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Enzyme replacement with transferrin receptor-targeted α-L-iduronidase rescues brain pathology in mucopolysaccharidosis I mice

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Mucopolysaccharidosis I (MPS I), a lysosomal storage disease caused by dysfunction of α-L-iduronidase (IDUA), is characterized by the deposition of dermatan sulfate (DS) and heparan sulfate (HS) throughout the body, which causes several somatic and central nervous symptoms. Although enzyme-replacement therapy (ERT) is currently available to treat MPS I, it does not alleviate central nervous disorders, as it cannot penetrate the blood-brain barrier. Here we evaluate the brain delivery, efficacy, and safety of JR-171, a fusion protein comprising humanized anti-human transferrin receptor antibody Fab and IDUA, using monkeys and MPS I mice. Intravenously administered JR-171 was distributed in major organs, including the brain, and reduced DS and HS concentrations in the central nervous system and peripheral tissues. JR-171 exerted similar effects on peripheral disorders similar to conventional ERT and further reversed brain pathology in MPS I mice. We found that JR-171 improved spatial learning ability, which was seen to deteriorate in the vehicle-treated mice. Further, no safety concerns were noted in repeat-dose toxicity studies in monkeys. This study provides nonclinical evidence that JR-171 might potentially prevent and even improve disease conditions in patients with neuronopathic MPS I without serious safety concerns.

INTRODUCTION

Mucopolysaccharidosis type I (MPS I) is a rare lysosomal storage disease caused by mutations of the gene encoding α-L-iduronidase (IDUA), a lysosomal enzyme that degrades glycosaminoglycans (GAGs), including dermatan sulfate (DS) and heparan sulfate (HS).[1,2] Dysfunction of this enzyme causes accumulation of these substrates throughout the body, resulting in various disorders, including skeletal deformities, cardiomyopathy, respiratory impairment, and central nervous system (CNS) anomalies.[1,2] The disease severity in MPS I varies widely. The most severe form is called Hurler syndrome, includes intellectual disability and the above-mentioned somatic manifestations, and death within the first decade of life. In the mildest form, Scheie syndrome, patients do not exhibit intellectual disability and have a normal life expectancy. Patients suffering from intermediate forms of MPS I, known as Hurler/Scheie syndrome, show high clinical variability depending on the different IDUA gene mutations.[1,2]

Currently, the approved treatments for MPS I include allogeneic hematopoietic stem cell transplantation (HSCT) and enzyme-replacement therapy (ERT).[3] HSCT, considered the first-choice treatment for Hurler syndrome, mitigates multiorgan morbidity and increases the patient's life span if initiated before the age of 2 years.[4–7] Critical obstacles to HSCT include difficulties in finding HLA-matched donors, failure of the graft, and graft-versus-host disease.[8] As for ERT, a recombinant human IDUA (rhIDUA, laronidase: ALDURAZYME, Sanofi Genzyme), available for MPS I, decreases urinary GAG levels, reduces hepatosplenomegaly, improves upper airway restriction and physical performance, and eases left ventricular hypertrophy.[9] However, it does not treat CNS manifestations because the blood-brain barrier (BBB) limits the delivery of the drug to the brain.

Lepunafusp alfa (JR-171), a fusion protein consisting of humanized anti-human transferrin receptor (hTfR) antibody Fab and IDUA, was designed to facilitate cellular uptake and BBB penetration by binding to hTfR.[10,11] The utilization of TfR-mediated transcytosis to cross the BBB for brain delivery of large molecule biologics was originally proposed and verified by Pardridge and colleagues.[12–17] We recently developed another BBB-penetrating fusion protein targeting TfR designated pabinafusp alfa (JR-141), which has been approved and marketed in Japan for treating all forms of MPS II, including neuronopathic.[18–23] Here, we conducted a nonclinical evaluation of JR-171 using a murine MPS I model and cynomolgus monkeys to elucidate its pharmacological effects on substrate deposition, histopathological changes, and neurobehavioral abnormality, as well as biodistribution, pharmacokinetics, and safety.

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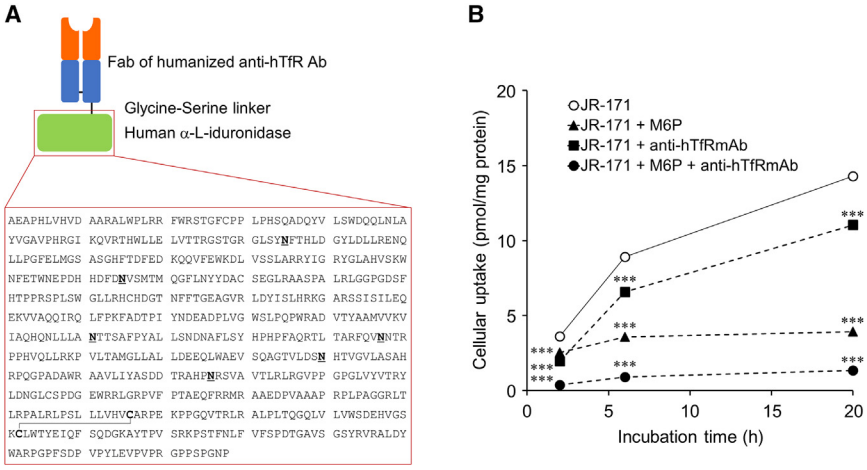


Figure 1. Molecular structure and receptor-targeting property of JR-171

(A) Depicted molecular structure of JR-171. JR-171 is a recombinant fusion protein consisting of two subunits: a V_H-C_H1 heavy chain of a humanized anti-human transferrin receptor antibody C-terminally fused to IDUA via a glycine-serine linker and the light chain of the same antibody. Its molecular weight is calculated to be 119,204 Da based on its amino acid composition ($C_{5337}H_{8209}N_{1479}O_{1578}S_{27}$). The amino acid sequence of the IDUA moiety is shown in the box. N, sugar chain binding asparagine; C-C, disulfide bond. (B) Receptor-mediated cellular uptake of JR-171 into human fibroblasts. JR-171 (160 nmol/L) was incubated with MRC-5 cells with or without M6P (10 mM) and/or the humanized anti-hTfR monoclonal antibody (400 μg/mL). Intracellular concentration of JR-171 was calculated from enzyme activity using an artificial substrate 4-MU-α-Ido. Endogenous enzyme activity was negligible in this assay. The values are represented as the mean ± SEM (n = 3). ***p < 0.001 (versus JR-171 alone, Dunnett's test).

RESULTS

Preparation and receptor-mediated cellular uptake of JR-171

JR-171 is a human IDUA fused to the Fab of a humanized anti-hTfR monoclonal antibody produced in Chinese hamster ovary (CHO) cells introduced with cDNA encoding IDUA fused with the V_H and C_H1 of a humanized antibody that specifically recognizes hTfR, and a cDNA encoding the V_L and C_k of the humanized antibody. The humanized variable region consists of complementarity-determining region amino acids from an anti-hTfR mouse antibody and framework residues from the human γ1 chain and κ chain V regions.[10,11] The resulting recombinant protein consists of two subunits, human IDUA fused with the V_H and C_H1 (869 amino acids) and the humanized antibody light chain (219 amino acids). The enzyme moiety has six N-binding sugar chains, and its amino acid sequence is identical to that of the clinically available agent laronidase (Figure 1A). JR-171 can bind to hTfR and cation-independent mannose-6-phosphate receptor (M6PR) with the K_D values of 1.88×10^{-10} M and 12.1×10^{-10} M, respectively.

