institutional Animal Use and Care Committee (IACUC) at the University of Minnesota. The MPS I knockout mice (*IDUA*$^{-/-}$) utilized in this study, a kind gift from Dr. Elizabeth Neufeld (University of California, Los Angeles [UCLA]), were generated by insertion of a neomycin resistance gene into exon 6 of the 14-exon *IDUA* gene on the C57BL/6 background [41]. All mice were genotyped by PCR by Transnetyx, Inc. and housed in specific pathogen-free conditions. Heterozygote mice were used as the control for this experiment, and as such are known to have approximately 50% IDUA tissue and plasma levels compared to wild type mice and have no significant difference in behavioral and motor skill phenotype compared to wild type [41–44]. As MPS I is inherited through an autosomal recessive pattern, it is not considered necessary to achieve normal or supranormal levels of IDUA in order to change the phenotype from diseased to a wild-type phenotype. Using heterozygote mice as the control reduces the number of research animal that must be bred and sacrificed and thereby satisfies the mandated humane and ethical requirements for animal research as put forth by IACUC (Institutional Animal Care and Use Committee) [45].

A single dose of 50 μg of plasmid was chosen in this study, which represents the maximum effective dose of a plasmid that is hydrodynamically injected into the tail vein. This dose has been used previously in this lab and other labs [32,46,47], and this dose had been shown to cause the highest expression levels for *IDUA*. That is, doses past 50 μg

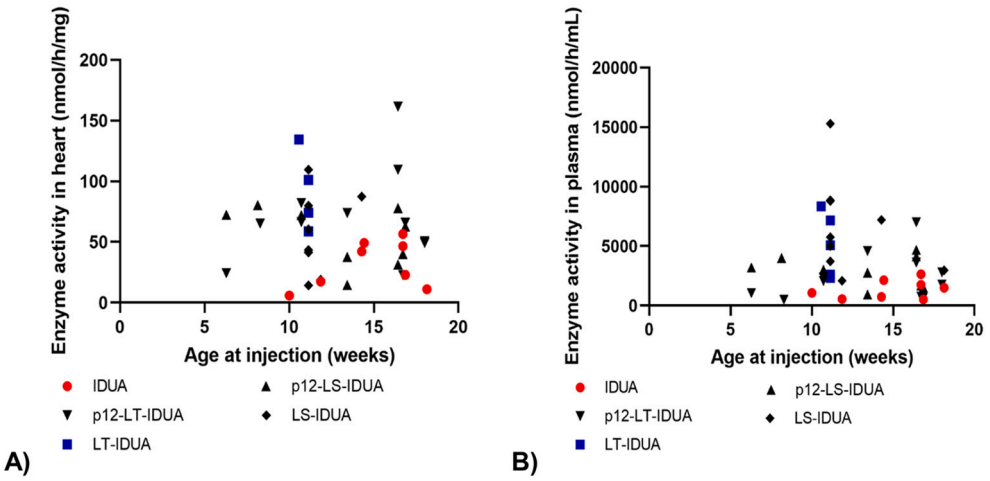

Fig. 3. No relationship between age at injection and enzyme activity levels.
Mice were hydrodynamically injected into the tail vein with 50 μg of plasmids encoding different iduronidase enzymes (IDUA, p12-LS-IDUA, p12-LT-IDUA, LS-IDUA, and LT-IDUA). Iduronidase levels were measured in the heart and plasma using an enzyme activity assay after 48 h. Pearson's correlation tests were performed for age at injection and enzyme activity levels. (A) Enzyme activity levels in the heart did not correlate with age at injection ($r^2 = 0.01640$, $p = 0.4190$). (B) Enzyme activity levels in the plasma did not correlate with age at injection ($r^2 = -0.04050$, $p = 0.2013$).

did not increase the activity levels of iduronidase.

Plasmid mixtures were prepared by mixing 50 μg of plasmids with a volume of Lactated Ringer's (LR) Injection, United States Pharmacopeia (USP), needed to achieve a total volume that is 10% of the mouse's body weight. For example, a mouse weighing 20 g corresponds to 2 mL of the plasmid mixture. The maximum volume of the plasmid mixture was 2.7 mL.

Mice were lightly sedated with ketamine/butorphanol/acepromazine cocktail. The sedative cocktail was prepared by the University of Minnesota's Research Animal Resources (RAR) as follows: 4.5 mL of 0.9% Sodium Chloride Injection, USP, 0.4 mL of 100 mg/mL ketamine, 0.05 mL of 1 mg/mL butorphanol, and 0.05 mL of 10 mg/mL acepromazine maleate injection were mixed to a final volume of 5 mL. Mice were administered 20 μL of the sedative cocktail intraperitoneally 30 min before hydrodynamic injection of the plasmid mixture intravenously through the lateral tail vein using a 27G butterfly needle.

2.3. Dissection and sample processing

Approximately 200 μL of blood was collected from the submandibular vein. The blood was mixed with 20 μL of 100 units/mL Heparin Lock Flush Solution, USP (manufactured by BD), then centrifuged for ten minutes at 3000 rpm (rpm) (Spectrafuge® 24D, Labnet International). The translucent layer corresponding to the plasma was transferred to a new tube and stored at −80 °C.

Mice were euthanized with CO_2 asphyxiation. Cardiac perfusion was performed with approximately 35 mL of phosphate-buffered saline (PBS) (Corning® ref. 21–040-CV). The brain, heart, lung, liver, kidney, and spleen were dissected and snap frozen on dry ice. Samples were stored at −80 °C.

Organs were mechanically homogenized with either a rotor-stator (Kinematica Polytron® CH-6010 Kriens-lu PT 10/35) or bead impact (Next Advance Bullet Blender® Storm). In both methods, 1 mL of PBS (Corning® ref. 21–040-CV) was added to the organ. After mechanical homogenization, 11 μL of 10% Triton in PBS (Triton X-100 Sigma T8787) was added. After ten minutes, samples were centrifuged at 13,300 rpm for 15 min (Spectrafuge® 24D, Labnet International). The supernatant was transferred to a new tube and stored at −80 °C.

