Quantitative Model Analysis and Simulation of Pharmacokinetics and Malat1 RNA Knockdown Effect After Systemic Administration of Cholesterol-conjugated DNA/RNA Heteroduplex Oligonucleotide Crossing Blood–Brain Barrier of Mice


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PK/PD model analysis of heteroduplex oligonucleotide

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Abbreviations

AAALAC, association for assessment and accreditation of laboratory animal care; ALS, amyotrophic lateral sclerosis; ASO, antisense oligonucleotide; BBB, blood-brain barrier; BCSFB, blood-cerebrospinal fluid barrier; CSF, cerebrospinal fluid; CHO-HDO, cholesterol conjugated HDO; FOCE-I, first-order conditional estimation method with interaction; FTD, frontotemporal dementia; HDO, heteroduplex oligonucleotide; LC/MS, liquid chromatography-tandem mass spectrometry; MAPT, microtubule-associated protein tau; PBS, phosphate-buffered saline; PK/PD, pharmacokinetics/pharmacodynamics; RNase H, ribonuclease H

Recommended section

Neuropharmacology
ABSTRACT

Cholesterol-conjugated heteroduplex oligonucleotide (Chol-HDO) is a double-stranded complex; it comprises an antisense oligonucleotide (ASO) and its complementary strand with a cholesterol ligand. Chol-HDO is a powerful tool for achieving target RNA knockdown in the brains of mice after systemic injection. Here, a quantitative model analysis was conducted to characterize the relationship between the pharmacokinetics (PK) and pharmacodynamics (PD), non-coding RNA metastasis-associated lung adenocarcinoma 1 (Malat1) RNA, of Chol-HDO, in a time-dependent manner. The established PK model could describe regional differences in the observed brain concentration-time profiles. Incorporating the PD model enabled the unique knockdown profiles in the brain to be explained in terms of the time delay after single dosing and enhancement following repeated dosing. Moreover, sensitivity analysis of PK exposure/persistency, target RNA turnover, and knockdown potency identified key factors for the efficient and sustained target RNA knockdown in the brain. The simulation of an adequate dosing regimen quantitatively supported the benefit of Chol-HDO in terms of achieving a suitable dosing interval. This was achieved via sufficient and sustained brain exposure and subsequent strong and sustained target RNA knockdown in the brain, even after systemic injection. The present study provides new insights into drug discoveries and development strategies for HDO in patients with neurogenic disorders.
SIGNIFICANCE STATEMENT

The quantitative model analysis presented here characterized the PK/PD relationship of Chol-HDO, enabled its simulation under various conditions or assumptions, and identified key factors for efficient and sustained RNA knockdown, such as PK exposure and persistency. Chol-HDO appears to be an efficient drug delivery system for the systemic administration of desired drugs to brain targets.
Introduction

Current practices regarding drug discovery and development are focused on new modalities such as antibodies, oligonucleotides, gene therapy products, cell therapy products, and microbiota (Valeur et al., 2017; Carter and Lazar, 2018; Nguyen et al., 2018; Pettit et al., 2018; Cavazzana et al., 2019; Setten et al., 2019). Oligonucleotide therapy using antisense oligonucleotides (ASO) has been extensively investigated as a therapeutic option for genetic diseases. Mipomersen, ASO for homozygous familial hypercholesterolemia (Waldmann et al., 2017), and nusinersen, ASO for spinal muscular atrophy (SMA) (Chiriboga, 2017) are examples of marketed ASO products. In addition to these products, various ASO candidates are currently being developed to treat neurological disorders that are caused by genetic and epigenetic changes, such as the huntingtin (HTT) gene for Huntington’s disease (Rinaldi and Wood, 2018), superoxide dismutase 1 (SOD1) gene for amyotrophic lateral sclerosis (ALS) (Smith et al., 2006; Miller et al., 2013), ataxin 2 gene for ALS (Scoles et al., 2017), microtubule-associated protein tau (MAPT) gene for tauopathy (DeVos et al., 2017), and C9ORF72 for ALS/frontotemporal dementia (FTD) (Riboldi et al., 2014; Tran et al., 2021).

One major obstacle to oligonucleotide therapeutics is ensuring their efficient delivery to target tissues other than the liver. In particular, it is difficult to deliver ASOs into the central nervous system (CNS) to treat neurological disorders, as they must be transported across the blood–brain
barrier (BBB). To overcome this delivery obstacle, ASOs are generally administered into the cerebrospinal fluid (CSF) by intrathecal (IT) or intracerebroventricular (ICV) injections. However, IT and ICV dosing are both invasive, and it is difficult to administer them frequently. Thus, systemic administration techniques, such as intravenous (IV) or subcutaneous (SC) injections, would be profitable in terms of patient quality of life. Heteroduplex oligonucleotide (HDO), which is a double-stranded complex of ASO and its complementary strand with conjugates, has recently been developed (Nagata et al., 2021). Although single-strand ASOs have not shown efficacy, cholesterol-conjugated HDO (Chol-HDO) has shown potent and persistent non-coding RNA metastasis-associated lung adenocarcinoma 1 (Malat1) RNA reduction effects in the brain after systemic injection in rodents. Therefore, a quantitative approach for interpreting HDO properties and translation is needed to develop a promising delivery technology using the HDO platform.