Next, we determined if JR-171 undergoes receptor-mediated cellular uptake via TfR and M6PR, using human fetal lung fibroblasts (MRC-5). We observed time-dependent incorporation of JR-171 (160 nmol/L) into MRC-5 cells (Figure 1B), which was significantly diminished in the presence of excess anti-hTfR monoclonal antibody (400 μg/mL) or M6P (10 mM) (Figure 1B). This indicates that both TfR and M6PR facilitate JR-171 uptake into the cells, which was reduced by the simultaneous addition of M6P and anti-hTfR monoclonal antibody (Figure 1B).

Pharmacokinetics and brain distribution of JR-171

As the anti-hTfR antibody used in JR-171 does not recognize mouse TfR, we conducted *in vivo* studies to examine the pharmacokinetics and pharmacodynamics in *Idua*-knockout (KO) mice (Figure S1) expressing hTfR (hTfR-KI/*Idua*-KO mice) as a murine model of MPS I. We intravenously injected a single dose of JR-171 or rhIDUA (laronidase) at 2 mg/kg to the MPS I mice and determined the drug concentrations in the plasma, liver, heart, spleen, and brain. The plasma drug concentrations in the JR-171 group were higher than those in rhIDUA group at all time points, probably due to lower distribution of JR-171 in the liver (Figure 2A). The area under the curve ($AUC_{0\text{-inf}}$) values of plasma drug concentrations after JR-171 and rhIDUA administration were 11.1 μg h/mL and 5.35 μg h/mL, respectively. The drug concentrations in the heart and spleen in the JR-171 group were higher than those in the rhIDUA group (Figure 2A). JR-171, but not rhIDUA, was detected in the brain of MPS I mice (Figure 2A), which was confirmed by the presence of JR-171 in the neurons in the brain, as seen using immunohistochemistry (Figure 2B). Moreover, after intravenous administration of radiolabeled JR-171 (^{125}I-JR-171, 2.5 MBq/kg) to cynomolgus monkeys, radioactivity was detected in the CNS tissues, including the cerebrum, cerebellum, and spinal cord (Figure S2). These results indicate that intravenous JR-171 crosses the BBB to reach the brain parenchyma.

Duration of efficacy after a single intravenous dose of JR-171

To investigate the duration of drug efficacy, we sequentially measured the IDUA enzyme activity and HS concentration after a single intravenous administration of JR-171 at 1 mg/kg to MPS I mice. The enzyme activity in the brain of JR-171-treated MPS I mice was close to that of wild-type (WT) mice (C57BL/6) 1 day after the administration and gradually decreased after that (Figure 3A). The activity remained at approximately 50% of the WT level 7 days after JR-171 administration, while rhIDUA administration at its clinical dose of 0.58 mg/kg (comparable to 1 mg/kg of JR-171 in terms of molar enzyme content) did not increase the brain enzyme activity in MPS I mice (Figure 3A). In the liver, JR-171 markedly enhanced the

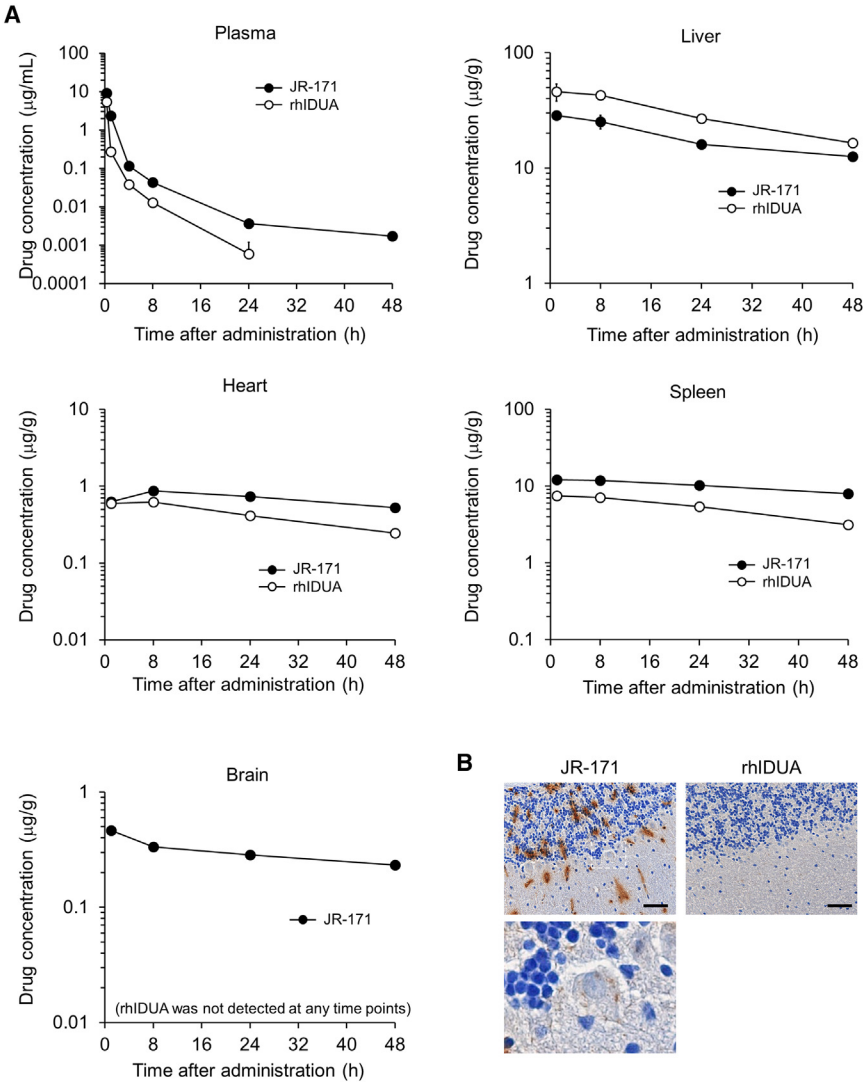


Figure 2. Pharmacokinetic and biodistribution of JR-171 and rhIDUA in MPS I mice

(A) Drug concentration profiles of JR-171 (2 mg/kg) or rhIDUA (2 mg/kg) administered intravenously to hTfR-KI/*Idua*-KO (MPS I) mice. The values are represented as the mean ± SEM for each group (n = 4). (B) Brain distribution of JR-171 (4 mg/kg) or rhIDUA (4 mg/kg) administered to the mice using cerebellum sections stained with an anti-IDUA antibody. The bottom panel is a high-power view of the white dashed rectangle shown in the upper panel. Dotted signals are observed in Purkinje neurons. Scale bars, 50 μm.

enzyme activity on day 1 after administration that remained higher than WT for up to 7 days later (Figure 3B). rhIDUA-treated mice also showed elevated liver enzyme activity (Figure 3B). Brain HS concentrations were lower in JR-171-treated MPS I mice than in untreated mice at all sampling points through 28 days, though the concentrations were not different between untreated and rhIDUA-treated mice (Figure 3C). Liver HS levels of MPS I mice were close to those of WT mice by treatment either with JR-171 or rhIDUA (Figure 3D). These results confirm the brain delivery of the active enzyme upon intravenous administration of JR-171 to MPS I. We also suggest that the maximum efficacy of JR-171, in terms of substrate reduction in both peripheral tissue and the brain, is attained when administered at weekly to every other week intervals.