2.4. Quantification of IDUA enzyme activity levels using fluorescent 4-Methylumbelliferyl-based assay

The enzyme activity assay for iduronidase was performed as previously described, with the modification that glycine-NaOH was used as a stopping buffer [44]. Samples were run in triplicate for the enzyme activity assay and the protein assay.

2.5. Quantification of IDUA protein levels using ELISA

A commercially available kit for human iduronidase was used for sandwich enzyme-linked immunosorbent assay (ELISA) (abx253700, Abbexa®) [49]. This ELISA kit was chosen over other commercially available assays because both sets of antigen-specific antibodies did not recognize near the N-terminal of iduronidase [50]. This allowed the kit to be used for both IDUA and LT-IDUA. Furthermore, this kit was compatible with plasma and tissue homogenates and had a sensitivity of 0.1 ng/mL and a range of 0.156 ng/mL and 10 ng/mL. Liver samples from mice treated with IDUA and LT-IDUA were used for ELISA. Livers were homogenized as previously described. One mouse from each plasmid-treated group was assessed at the 0.5, 1, 2, 4, 8, and 12 h post-injection. Two mice from each plasmid-treated group were assessed at 24, 48, 96, and 168 h post-injection.

2.6. Statistical analysis

Statistical tests were performed using GraphPad Prism version 9.2.0 for Windows, GraphPad Software (San Diego, California, USA). In the identification of fusion IDUA candidates, the initial statistical plan was to compare the enzyme activity levels of all seven groups. However, it became evident that the hydrodynamic injection of plasmids caused supraphysiological enzyme activity levels in the plasma and organs, except the brain. Therefore, for enzyme activity levels in the brain (Fig. 2A), all seven groups were compared. The enzyme activity levels in the brain were natural log transformed, and a Welch one-way ANOVA test followed by Dunnett's T3 multiple comparisons test was performed. For the enzyme activity levels in the plasma and non-brain tissues (Fig. 2B through Fig. 2G), only the plasmid-treated groups were compared statistically to conserve power. Data were natural log-transformed, and an ordinary one-way ANOVA followed by Tukey's multiple comparisons test was performed.

Three Pearson's correlation tests were performed: (1) age at injection and enzyme activity levels in the heart (Fig. 3A), (2) age at injection and enzyme activity levels in the plasma (Fig. 3B), (3) enzyme's physical levels in the liver and the enzyme's activity levels in the liver (Fig. 5).

2.7. Noncompartmental analysis

A noncompartmental analysis (NCA) of enzyme activity levels in the plasma and liver was performed for mice treated with IDUA and LT-IDUA. NCA was performed using Phoenix® 64 (Phoenix 64, Build 8.3.3.33, Certara L.P., 100 Overlook Center, Suite 101, Princeton, NJ 08540 USA). The below quantification limit (BQL) was inputted for samples with fluorescence readings less than twice the "blank" wells.

The lower limit of quantification (LLOQ) was substituted for BQL for the conditions before T_{max}, after T_{max}, and the first consecutive after T_{max}. The LLOQ value for enzyme activity in plasma was 0.93 nmol/h/mL, which was calculated from "blank" wells. The LLOQ value for enzyme activity in the liver was 2.20 nmol/h/mg, which was calculated from "blank" wells in the enzyme activity assay and Pierce protein assay.

For the therapeutic response settings, a lower boundary was set, which was the average levels from heterozygous mice. The lower boundary represented the minimum enzyme activity needed for efficacy and thus minimum effective concentration (MEC). The lower boundary of 6.10 nmol/h/mL for enzyme activity in the plasma. The lower boundary was 3.89 nmol/h/mg for enzyme activity in the liver.

The NCA model type of plasma (200−202) was chosen for the enzyme activity in the plasma and the liver. Nominal timepoints and uniform weighting were used due to sparse sampling. The mean concentration curve is used to calculate the parameters. The following parameters were calculated: T_{max} of activity, C_{max} of activity, Lambda_z (reported as k_e of activity), HL_Lambda_z (reported as $t_{1/2}$ of activity or half-life of enzyme activity), AUC_{last} (reported as $AUC_{0–168\ h}$ of activity), AUCINF (AUC from time of dosing extrapolated to infinity based on last observed concentration; reported as $AUC_{0-\infty}$ of activity), TimeHigh (reported as $Time_{above\ MEC}$ of activity), AUCHigh (reported as $AUC_{above\ MEC}$ of activity).

A linear trapezoidal-linear extrapolation method was used to calculate the AUC of enzyme activity in the plasma and the liver. A linear-linear method was chosen because Bailer's method, with modifications described by Phoenix, was planned to compare the AUC of activity between enzymes [51–53]. Bailer's method allows testing of equality in AUCs in study designs that use destructive measurements, and Bailer's method relies on AUCs calculated by linear trapezoidal rules [51]. The AUC of activity's mean and standard error of the mean was obtained from Phoenix [47–49]. The AUC of activity's mean and the standard error of the mean were then entered into GraphPad to perform an unpaired, two-tailed, *t*-test to compare the AUC of activity between enzymes.

To calculate k_e and $t_{1/2}$ of enzyme activity levels in the plasma, timepoints from 24 to 168 h were used to calculate a slope. From k_e of activity, $t_{1/2}$ of activity was calculated using the equation $t_{1/2}$ of activity $= 0.693 * k_e$ of activity. The k_e and $t_{1/2}$ of activity using only the 96 and 168 h were also calculated. The timepoints of 96 and 168 h were chosen because LT-IDUA appeared to be going through a different phase at 48 h and to maintain consistency with IDUA. Graphs were generated with GraphPad Prism version 9.2.0 for Windows, GraphPad Software (San Diego, California USA). The names of PK parameters were denoted with "of activity" (e.g., AUC of activity) when possible to indicate that enzyme activity levels were analyzed.

2.8. Identification of fusion IDUA candidates: Study design

Seven groups were tested in the experiments to identify a fusion iduronidase candidate (Fig. 1). MPS I mice (*IDUA*$^{-/-}$) injected with Lactated Ringer's (LR) served as a negative control group, and heterozygous mice (*IDUA*$^{+/-}$) served as a positive control group. Five groups were administered plasmids, each encoding a different iduronidase. The IDUA group was administered a plasmid containing the human IDUA cDNA, which encoded for the unmodified human iduronidase, to serve as a comparator group. Four fusion IDUAs were tested: p12-LS-IDUA, p12-LT-IDUA, LS-IDUA, and LT-IDUA.

Two fusion IDUA constructs, p12-LS-IDUA and p12-LT-IDUA, contained sequences for a peptide ligand for the CB1 receptor, specifically, pepcan-12 (p12). Pepcan-12 is part of a class of peptides called pepcans, abbreviated for peptide endocannabinoids, whose sequences are derived from the α chain of hemoglobin and can bind to the CB1 receptor [54,55]. Pepcan-12 was chosen as this study's ligand for the CB1 receptor because pepcan-12 has a higher affinity for the CB1 receptor than other pepcans [55]. Previous studies reported pepcan-12 could induce internalization of the CB1 receptor after binding, had limited cross-reactivity to other receptors, and had manageable physiological effects after exogenous administration [56–60]. Because pepcan-12's N-terminal was reported to be important for binding to CB1 [51,56], pepcan-12 would need to be expressed at the N-terminal of iduronidase (i.e., 5′ end of the IDUA cDNA). However, the 5′ end of the IDUA cDNA contained a 57 bp (19 aa) signal sequence that ensures proper protein processing [50,61,62]. This signal sequence is cleaved off during protein processing and absent in the mature form of iduronidase. Therefore, pepcan-12 was inserted immediately downstream of IDUA's signal sequence, allowing pepcan-12 to have a free N-terminal on the mature form of the fusion iduronidase. Since literature and database searches did not report structural or functional domains near iduronidase's N- or C-terminal, aside from the above mentioned signal sequence [50, 61–63], an insertion near the N-terminal of iduronidase seemed unlikely to affect the enzymatic function.

All four fusion IDUA constructs also contained sequences for linkers between pepcan-12 and the mature enzyme (Fig. 1). Because iduronidase is considerably larger than pepcan-12 (634 aa vs. 12 aa), it seemed plausible that iduronidase could sterically hinder pepcan-12 from interacting with the CB1 receptor. It also seemed plausible, though to a lesser extent, that pepcan-12 could interfere with the activity of iduronidase. To decrease the likelihood of functional interference between the pepcan-12 and iduronidase domains, flexible linkers were placed between the sequences for pepcan-12 and the mature IDUA enzyme (Fig. 1). Because the length and sequence of linkers can impact the activity of fusion enzymes [59], two linkers were tested, Linker S (LS) and Linker T (LT). These linkers encoded primarily glycine residues which allow for flexible movement between domains. Linker S was encoded by a 45 bp sequence, which translates into the 15 aa sequence (GGGGS)3. Linker T was encoded by a 18 bp sequence, which translates into the six aa sequence GGGGTG. To assess if the linkers were affecting enzymatic activity, two modified IDUAs contained sequences for linkers but not pepcan-12: LS-IDUA and LT-IDUA.

Table 1
Age and Sex of MPS I Knockout Mice (*IDUA*$^{-/-}$) in Experiments to Identify Fusion IDUA Candidates.

Group	Mean age at injection in weeks (minimum and maximum age)	Number of females	Number of males	Total number of mice
Lactated Ringer's	17.3 (6.3–24.1)	5	5	10
IDUA	14.9 (10.0–18.1)	4	4	8
p12-LS-IDUA	13.2 (6.3–16.9)	6	3	9
p12-LT-IDUA	13.8 (6.3–18.0)	7	4	11
LS-IDUA	12.4 (11.1–18.1)	5	4	9
LT-IDUA	11.0 (10.6–11.1)	3	2	5
Heterozygotes	15.1 (8.1–18.3)	6	6	12

The age, sex, and number of mice used in the experiments to identify fusion IDUA candidates are shown. All the mice were IDUA−/−, aside from the heterozygotes.

3. Results

3.1. Identification of fusion IDUA candidates

The experimental groups in the identification of fusion IDUA candidates are shown in Fig. 1, the age and sex of the 64 mice used in these experiments are summarized in Table 1. The mean age at which a single injection of plasmid was given was 14.2 weeks, ranging between 6.3 weeks to 24.1 weeks. The age at injection was generally similar across groups, with some exceptions. The LR group was older than the rest of the groups, and the LT-IDUA group was on average four weeks younger than the IDUA group. Sex was generally balanced across groups, although there was a greater proportion of females in the p12-LS-IDUA

Table 2
Age, Sex, and Number of Mice at Each Timepoint in the Quantitative Pharmacology Study.