The substrate-lowering ability of JR-171

Next, we examined the dose-dependent effect of JR-171 on substrate accumulation in MPS I mice. JR-171 was intravenously administered to the mice for 12 weeks, once every week (EW) or once every other week (EOW), based on the data obtained from the above-mentioned experiments. The HS and DS concentrations in the peripheral (serum, liver, heart, and spleen) and CNS (brain and cerebrospinal fluid [CSF]) tissues were measured 1 week (for EW groups) or 2 weeks (for EOW groups) after the last dose. These concentrations were significantly elevated in all tissues of the disease mice compared with WT mice (hTfR-KI/*Idua*$^{+/+}$) (Figure 4). JR-171 dose-dependently decreased HS and DS concentrations at 1, 2, and 4 mg/kg in all peripheral tissues, and the EW dosing regimen was slightly more effective than the EOW one (Figures 4A–4D). In the peripheral tissues, the substrate-reducing effect of JR-171 at 1 mg/kg/wk was similar to or greater than that of rhIDUA (0.58 mg/kg/wk) (Figures 4A–4D). Importantly, although rhIDUA exerted only a limited effect on the brain and CSF, JR-171 markedly decreased HS and DS concentrations at the equivalent molar base dose to rhIDUA and nearly normalized the levels at maximum in these tissues (Figures 4E and 4F).

Effect of JR-171 on pathological changes in peripheral tissues

We examined the peripheral tissue pathology using MPS I mice in a 12-week repeat-dose study. JR-171 was intravenously administered to the mice at 1, 2, and 4 mg/kg/wk, using rhIDUA (0.58 mg/kg/wk) as a control. Vacuolation and/or swelling of cells, likely due to accumulation of storage materials, was a characteristic finding in various peripheral tissues of vehicle-treated MPS I mice, which were normalized in most tissues by either JR-171 or rhIDUA (Figure S3). Regarding the aortic wall of the heart, rhIDUA at its clinical dose of 0.58 mg/kg and JR-171 at 1 mg (the equivalent molar base dose to rhIDUA) similarly and partially suppressed vacuolation and swelling of interstitial and smooth muscle cells. JR-171 dose-dependently suppressed the changes and normalized at 4 mg/kg (Figures 5A and S3). Additionally, JR-171 dose-dependently decreased the liver weight (Figure 5B).

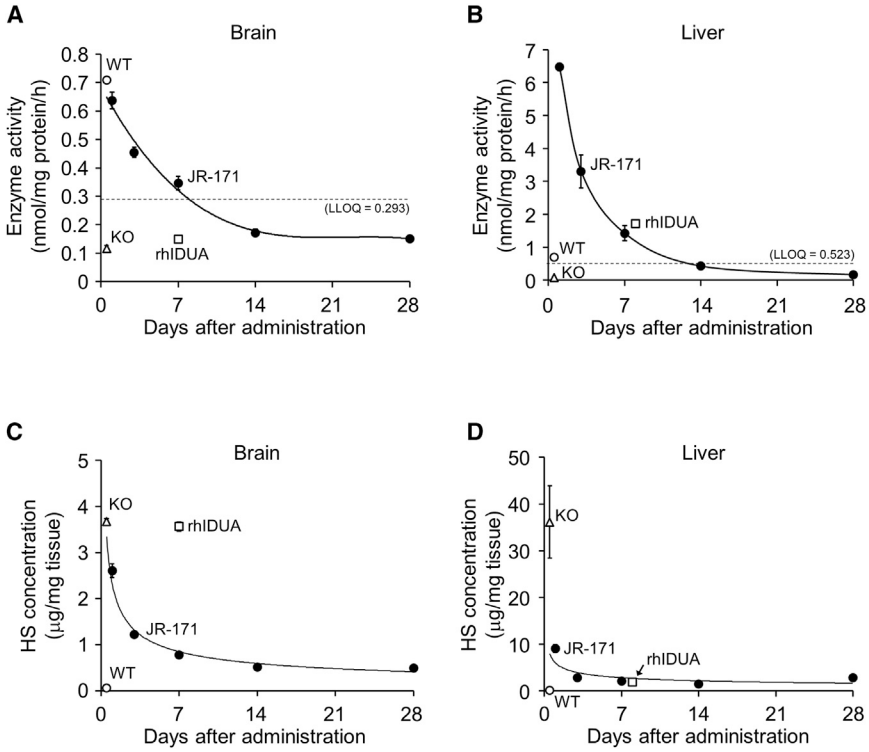


Figure 3. Enzyme activity and HS concentration in the brain and liver after intravenous administration of JR-171 to MPS I mice

MPS I mice were intravenously injected with JR-171 (1 mg/kg) or rhIDUA (0.58 mg/kg). The amount of injected IDUA enzyme is almost equivalent at molar basis. IDUA enzyme activity in the brain (A) and liver (B), and HS concentrations in the brain (C) and liver (D) are shown. Enzyme activity in KO mice was below the quantification limit. Data represent the means ± SEM (n = 3 at each time point). WT, wild-type mice; KO, untreated hTfR-KI/*Idua*-KO (MPS I) mice; LLOQ, the lower limit of quantification.

Effects of JR-171 on pathological changes in the brain

We investigated and compared the long-term effects of JR-171 (1 mg/kg/wk) and rhIDUA (0.58 mg/kg/wk) on brain pathology in a 31-week treatment schedule using MPS I mice. At 11 weeks of age, when the treatment was started (baseline), cells strongly positive for Lamp1, a hallmark of lysosomal storage and hypertrophy, were already increased in MPS I mouse brains (cerebral cortex, hippocampus, diencephalon, mid brain, medulla oblongata, and cerebellum) (Figures 6 and S4). GFAP-positive activated astrocytes and Iba1-positive vacuolated microglial cells were frequently detected in most brain regions in 11-week-old MPS I mice (Figures 6, S5, and S6), indicating neuroinflammation before treatment was started. Moreover, neural cell degeneration manifested as vacuolation or swelling, which are considered as the signs of excessive lysosomal accumulation of undigested materials, was also observed throughout the brains of MPS I mice at baseline (Figures 6 and S7). After 31 weeks of treatment, these histopathological changes still existed or worsened in vehicle-treated and rhIDUA-treated MPS I mice (Figure 6). Intriguingly, JR-171-treated MPS I mice showed no pathological changes and near-normal HS concentrations in the brain (Figures 6 and S8).