	Mean age at sample collection in weeks		Percent of females		Total number of mice	
Time post-injection (hours)	IDUA	LT-IDUA	IDUA	LT-IDUA	IDUA	LT-IDUA
0.5	10.9	11.1	50%	33.3%	4	3
1	10.9	11.2	75%	66.7%	4	3
2	8.4	10.2	50%	33.3%	4	3
4	7.9	8.6	33.3%	66.7%	3	3
8	8.1	10.1	50%	66.7%	4	3
12	7.6	7.9	50%	66.7%	4	3
24	8.6	8.0	66.7%	50%	3	4
48	7.9	9.2	50%	33.3%	4	3
96	8.8	8.7	50%	25%	4	4
168	9.4	9.5	25%	50%	4	4
All timepoints	8.9	9.4	50%	48.5%	38	33

The age, sex, and number of mice used in the quantitative pharmacology experiments are shown. In addition to the IDUA$^{-/-}$ mice injected with IDUA or LT-IDUA, there were four IDUA$^{-/-}$ mice injected with Lactated Ringer's and four heterozygotes as controls.

and p12-LT-IDUA groups.

The enzyme activity levels in the tissues and plasma are shown in Fig. 2. In the brain, all plasmid-treated groups had statistically significantly higher activity than the Lactated Ringer's group (Fig. 2A). However, all plasmid-treated groups had statistically significantly lower activity than heterozygotes in the brain. There were no statistically significant differences between the plasmid-treated groups in the brain, suggesting that none of the fusion IDUAs had improved delivery into the CNS than IDUA.

Two modified IDUAs had higher enzyme activity levels than IDUA in homogenized heart tissue (Fig. 2B). The p12-LT-IDUA group had higher activity than the IDUA group in homogenized heart tissues (mean: 70.18 vs. 31.50 nmol/h/mg, $p = 0.0481$) (Fig. 2B) compared to Lactaed Ringer's group. The LT-IDUA group had an unexpectedly significantly higher activity level than the unmodified IDUA in homogenized heart tissues (mean: 88.56 vs. 31.50 nmol/h/mg, $p = 0.0211$) (Fig. 2B).

The LT-IDUA group also had significantly higher activity levels than the unmodified IDUA in the plasma (mean: 5100 vs. 1369 nmol/h/mL, $p = 0.0092$) (Fig. 2C). Additionally, the LS-IDUA had significantly higher activity levels than the unmodified IDUA in the plasma (mean: 6635 vs. 1369 nmol/h/mL, $p = 0.0002$) (Fig. 2C). As these linkers had no known biological activity or function, they were not expected to affect iduronidase's activity levels.

The enzyme activity levels in the homogenized liver, spleen, kidneys, and lungs are shown in Fig. 2D through Fig. 2G. No statistically significant differences were seen between any of the fusion IDUAs and the unmodified IDUA in these tissues. Instead, the IDUAs containing the linkers tended to have higher activity levels than the fusion IDUAs containing pepcan-12. For example, LT-IDUA had significantly higher activity levels than p12-LT-IDUA in the spleen (mean: 441.80 vs. 196.00 nmol/h/mg, $p = 0.0275$) (Fig. 2E). Likewise, LT-IDUA had significantly higher activity levels than p12-LT-IDUA in the kidneys (mean: 110.70 vs. 47.43 nmol/h/mg, $p = 0.0251$) (Fig. 2F).

No supranormal levels were observed in the plasma nor in the homogenized heart, liver, spleen, kidneys and lungs for any mouse groups.

The LT-IDUA group were on average four weeks younger than the IDUA group (11.0 weeks vs. 14.9 weeks, respectively). A Pearson's correlation test was performed for plasmid-treated mice with the variables of age at injection and enzyme activity levels in the heart. There was no correlation between age at injection and enzyme activity levels in the heart ($r^2 = 0.01640$, $p = 0.4190$) (Fig. 3A). There was also no correlation between age at injection and enzyme activity levels in the plasma ($r^2 = -0.04050$, $p = 0.2013$) (Fig. 3B), consistent with previous studies on hydrodynamic injection [32,64,65].

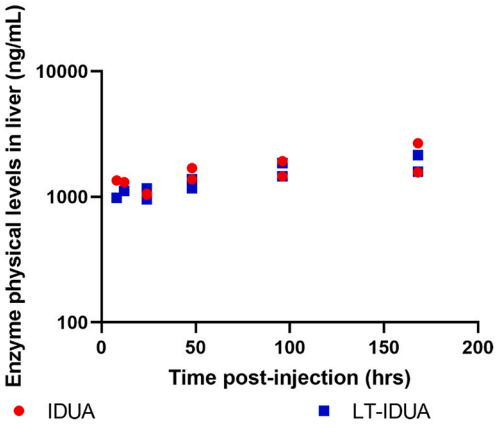

Fig. 4. Enzyme's physical levels in the liver over time.
Mice were hydrodynamically injected into the tail vein with 50 μg of plasmids encoding either IDUA or LT-IDUA. Enzyme physical levels in the liver were measured with ELISA at ten timepoints ranging from 0.5 to 168 h post-injection. Each data point represents one mouse. Data points from 8 to 168 h post-injection are shown in this figure, as time points earlier than 8 h yielded absorbance readings outside the standard curve.

3.2. Quantitative pharmacology comparison of IDUA and LT-IDUA: age and sex of mice

As LT-IDUA had higher activity levels than the unmodified IDUA in the homogenized heart and in the plasma, LT-IDUA was chosen for further characterization. LT-IDUA and IDUA were further compared in a series of quantitative pharmacology studies to characterize LT-IDUA's therapeutic potential and to study the mechanism of the LT peptide.

A total of 79 mice were utilized for the quantitative pharmacology studies, including the four mice injected with LR and four heterozygous controls. The age, sex, and number of plasmid-treated mice at each timepoint are shown in Table 2. Each timepoint contained three to four mice, and age and sex were generally balanced between groups.

3.3. Physical levels of enzymes over time in the liver

In a previous experiment, LT-IDUA had a higher enzyme activity level than IDUA in the liver (Fig. 2D). While this was not a statistically significant difference, levels in the liver were particularly important because the liver was the site of transgene expression. Therefore, the possibility of LT impacting transgene expression was further investigated. One potential way LT could increase transgene expression is if LT contained an alternative start codon, allowing translation of multiple new opening reading frames (ORFs). However, this did not seem likely, because two programs did not detect any ORFs that began in LT (National Center for Biotechnology Information (NCBI) ORF program; SnapGene) [66,67].

The possibility of LT impacting transgene expression was further investigated by measuring enzyme levels at multiple timepoints. Because enzyme activity assays cannot differentiate between the catalytic activity vs. physical levels of an enzyme, an ELISA was performed on liver samples from mice hydrodynamically injected with IDUA and LT-IDUA to quantify the iduronidase protein levels. As seen in Fig. 4, the physical levels of iduronidase and LT-iduronidase in the liver appeared similar from 8 to 168 h post-injection. The similar time-enzyme physical level profiles between IDUA and LT-IDUA suggested that LT does not increase transgene expression.

One common practice in pharmacokinetic studies of enzyme replacement therapies for lysosomal diseases is the conversion of

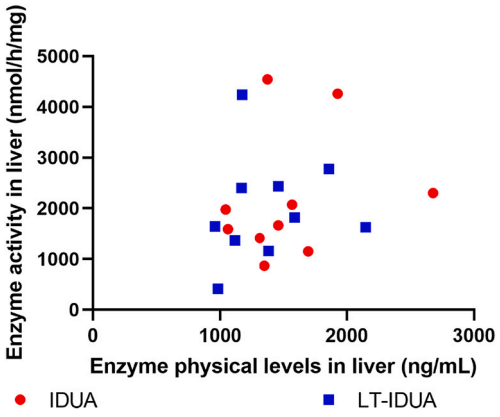

Fig. 5. No Relationship Between Enzyme's Physical Levels in the Liver and Enzyme's Activity Levels in the Liver.
Mice were hydrodynamically injected into the tail vein with 50 μg of plasmids encoding either IDUA or LT-IDUA. Enzyme levels in the liver were measured with enzyme-linked immunosorbent assay (ELISA) and an enzyme activity assay at timepoints ranging from 8 to 168 h post-injection. Each data point represents one mouse. In the IDUA group, there was no correlation between the enzyme's physical levels in the liver and the enzyme's activity levels in the liver (IDUA $r^2 = 0.05341$, $p = 0.5206$). Similarly, in the LT-IDUA group, there was no correlation between the enzyme's physical levels in the liver and the enzyme's activity levels in the liver (LT-IDUA $r^2 = 0.01666$, $p = 0.7223$).

enzyme activity levels to enzyme physical levels. This conversion allows enzyme levels to be expressed in the same units as the dose (e.g., mg of protein/mL), which is necessary for solving the majority of mathematical equations that underlie pharmacokinetics. Therefore, there was some curiosity as to whether enzyme activity levels correlated with enzyme physical levels in this study. Pearson's correlation tests were performed for the amount of IDUA protein and the catalytic activity in liver samples (Fig. 5). In the IDUA group, there was no correlation between enzyme's physical levels in the liver and its activity levels in the liver ($r^2 = 0.05341$, $p = 0.5206$) (Fig. 5, red data points). Similarly, in the LT-IDUA group, there was no correlation between enzyme's physical levels in the liver and its activity levels in the liver ($r^2 = 0.01666$, $p = 0.7223$, Fig. 5, blue data points).

3.4. Enzyme activity levels over time in the liver

Enzyme levels in the liver from mice hydrodynamically injected with IDUA and LT-IDUA were also quantified at multiple timepoints using an enzyme activity assay. As seen in Fig. 6, IDUA and LT-IDUA had similar time-enzyme activity levels in the liver. Both enzymes had no detectable activity levels until 4 h post-injection. At 48 h, both enzymes had a peak in enzyme activity levels, and their levels remained steady up to the last timepoint of 168 h. Enzyme activity levels in the liver were entered into WinNonLin, a PK computational program, and a NCA was performed to generate PK parameters (Table 3). The resultant PK parameters were similar between the two enzymes, further supporting the trend seen in the graph. The AUC of activity was similar between the two enzymes ($p = 0.6372$). The k_e and $t_{1/2}$ of activity could not be calculated as there were no negative slopes in LT-IDUA's or IDUA's time profile.

3.5. Enzyme activity levels over time in the plasma

In contrast to LT-IDUA and IDUA exhibiting similar time profiles in the liver, their profiles in the plasma exhibited marked differences. A graph of enzyme activity levels in the plasma over time is shown in