Maintenance of spatial learning ability by JR-171

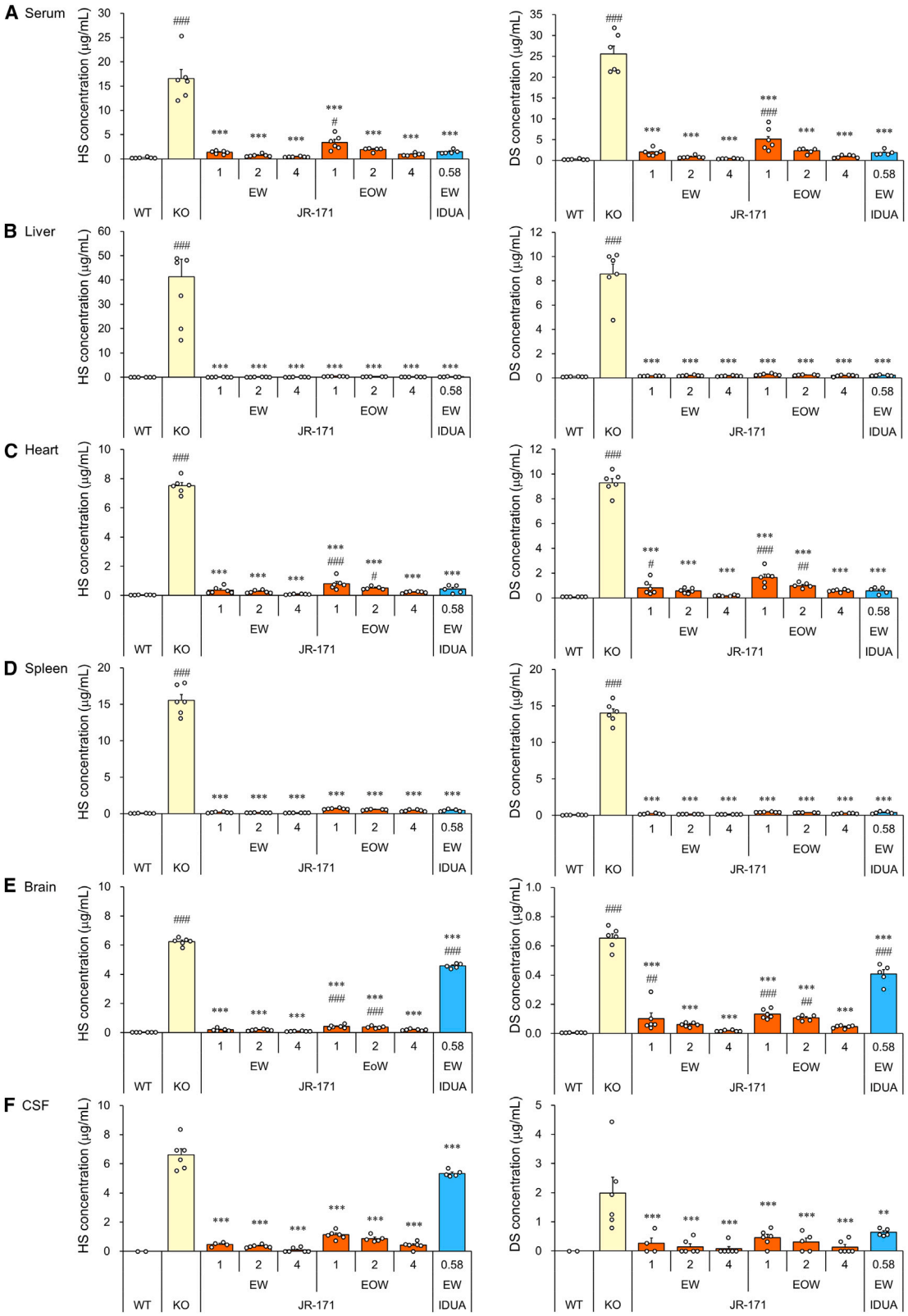
We performed the Morris water maze test to assess spatial learning ability after a 24-week treatment of the mice with JR-171 at 1 mg/kg/wk. The time to reach the platform (goal latency) tended to be longer in the MPS I model mice than in WT mice (hTfR-KI/*Idua*$^{+/+}$) on the first trial day. While the goal latency was not reduced after repeated trials in vehicle-treated or rhIDUA-treated MPS I mice, the long-term treatment with JR-171 significantly shortened the latency after a 3-day trial to a level similar to that of WT (Figure 7A). Additionally, JR-171 tended to improve the performance in probe trials (the time spent in the target quadrant, the number of platform crossings, and the moved distance). However, these differences were not statistically significant due to large parameter variations and small sample sizes (Figures 7B–7D). These results indicate that JR-171 can preserve the spatial learning ability of MPS I mice to a level comparable to that of WT mice.

Safety evaluation of JR-171

We conducted three toxicity studies of JR-171 using sexually mature and juvenile cynomolgus monkeys using weekly doses at 10, 20, and 40 mg/kg for 13 weeks (for mature only) or 4 weeks (for both mature and juvenile). We did not observe any test article-related changes in clinical signs, general behavior, body weight, food consumption, ophthalmology, urinalysis, hematology, blood chemistry, necropsy, organ weight, or histopathology (Tables S1–S33). Additionally, no adverse effects were observed on the CNS, cardiovascular, or respiratory systems (Tables S1–S33). As a result, no animals died or became moribund at any dose. At the end of the dosing period in the 13-week study, all JR-171-treated animals were positive for anti-drug antibody (ADA). Some animals were positive for neutralizing antibody (Nab) against TfR, while all were positive for Nab against M6PR (Table S34). There was no clear correlation between the dose and ADA titer, and no sex-related difference was observed in the ADA analysis. The production of ADA did not induce any toxic reactions. Accordingly, these studies estimated the no-observed-adverse-effect level (NOAEL) as 40 mg/kg.

DISCUSSION

Here, we have shown that JR-171, a fusion protein consisting of human IDUA and a Fab targeting hTfR designed for penetrating the BBB, was distributed in the CNS and peripheral tissues after intravenous administration to monkeys and MPS I mice. The fusion protein



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degraded the IDUA substrates, DS and HS, deposited throughout the body, including the brain, recovered neuroinflammation and neurodegeneration, and improved neurocognitive performance in MPS I mice. This nonclinical study showed a proof-of-concept for developing a novel therapy for neuronopathic MPS I.

The basic concept of BBB penetration of large molecule drugs using receptor-mediated transcytosis was proposed and verified by Pardridge and colleagues.[12–17] Although the BBB restricts molecular and cellular transport from the bloodstream into the brain parenchyma, several proteins, including leptin, insulin, and transferrin, penetrate the BBB by binding to their specific receptors on the surface of the capillary endothelial cells in the brain.[24–26] Anti-insulin receptor antibody and anti-TfR antibody can be used as a carrier/vehicle of drugs to be delivered to the brain.[15,16,27,28] We developed the anti-TfR antibody-based technology to enable BBB penetration of CNS-targeting drugs.[18] Pabinafusp alfa (JR-141), which uses the first generation of this technology, is currently available for clinical use for treating patients with all, including neuronopathic, types of MPS II in Japan. JR-171 is a second-generation molecule, in which only the Fab domain of the antibody is used as the TfR binding portion. Removing the Fc domain from the antibody can reduce its size and avoid the potential risk of cytotoxic effector function of antibodies mediated by the Fc region, which might enable efficient distribution of the drug to target organs and reduce safety concerns.

Lysosomal enzymes are stable and active under acidic lysosomal conditions.[29] Consistent with this, IDUA enzyme activity in the brain and liver was maintained higher than the detectable levels at least 7 days after administration of JR-171 to MPS I mice, resulting in a long-lasting (at least for 28 days) reduction of HS concentration in these tissues. These findings might indicate that monthly dosing frequency could be applied to maintain decreased substrate levels, but more frequent administration regimens can additionally restore the substrate concentrations to near-normal levels, as shown in the 12-week repeat-dose study. Accordingly, our nonclinical results using MPS I mice support a weekly or EOW dosing frequency for the drug in human patients. Concerning the dose, the clinically approved amount of rhIDUA (laronidase) is 0.58 mg/kg, corresponding to approximately 1 mg/kg of JR-171 at a molar basis (molecular weights of JR-171 and rhIDUA are 119,204 and 70,106, respectively, based on their amino acid composition). We showed that JR-171 at 1 mg/kg/wk reduced DS and HS concentrations in the peripheral tissues almost as efficiently as rhIDUA at 0.58 mg/kg/wk. Moreover, only JR-171 reduced the substrate concentrations in the CNS. Based on these results, intravenous JR-171 might benefit somatic and CNS disorders in MPS I patients at a dose of 1 mg/kg. However, a higher dose tended to be more effective against substrate accumulation in MPS I mice. Its effect on the histopathological changes of the aortic wall of the heart showed clear dose dependency, maximized at 4 mg/kg. Therefore, appropriate clinical dose setting should be determined based on the results from human studies considering the balance between efficacy and safety.

The initial pathogenic event of MPS I is DS and HS deposition throughout the body. The pathogenic roles of DS and HS may vary across organs.[30,31] In the brain, HS deposition in neurons and glial cells induces neuroinflammation and neurodegeneration,[32,33] triggering CNS manifestations and progressive deterioration of disease conditions.[34] Thus, clearance of HS from the brain at early disease stage might prevent the initiation of this pathogenic cascade.[20,31] In fact, the removal of deposited HS by JR-171 suppressed pathological changes in the brain and further prevented the loss of spatial learning ability in the Morris water maze test. Notably, in the 31-week treatment study, neuroinflammation and neurodegeneration were already present at the baseline (11 weeks of age), indicating that the pathogenic cascade was initiated before starting the treatment. Even under these conditions, the normalization of HS concentration by JR-171 reversed the brain pathology preventing the onset of CNS symptoms. Moreover, ongoing neurological symptoms might also be reversed, at least partially, by normalizing HS concentration in the brain before irreversible damage to neurons occurs.[20] Further elucidation of this concept is necessary for future studies.

Based on our findings, the brain HS level can most likely reflect the CNS effect of JR-171 in MPS I model mice and in patients with MPS I. However, it is impractical to perform brain biopsy to measure HS concentration in the patients. In this regard, we previously found that HS concentration in the brain correlated well with that in the CSF in MPS II mice treated with pabinafusp alfa.[20,35] A correlation between the HS concentrations in the brain and CSF was also observed in MPS I mice treated with JR-171 (Figure S9), suggesting that CSF HS levels might be an alternative marker for brain HS levels, which reflect the CNS drug efficacy of JR-171 in MPS I.

Since JR-171 binds to TfR, *in vivo* administration might perturb iron metabolism to induce anemia. We found that JR-171 only partially suppressed the interaction between transferrin and TfR and was mainly preserved even in excess of JR-171 *in vitro* (unpublished observation). More importantly, toxicity studies using cynomolgus monkeys revealed that JR-171 did not cause any change suggestive of anemia in hematology, blood chemistry, or histopathology assessments after repeated administration for up to 13 weeks at 40 mg/kg.

Figure 4. Substrate-lowering efficacy of JR-171 in MPS I mice

Graphs showing HS (right) and DS (left) concentrations in the serum (A), liver (B), heart (C), spleen (D), brain (E), and (F) CSF. JR-171 or rhIDUA was administered intravenously to 11-week-old MPS I mice once every week (EW) or once every other week (EOW) for 12 weeks at doses indicated on the horizontal axis of each graph (in mg/kg). Tissues were collected 1 week (for EW groups) or 2 weeks (for EOW groups) after the last dose. Values are represented as the mean with SEM bar for each group (n = 5–6). Individual values are also plotted. $^{###}p < 0.001$; $^{##}p < 0.01$; $^{#}p < 0.05$ (versus WT, unpaired t test), $^{***}p < 0.001$; $^{**}p < 0.01$ (versus KO, Dunnett's test). WT, wild-type mice; KO, untreated hTfR-KI/*Idua*-KO (MPS I) mice.

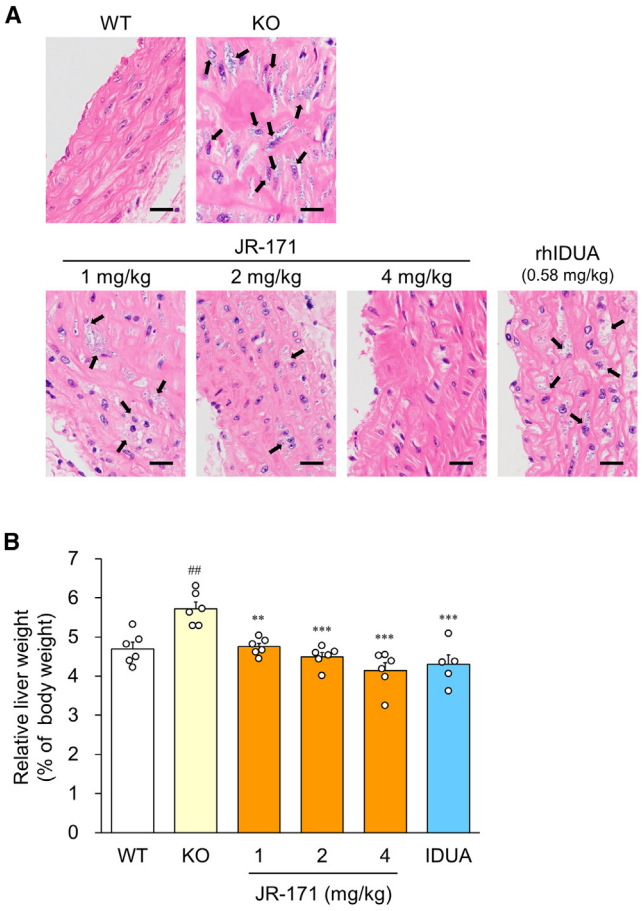


Figure 5. Effect of JR-171 on peripheral pathology in MPS I mice

JR-171 (1, 2, or 4 mg/kg) or rhIDUA (0.58 mg/kg) was administered intravenously to 11-week-old MPS I mice once every week for 12 weeks. (A) H&E-stained sections of the aortic wall of the heart. Vacuolation and swelling of interstitial and smooth muscle cells are observed. Scale bars, 20 μm. (B) Relative liver weight. Values are presented as the mean with SEM bar for each group (n = 5–6). Individual values are also plotted. $^{\#\#}p < 0.01$ (versus WT, unpaired t test), $^{***}p < 0.001$; $^{**}p < 0.01$ (versus KO, Dunnett's test). WT, wild-type mice; KO, hTfR-KI/*Idua*-KO (MPS I) mice.