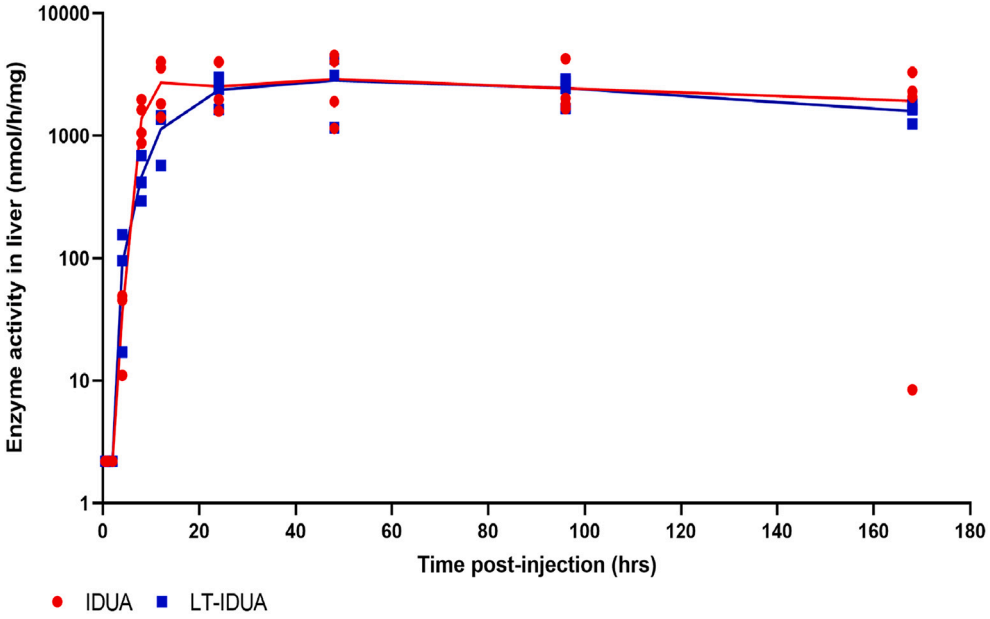

Fig. 6. Enzyme activity levels over time in liver.
Mice were hydrodynamically injected into the tail vein with 50 μg of plasmids encoding either IDUA or LT-IDUA, and enzyme activity levels were measured in the liver at ten timepoints ranging from 0.5 to 168 h post-injection. Each data point represents one mouse, and the line is the mean enzyme activity for a timepoint. Standard deviation bars are not shown in the figure because each timepoint had three to four mice per group. The lower limit of quantification for the noncompartmental analysis was set as 2.198 nmol/h/mg.

Table 3
Pharmacokinetics of enzyme activity levels in the liver.

Treatment	T_{max}(hr)	C_{max}(nmol/h/mg)	AUC_{0-168h}(hr*nmol/h/mg)	$Time_{above\ MEC}$(hr)	$AUC_{above\ MEC}$(hr*nmol/h/mg)
IDUA	48	2908.25	393,168.77	165.90	392,518.95
LT-IDUA	48	2835.88	360,217.10	165.96	359,567.22
			p = 0.6372		

Mice were hydrodynamically injected into the tail vein with 50 μg of plasmids encoding either IDUA or LT-IDUA, and enzyme levels were measured in the liver with an enzyme activity assay at ten timepoints ranging from 0.5 to 168 h *post-injection. Noncompartmental analysis was performed on enzyme activity levels in the liver.*

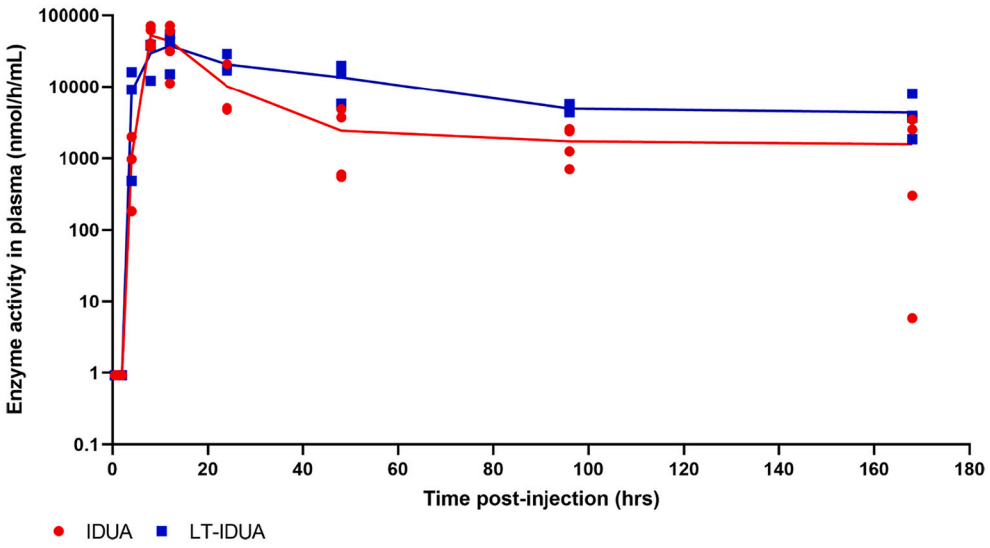

Fig. 7. Enzyme activity levels over time in plasma.
Mice were hydrodynamically injected into the tail vein with 50 μg of plasmids encoding either IDUA or LT-IDUA, and enzyme activity levels were measured in the plasma at ten timepoints ranging from 0.5 to 168 h post-injection. Each data point represents one mouse, and the line is the mean enzyme activity for a timepoint. Standard deviations are shown in Table 4. The lower limit of quantification for the noncompartmental analysis was set as 0.926 nmol/h/mL.

Table 4
Enzyme activity levels over time in the plasma (exact values).

	Plasmid			
	IDUA		LT-IDUA	
Time post-injection (hours)	Mean (nmol/h/mL)	SD	Mean (nmol/h/mL)	SD
0.5	0.93	0	0.93	0
1	0.93	0	0.93	0
2	0.93	0	0.93	0
4	1046.56	903.01	8581.39	7833.05
8	52,717.78	16,902.84	29,718.45	15,147.67
12	43,831.24	27,506.93	37,624.96	20,421.59
24	10,226.86	9274.89	20,645.78	5564.81
48	2423.87	2188.68	13,620.37	7142.28
96	1716.07	888.09	4941.72	583.67
168	1571.69	1686.65	4356.35	2602.53

Mice were hydrodynamically injected into the tail vein with 50 μg of plasmids encoding either IDUA or LT-IDUA, and enzyme activity levels were measured in the plasma at ten timepoints ranging from 0.5 to 168 h post-injection. The mean and standard deviation (SD) values of the enzyme activity levels in the plasma are reported. The lower limit of quantification for the noncompartmental analysis was set as 0.926 nmol/h/mL.

Fig. 7, and the exact values are shown in Table 4. The PK parameters generated from plasma enzyme activity levels are shown in Table 5.