The limitations of this study include a lack of morphological and functional analyses of the peripheral organs, including the heart and bones/joints. Patients with MPS I suffer from progressive severe skeletal dysplasia and heart disease. Particularly, heart valve dysfunction could contribute to early mortality. These abnormalities largely affect the quality of life of patients and caregivers. We will address whether JR-171 affects cardiovascular dysfunction and bone morphological changes using the MPS I mouse strain in our future studies. Concerning CNS manifestations, the dose-dependent effect on neurological function has not been shown in this study. Although JR-171 cleared HS from the brain and improved spatial learning ability, the quantitative relationship between CNS HS concentration and neurological outcome in MPS I mice remains unclear, and additional studies are required to elucidate this.

In conclusion, this study confirmed the nonclinical proof-of-concept that applying TfR-targeting technology enables delivery of IDUA enzyme to the brain parenchyma, by crossing the BBB after intravenous administration, where the enzyme reduces accumulated substrates to prevent and/or recover pathological changes. These results, along with a comprehensive nonclinical safety assessment, support the initiation of clinical trials, currently in a phase I/II study.

MATERIALS AND METHODS

Recombinant proteins

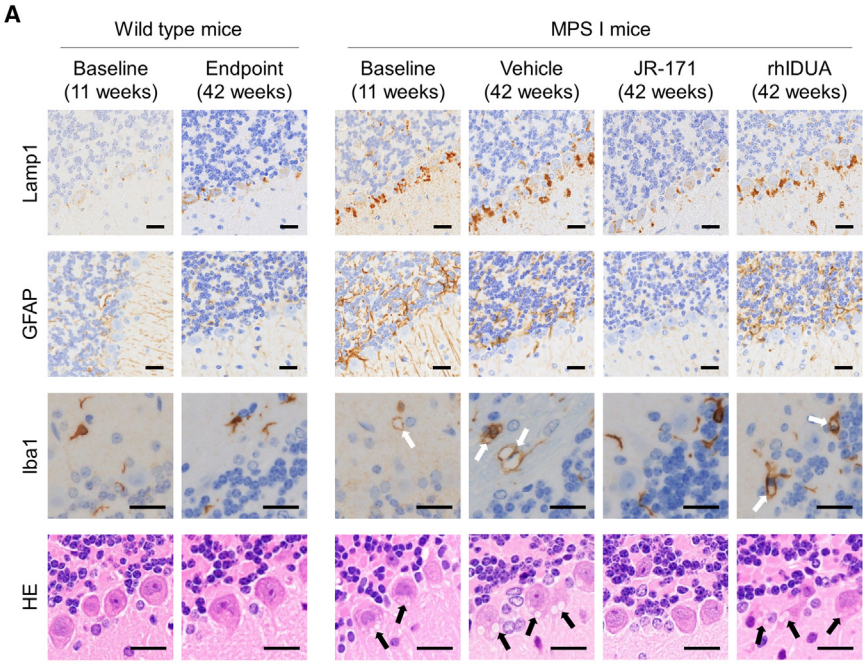
JR-171 (lepunafusp alfa), a fusion protein consisting of humanized hTfR antibody Fab and IDUA (Figure 1A), was produced using transformed CHO cells under regular conditions without any animal-derived culture materials.[10,11,18] The calculated molecular weight of JR-171 is 119,204 Da. Laronidase (rhIDUA: ALDURAZYME) was purchased from Sanofi Genzyme.

Animals

The murine MPS I model used in this study was hTfR-KI/*Idua*-KO with a C57BL/6 background. hTfR-KI mice were established as described previously.[18] The hTfR-KI used in this study was monoallelic. The *Idua*-KO mice were generated by inserting a neomycin-resistance (PGK *neo*) cassette containing a stop codon into exon 6 of the *Idua* gene (Figure S1A).[36] C57BL/6 ES cells were electroporated with the targeting vector, and positive clones were selected by southern blotting (Figure S1B). The resulting *Idua*-KO mice lost the enzyme activity (Figure 3) and showed elevated DS and HS concentrations in major tissues and organs (Figure 4), validating it as an animal model of MPS I. *Idua*-KO mice were crossbred with hTfR-KI mice to obtain hTfR-KI/*Idua*-KO mice. Nearly 10% of the MPS I mice died of intrathoracic hemorrhage due to cardiovascular abnormalities before 30 weeks of age. C57BL/6 mice were purchased from Charles River, Japan (Yokohama, Japan). The WT control used in this study was hTfR-KI/WT ($Idua^{+/+}$) strain, except in the experiment presented in Figure 3, where C57BL/6 strain (without hTfR-KI) was used. Genotypes of mice were determined by PCR analysis of genomic DNA from the tail. The mice were housed under 12-h light-dark cycles with free access to water and a standard rodent chow diet. All animal experiments were performed under protocols approved by the Animal Care and Use Committees of JCR Pharmaceuticals.

Cellular uptake assay

The cellular uptake of JR-171 was determined based on the cellular IDUA enzyme activity after incubation with the agent. Human fetal lung fibroblasts (MRC-5) were purchased from ATCC (Manassas, VA). Cells were cultured in Eagle's Minimum Essential Medium with 10% fetal bovine serum on 96-well microplates at a density of 1.8×10^4 cells/well for 3 days. After incubation, the culture medium was replaced with either JR-171 solutions (160 nmol/mL) or only culture medium. The cells were incubated for 2, 6, and 20 h at 37°C in a humidified atmosphere with 5% CO_2. M6P (10 mM) and/or anti-TfR monoclonal antibody (400 μg/mL) were also added to the corresponding wells. Cells were washed thrice with PBS and then dissolved



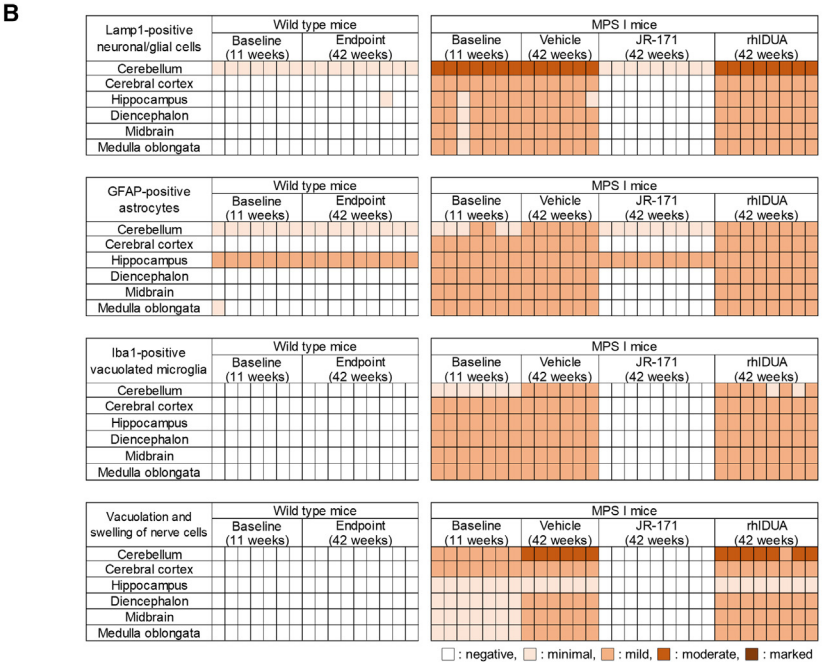


Figure 6. Effects of JR-171 on brain pathology in MPS I mice

JR-171 (1 mg/kg) or rhIDUA (0.58 mg/kg) was administered intravenously to 11-week-old MPS I mice once every week for 31 weeks. (A) Representative photomicrographs of cerebellum sections are shown. Brown signals indicate positive staining for Lamp1, GFAP, or Iba1. White arrows indicate vacuolated Iba1-positive microglial cells, and black arrows indicate vacuolation or swelling of Purkinje neurons (H&E). Scale bars, 20 μm. (B) Grading of histopathological changes. Each square indicates an individual animal.

in lysis buffer containing M-PER (Thermo Fisher Scientific, Waltham, MA) with a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO). The cell lysates were aliquoted for protein quantification and IDUA activity assay. The IDUA activity assay was performed using 4-methylumbelliferyl α-L-iduronide (4-MU-α-IdoA) (Glycosynth, Cheshire, UK) as an artificial substrate. The cell lysates and 4-MU-α-IdoA solutions were added to a black 96-well microplate and incubated for 90 min at 37°C with continuous shaking. The reaction was terminated by adding an alkaline solution. The 4-MU release was quantified by measuring fluorescence (excitation at 355 nm and emission at 450 nm) using standard 4-MU solutions as controls. The results were presented as average values from three independent plates for each duplicate measurement.

Pharmacokinetics and biodistribution

For evaluating the pharmacokinetics in MPS I (hTfR-KI/*Idua*-KO) mice, the same dose (2 mg/kg) of JR-171 or rhIDUA was intravenously administered to male mice to compare the mass concentration between the drugs. Blood samples were obtained at 0.25, 1, 4, 8, 24, and 48 h, and the tissues (liver, heart spleen, and brain) were collected at 1, 8, 24, and 48 h after administration (n = 4/time point). The blood and tissue samples were processed as described previously.[18] JR-171 and rhIDUA were quantified with a validated in-house electro-chemiluminescent (ECL) assay system. Briefly, each well of an MSD GOLD Streptavidin plate (Meso Scale Diagnostics, Rockville, MD) was blocked with SuperBlock Blocking Buffer (Thermo Fisher Scientific), and biotin-labeled chicken anti-IDUA immunoglobulin (Ig)Y solution was added to immobilize the antibody. After washing, a secondary antibody solution containing rabbit anti-IDUA polyclonal antibody was added to each well and incubated at 25°C for 60 min. The plate was washed, and SULFO-Tag goat anti-rabbit IgG (Meso Scale Diagnostics) was added to each well and incubated at 25°C for 60 min. Then 2× Read buffer T (Meso Scale Diagnostics) was added, and the plate was read with an ECL microplate reader (Model SECTOR S600: Meso Scale Diagnostics). Each sample was measured in duplicate together with JR-171 standard samples on the same plate, and the concentration was calculated using a standard curve. The average values were used.

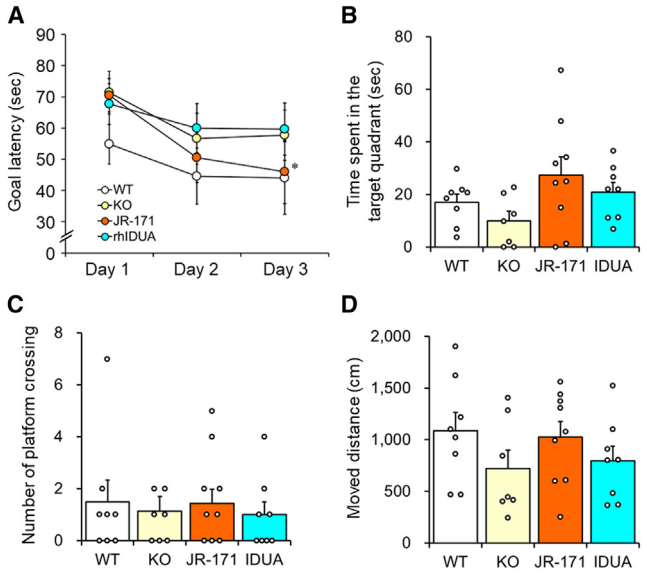


Figure 7. Morris water maze performance in MPS I mice treated with JR-171

(A) Goal latency. (B–D) Probe trial performed on day 4. The time spent in the target quadrant (B), the number of platform crossings (C), and the moved distance (D) were measured. MPS I mice were chronically treated with JR-171 (1 mg/kg/wk) or rhIDUA (0.58 mg/kg) for 24 weeks. The time to reach the platform (goal latency) was measured thrice daily and the means were calculated daily for individual animals. Values are represented as the mean with SEM bar for each group (n = 7–9). *$p < 0.05$ (compared with the value on day 1, paired t test). The probe trial was performed on day 4 after removing the platform. Differences are not statistically significant between any two groups (one-way ANOVA). WT, wild-type mice; KO, untreated hTfR-KI/*Idua*-KO (MPS I) mice.

Immunohistochemistry was performed to detect IDUA enzyme molecule using MPS I mice 6 h after administration of 4 mg/kg JR-171 or rhIDUA. Frozen brain sections (7-μm thick) of the mice were fixed in 4% paraformaldehyde and blocked with Avidin Block (Avidin/Biotin Blocking kit: Abcam, Cambridge, UK) and Starting Block (Thermo Fisher Scientific) solutions. After reaction with primary antibody (horseradish peroxidase [HRP]-labeled anti-IDUA monoclonal antibody), Biotin XX-Tyramide SuperBoost kit (Thermo Fisher Scientific) was used for signal amplification, according to the manufacturer's instruction, but biotin-tyramide and HRP-conjugated streptavidin were replaced with biotin-styramide (AAT Bioquest, Pleasanton, CA) and Streptavidin-Peroxidase Polymer (Sigma-Aldrich, St. Louis, MO), respectively. The signal was visualized using 3,3′-diaminobenzidine as a chromogen.

The biodistribution of JR-171 in cynomolgus monkeys was examined at the Pharmacokinetics and Bioanalysis Center, Shin Nippon Biomedical Laboratories. ^{125}I-labeled JR-171 (2 mg/kg/2.5 MBq/kg) was intravenously administered once to male and female cynomolgus monkeys (n = 5/sex) by continuous infusion for 1 h. Blood samples were collected from the femoral vein and centrifuged with EDTA-2K to obtain plasma. Radioactivity was measured in trichloroacetic acid-precipitated plasma. For quantitative whole-body autoradiolu-

minography, the animals were euthanized by injecting thiopental solution for necropsy at 2, 8, 24, 48, and 72 h after the initiation of administration. One male and one female animal were used at each time point. The frozen bodies were embedded in 5% Na-carboxymethyl cellulose solution and placed in dry ice-cold n-hexane. Whole-body sections (40-μm thick) at the midline, middle of the kidney, and middle of the eyeball were prepared using a cryomicrotome (Leica CM3600, Leica, Wetzlar, Germany). The sections were freeze-dried at −20°C and were covered with Mylar sheets, kept in close contact with the imaging plate, and exposed for 24 h. The imaging plates were analyzed using a bioimaging analyzer (BAS-2500, GE Healthcare, Buckinghamshire, UK).[22]