Both enzymes had no detectable enzyme activity levels until 4 h. However, IDUA's activity peaked at 8 h, whereas LT-IDUA peaked at 12 h. IDUA also had a higher peak than LT-IDUA. Beginning at 12 h, a clear separation between the two enzymes' activity levels was seen. Between 12 and 48 h, LT-IDUA had a smaller decline (i.e., less negative slope) than IDUA. From 96 to 168 h, LT-IDUA and IDUA had similar declines (i.e., parallel slopes). When the $t_{1/2}$ of activity was calculated using timepoints from 24 to 168 h, the $t_{1/2}$ of activity were similar between the two enzymes (Table 5).

However, LT-IDUA had a two-fold higher AUC of activity than IDUA, which was a statistically significant difference ($p = 0.0034$) (Table 5). LT-IDUA also exhibited a two-fold higher $AUC_{above\ MEC}$ of activity than IDUA, whereas $Time_{above\ MEC}$ of activity were similar between the two enzymes. The minimum effective concentration (MEC) of activity was defined as the enzyme activity level in heterozygotes.

4. Discussion

This study is the first to report a six amino acid peptide that improves the activity of iduronidase in homogenized heart tissue of the MPS I knockout mice (*IDUA*$^{-/-}$). Compared to IDUA, LT-IDUA had significantly higher enzymatic activity in the heart ($p = 0.0211$). The 2-fold higher AUC in the plasma of LT-IDUA compared to IDUA ($p = 0.0034$) suggest LT-IDUA may have a lower dose requirement than IDUA. Current challenges in manufacturing gene therapy include difficulties manufacturing adequate amounts of gene therapy product and this in turn can delay clinical trials [68]. Thus new methods to improve manufacturing scalability are needed and a lower gene therapy dose requirement would be beneficial towards this end. A lower gene therapy dose requirement may also mitigate risk of immunogenicity, as T-cell responses to adeno-associated virus (AAV) appear to be dose-dependent. It is important to note that estimations of AUC of activity may change at different doses and/or different gene delivery systems. Therefore, more information is needed on whether LT-IDUA retains a higher AUC of activity than IDUA when delivered by a long-term vector like AAV and at different doses. Additional studies with LT-IDUA would provide information on its long-term safety and efficacy, including its impact on glycosaminoglycan levels and cardiovascular function.

Table 5
Pharmacokinetics of Enzyme Activity Levels in the Plasma.

Treatment	T_{max}(hr)	C_{max}(nmol/h/mL)	k_e(1/h)	$t_{1/2}$(hr)	$AUC_{0–168h}$(hr*nmol/h/mL)	$AUC_{0-\approx}$(hr*nmol/h/mL)	$Time_{above\ MEC}$(hr)	$AUC_{above\ MEC}$(hr*nmol/h/mL)
IDUA	8	52,717.78	0.0105	66.03	995,551.23	1,145,347.65	165.99	994,537.88
LT-IDUA	12	37,624.96	0.0110	63.10	1,760,909.40 p = 0.0034	2,157,474.36	166.00	1,759,896.02

Mice were hydrodynamically injected into the tail vein with 50 μg of plasmids encoding either IDUA or LT-IDUA, and enzyme activity levels were measured in the plasma at ten timepoints ranging from 0.5 to 168 h post-injection. Noncompartmental analysis was performed on enzyme activity levels in the plasma. The k_e, $t_{1/2}$, and $AUC_{0-\approx}$ reported in this table used the timepoints from 24 to 168 h. When only the 96 and 168 h were used, the following parameters were generated for IDUA and LT-IDUA, respectively: (k_e 0.00122 1/h vs 0.00175 1/h), ($t_{1/2}$ 567.83 h vs 395.83 h), ($AUC_{0-\approx}$ 2,283,093.03 vs 4,248,687.12 h*nmol/h/mL).

Table 6
Biological processes that would increase AUC of plasma enzyme activity levels and their plausibility as LT peptide's mechanism.

Biological Process/ Mechanism	Supporting or Refuting Evidence as LT's Mechanism	Plausibility for LT's Mechanism
↑ enzyme production in liver (e.g., transgene expression)	LT-IDUA and IDUA had similar enzyme physical levels in liver (Fig. 4). Alternative start codons or alternative open reading frames were not detected in LT-IDUA.	Unlikely
↑ enzyme activity in liver (e.g., post-translational modification in hepatocytes)	LT-IDUA and IDUA had similar enzyme activity levels in liver (Fig. 2D, Fig. 6)	Unlikely
↑ distribution of enzyme from the liver to plasma	Uncertain. Cannot be ruled out with the currently available data.	Possible
↑ enzyme activity in plasma	LT-IDUA and IDUA had similar enzyme activity levels in liver (Fig. 2D, Fig. 6). In contrast, LT-IDUA and IDUA had different enzyme activity levels in the plasma (Fig. 2C, Fig. 7). A difference in the plasma, but not the liver, suggests that there is some new process occurring in the plasma, but the causative biological process is unknown. One theoretical possibility might be a change in protein folding, possibly due to the blood's pH. However, this would have to offset the decrease in enzyme activity caused by the blood's high pH relative to the lysosome's low pH.	Uncertain. This explanation/mechanism is consistent with the results, but the biological process that would cause this is unknown.
↓ enzyme uptake into organs	LT-IDUA's enzyme activity levels were similar or greater than IDUA in tissues (Fig. 2A through Fig. 2G).	Unlikely
↓ elimination and/or ↓ inactivation of enzyme in plasma (e.g., stabilization of activity)	The time-enzyme activity profile of LT-IDUA and IDUA showed different slopes between enzymes in the plasma (Fig. 7). Decreased inactivation would be seen as sustained activity of LT-IDUA in the plasma (e.g., less negative slope). Fig. 7 shows a biphasic profile in both enzymes. That is, between 12 and 48 h, LT-IDUA had a smaller decline (less negative slope) than IDUA. Between 96 and 168 h, LT-IDUA and IDUA had similar declines (parallel slopes). Decreased elimination should be reflected as a higher $t_{1/2}$ of activity. However, enzymes had similar calculated $t_{1/2}$ of activity in this study because noncompartmental analysis was performed for a biphasic profile and later timepoints are needed to capture the terminal elimination phase.	Likely