Duration of JR-171 efficacy in MPS I mice

JR-171 (1 mg/kg) or rhIDUA (0.58 mg/kg) was intravenously administered by bolus injection over 10 s to 15-week-old male MPS I mice (n = 15 for JR-171 group; n = 3 for rhIDUA group). The doses used were equivalent on the basis of enzyme content. The JR-171 group was subdivided into five groups (n = 3/group) for tissue sampling on days 1, 4, 7, 14, and 28 after the administration. Tissue sampling from the rhIDUA-treated mice was done on day 7 after the administration. Untreated MPS I and WT mice were used as the disease control (n = 3) and normal control (n = 3), respectively. The mice were grouped randomly to ensure that the mean body weight of each group was approximately equal. Tissues (brain and liver) were collected after systemic perfusion of physiological saline to measure enzyme activity and HS concentration. To measure the enzyme activity, tissues were homogenized in an extraction buffer (20 mM Tris-HCl containing 1 mM EDTA, 1 mM DTT, and 1% Triton X-100) and centrifuged to obtain supernatants. The total protein concentration was determined using the BCA protein assay (Thermo Fisher Scientific), and samples were diluted to the same concentration. IDUA enzyme activity was measured as described above. As reported previously, the HS concentration was quantified by a validated method using liquid chromatography-tandem mass spectrometry.[35]

Twelve-week efficacy study in MPS I mice

Male MPS I mice were given weekly or EOW intravenous injections of JR-171 at 1, 2, and 4 mg/kg for 12 weeks (12 times weekly and six times EOW). rhIDUA (0.58 mg/kg) was intravenously administered weekly to the mice. The dose selection of JR-171 was based on the molar mass of the enzyme. Disease control mice were treated weekly with the vehicle (physiological saline). The mice were pretreated with diphenhydramine (5 mg/kg, intraperitoneally [i.p.]) to prevent infusion reactions. Untreated WT mice were used as the normal control. The treatment was started at 11 weeks of age. On the first day of treatment, the mice were grouped randomly to ensure that the mean body weight of each group was approximately equal. The number of animals used (n = 6/group) was determined based on pilot studies. One animal each died from the JR-171 (2 mg/kg EOW) and rhIDUA groups on day 17 and on day 55 of administration, respectively, due to cardiovascular abnormalities. One week after the final administration of weekly dosing groups, samples (CSF, blood, and tissues) were collected and processed for downstream analyses (liver weight

measurement, DS/HS quantification, and histopathology), as described previously.[18,20,23] The grading of histopathological changes was performed based on the severity of the changes by a registered pathologist (Diplomate of Japanese Society of Toxicologic Pathology) without blinding.

Long-term efficacy study on brain pathology in MPS I mice

JR-171 (1 mg/kg) or rhIDUA (0.58 mg/kg) was administered intravenously to MPS I mice weekly, beginning from the age of 11 weeks, for the complete 31 weeks. WT and vehicle-treated MPS I mice were used as normal control and disease control, respectively. The mice were pretreated with diphenhydramine (5 mg/kg, i.p.) to prevent infusion reactions. Thirteen animals were used per group, which was determined concerning pilot studies, including four animals for DS/HS measurement and the animals remaining alive at the end of the test period (n = 9 for the normal control group, n = 6 for disease control group, n = 9 for JR-171 group, and n = 8 for rhIDUA group) for brain pathology. As described previously, 1 week after the final administration, CSF and brain tissues were collected and processed for downstream analyses (DS/HS quantification and histopathology).[18,20,23]

Long-term efficacy study on spatial learning ability in MPS I mice

JR-171 (1 mg/kg) or rhIDUA (0.58 mg/kg) was administered intravenously to MPS I mice weekly, beginning from the age of 10 weeks, for the complete 24 weeks. WT and vehicle-treated MPS I mice were used as normal control and disease control, respectively. Ten animals were allocated to each treatment group, and alive animals at the end of the test period (n = 8 for the normal control group, n = 7 for the disease control group, n = 9 for JR-171 group, and n = 8 for rhIDUA group) were subjected to the Morris water maze test to assess spatial learning ability. Two mice from the normal control group were euthanized because of malocclusion and hindlimb plegia. The time to reach the hidden platform (goal latency) was measured as described previously.[20,23] On day 4 of the water maze test, a probe trial (measurement of the time spent in the target quadrant, the number of platform crossing, and moved distance) was performed after removing the platform.

Toxicity studies in cynomolgus monkeys

Because JR-171 recognizes monkey TfR with a similar affinity range as that with hTfR, we selected cynomolgus monkeys for the toxicity studies. All toxicity studies were conducted under GLP compliance and related International Council for Harmonization guidelines.

For the 4-week repeat-dose toxicity studies in sexually mature and juvenile cynomolgus monkeys, 3- to 6-year-old and 4- to 7-month-old animals (both male and female) were used, respectively. In both studies, JR-171 was injected into the monkey's cephalic vein at doses of 10, 20, and 40 mg/kg once weekly (n = 3 for each sex for 10 and 20 mg/kg groups; n = 5 for each sex for control and 40 mg/kg groups). In the 13-week repeat-dose toxicity study, 2- to 3-year-old male and female cynomolgus monkeys were administered JR-171 intravenously through the cephalic vein at doses of 10, 20, and 40 mg/kg once weekly (n = 4 for each sex for 10 and 20 mg/kg groups; n = 6 for each sex for control and 40 mg/kg groups). Necropsy was performed after euthanization by exsanguination under anesthesia with intravenous sodium pentobarbital, 2 days after the last dose. We performed general toxicity assessment, safety pharmacology, blood and urine analyses, and histopathology as described previously.[22] Although a 4-week recovery period to evaluate the reversibility of toxicity was included in each study, the results were not reported in this manuscript since no test article-related changes were noted in any examination during the dosing period.

Statistics

Data are presented as mean ± SEM. Statistical analysis was conducted using KyPlot 6.0 statistics software (KyensLab, Tokyo, Japan). The differences between the WT and the disease control (KO) groups were compared using an unpaired t test to confirm the validity of the disease model. The Dunnett's test was used to compare the differences in the results of the cellular uptake study, substrate-reducing efficacy study, and probe trial using the Morris water maze test between the control and test groups. For goal latency in the water maze test, a paired t test was used to compare the difference from the trial on day 1 within each group. Statistical significance was set at $p < 0.05$.

DATA AVAILABILITY

All relevant data are within the manuscript and its supplemental information.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.omtm.2023.05.010.

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AUTHOR CONTRIBUTIONS

T.H., K.T., H.S., H.M., R.Y., and K.M. designed and supervised the study. S.K., Y.K., E.Y., A.Y., H.M., A. Imakiire, N.T., S.T., A.M., J.I., and A. Inoue performed the experiments and analyzed the data. K.M. analyzed data and wrote the manuscript. All the authors have read and approved the final manuscript.

DECLARATION OF INTERESTS

S.K., Y.K., E.Y., A.Y., H.M., A. Imakiire, N.T., S.T., A.M., J.I., A. Inoue, R.Y., K.M., T.H., K.T., and H.S. are employees and/or stockholders of JCR Pharmaceuticals Co., Ltd.

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