Although none of the modified IDUAs had higher enzyme activity levels in the homogenized brain compared to the unmodified IDUA, the modified IDUA containing only Linker T had higher activity levels in the heart and plasma. Considering that LT is a six amino acid peptide primarily containing glycine, LT was not expected to affect the catalytic activity, much less cause an increase in activity. While a modified enzyme containing only the LT peptide has not been investigated prior to this study, LT has been included in a fusion enzyme. In a study by Grubb et al., the LT sequence was used to join the lysosomal enzyme β-glucuronidase and an IgG Fc region [69]. When plasmids encoding either this fusion β-glucuronidase or an unmodified enzyme were administered, a 50% decrease in the enzyme activity was seen at approximately 36 min vs. 1.7 min post-injection, respectively [69]. Furthermore, fusion β-glucuronidase activity levels were higher than the unmodified enzyme in several tissues of prenatally treated pups [69]. While these results can be attributed to the Fc region, it is also possible that LT is playing some role. However, it is difficult to distinguish the effects of Fc vs. LT because a β-glucuronidase containing the LT sequence without the Fc region was not tested.

Several potential mechanisms of LT were considered in this study. Database searches did not detect alternative start codons or alternative open reading frames on LT-IDUA [66,67]. Likewise, database searches did not detect any differences in protein domains/motifs or signal sequences between LT-IDUA and unmodified IDUA [70–75]. Of note, LT's sequence is similar to linkers found in native proteins. In a review of naturally occurring linkers by Argos, "pentapeptides with only Gly, Ser and Thr would make the best general linkers as these residues occur most often in and are strongly preferred by natural linkers" [76]. Table 6 discusses LT's potential mechanism in further detail and how it was deduced using quantitative pharmacology approaches.

As database and literature searches yielded little information on LT's mechanism, the higher enzyme activity levels of LT-IDUA in the heart was then hypothesized to be caused by a longer $t_{1/2}$ in the plasma. This hypothesis was supported by LT-IDUA having a higher enzyme activity level than the unmodified IDUA in the plasma at 48 h (Fig. 2C). Previous studies have suggested that prolonging a lysosomal enzyme's "half-life" in the plasma can improve its uptake into tissues [69,77,78]. In these studies, $t_{1/2}$ was estimated directly with enzyme activity levels (i.e., enzyme activity levels were not converted to enzyme physical levels) [69,77,78]. In these studies, most graphs of enzyme activity levels over time had different slopes that would have corresponded to different $t_{1/2}$ of activities [69,77,78]. However, it is unclear how the $t_{1/2}$ was calculated in these studies. That is, there is no mention of conversion factors, equations, or PK computational programs, and a 50% decrease in activity was not seen in some graphs [69,77,78].

Half-life, and other parameters of how a therapy changes in the body over time, are routinely quantified in the field of pharmacokinetics. PK methodologies use statistics and computational modeling that allow rigorous and reproducible estimations of parameters, and PK studies have played important roles in drug development. However, PK methodologies assess therapies with respect to the dosage form. Since the modified iduronidase was delivered as a plasmid (i.e., DNA), the conventional PK approach would be to perform a PK analysis on plasmid levels and couple this to a PD model of enzyme activity levels. However, as stated in a guidance by the Food and Drug Administration (FDA), "traditional PK study designs are generally not feasible for [cell and gene therapy] products" in pre-clinical studies [64]. One challenge is that gene therapy studies use several different units, whereas PK analysis requires uniform or convertible units [79]. Another major challenge is that the fundamental PK concepts of absorption, distribution, metabolism, and elimination (ADME), which are well-characterized for small molecular drugs, have yet to be adapted for gene therapy [79].

In an attempt to address these issues, this study explored a novel analytical approach by directly analyzing enzyme activity levels, measured after gene delivery, using a computational model that is traditionally reserved for PK. This approach differs from other studies

that directly estimate $t_{1/2}$ using enzyme activity levels [69,77,78,80], by using a PK computational program, which lends rigor and reproducibility found in traditional PK methodologies. This study's approach also differs from previous PK studies for gene therapy by analyzing enzyme activity levels rather than DNA levels [79]. As a result, the estimated parameters were interpreted by integrating concepts from PK, molecular biology, gene therapy, and lysosomal diseases.

In this study, LT-IDUA had a two-fold higher AUC of plasma activity than the unmodified IDUA. In traditional PK, the AUC represents the body's total exposure to the drug. Therefore, the AUC of activity in the plasma may reflect the receptors' exposure to functional enzyme. For lysosomal enzymes, this may include the mannose 6-phosphate receptor, which plays a well-known role in transporting enzymes from the circulatory system and into tissues. While it may be tempting to equate a higher AUC of plasma activity to an increase in efficacy, the AUC generated from this PK approach encompasses multiple biological processes and must be carefully considered in the context of other data. These biological processes and their possibilities of occurring in LT-IDUA are discussed in Table 6.

5. Conclusion

In summary, the addition of a six amino acid peptide, termed LT, improved the catalytic activity of iduronidase in heart tissue and plasma of MPS I mice. This modified LT-IDUA was further characterized using a noncompartmental pharmacokinetic approach for gene therapy that suggests LT-IDUA may have a lower dose requirement than IDUA. Future studies with LT-IDUA delivered in a long-term expression vector, such as AAV, and at different doses, would provide more information on its therapeutic potential, including its impact on gene therapy dosing requirements and impact on cardiovascular function and biomarkers such as glycosaminoglycans.

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Author statement

None.

Data availability

The data that has been used is confidential.

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