Comprehensive evaluations of pharmacokinetic and pharmacodynamic (PK/PD) relationships are useful for conducting quantitative interpretation, simulating the appropriate dose regimen, and predicting human PK/PD relationships. Modeling and simulation (M&S) is a widely used tool for interpreting, simulating, and predicting PK/PD relationships (Ploeger et al., 2009; Goto et al., 2019). Although several studies have applied quantitative PK/PD model analysis using the M&S technique for ASO (Shimizu et al., 2015; Biliouris et al., 2018; Tabrizi et al., 2019; Willmann et al., 2021), to date, no such brain PK/PD M&S analysis of systemically injected ASO has been reported.
The objective of the present study was to characterize the relationship between ASO concentrations and target Malat1 RNA levels in a time-dependent manner following the IV injection of Chol-HDO, using quantitative PK/PD model analysis. A quantitative model was established to interpret the unique PK/PD profiles of Chol-HDO, and sensitivity analysis was conducted to determine the key factors for efficient and sustained Malat1 RNA knockdown.

Material and Methods

Materials

ASO targeting Malat1 and its Chol-HDO were synthesized by Takeda Pharmaceutical Company (Osaka, Japan), as previously described (Nagata et al., 2021). The structures of ASO and its complementary RNA with cholesterol conjugation are described in the Supp. Fig. 1. All the other reagents were purchased from commercial vendors.

Animal experiment

C57BL/6J mice (male, 6–8 weeks of age, n = 3, The Jackson Laboratory Japan, Kanagawa, Japan) were used in this study; the study conditions are summarized in Table 1. Briefly, Chol-HDO was intravenously administered to mice, and plasma and tissues were collected at designated time points under anesthesia with isoflurane (Pfizer, New York City, NY). The dose amount of Chol-HDO is expressed as the amount of single strand ASO (e.g., 50 mg·kg⁻¹ is equivalent to 105.5 mg·kg⁻¹ as
Phosphate-buffered saline (PBS) was administered to control animals. The brain of each animal was split into four regions (cortex, striatum, cerebellum, and hippocampus), and the collected samples were immediately frozen until analysis.

All animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee of Takeda Pharmaceutical Company. The site of animal studies obtained accreditation from the association for assessment and accreditation of laboratory animal care (AAALAC) international.

**PK/PD assessment**

The concentration of single-stranded or parent ASO of intact double-stranded Chol-HDO was measured using liquid chromatography-tandem mass spectrometry (LC-MS/MS). The LC system equipped with two pumps (LC-20AD), an autosampler (SIL-20AC), and a column oven (CTO-20AC) (Shimadzu, Kyoto, Japan) was used for the chromatographic separation. Separation for the analytes was accomplished on a GL Science Inertsustain C18-AQ Guard column (5 μm 2.1 × 10 mm, GL Science, Tokyo, Japan). The ASO concentrations in biological samples from Chol-HDO-treated mice represent the total concentration of parent ASO of intact double-stranded Chol-HDO and ASOs separated from Chol-HDO. Tissue samples were homogenized in 20% (w/v) ultrapure water. The standard samples for the calibration curve were prepared using a standard solution of single strand ASO as well as control plasma and tissue homogenate. The quality samples
were prepared for Chol-HDO to confirm the accuracy and precision of the bioanalytical assay. The single-stranded or parent ASO in plasma and tissue homogenate analytical samples, standard curve samples, and quality control samples were extracted using the phenol-chloroform isoamyl alcohol (PCI, 25/24/1, v/v/v) liquid-liquid extraction method. Briefly, 100 μL of tissue homogenate was mixed with 150 μL 70% of lysis buffer (A8261, pH8, Promega, WI, US) and incubated at 60°C for 1 h. Two hundred microliter of PCI was added and mixed well. After centrifugation (216000 × g, 40°C, 1.5 h), an aliquot of the supernatant (100 μL) was transferred to a new tube and mixed with mobile phase A. The mobile phase comprised H₂O/Hexafluoro-2-propanol (HFIP)/triethylamine (TEA) (1000/30/0.4, v/v/v; mobile phase A) and methanol/HFIP/TEA (1000/30/0.4, v/v/v; mobile phase B). The initial flow rate was 0.3 mL/min and increased to 0.5 mL/min from 3.1 to 6.1 min after injection.

The initial mobile phase composition of 100% A was maintained for 0.5 min, and then the percentage of B was linearly increased up to 40% over the next 2.0 min, followed by an immediate increase to 95% in 0.1 min. After the maintenance of 95% B for 1.9 min, the percentage of B was brought back to 0% in 0.1 min, followed by re-equilibration for 2.4 min. The total cycle time for each injection was 7.0 min. MS detection was performed using an API-4000™ triple quadrupole mass spectrometer (SCIEX, Framingham, MA) equipped with a turbo electrospray ion source that was operated in positive selected reaction monitoring (SRM) mode. The SRM transitions for full-length ASO targeting *Malat1* as an analyte, and its structural analogs, as an internal standard
were set at 755.3/736.0 and 653.5/673.3, respectively. Analytical data were acquired and analyzed using the Analyst software (SCIEX). The terminal half-life in each brain region was calculated by non-compartment model analysis using Phoenix WinNonlin 8.1 (Cetera, Princeton, NJ).

For the PD assessment, total RNA was extracted from mouse tissues using a QuickGene RNA tissue kit (Kurabo Industries, Okayama, Japan). Total RNA was reverse transcribed using a SuperPrep II RT kit (Toyobo, Osaka, Japan). Three microliter of reverse-transcribed cDNA was used for quantitative polymerase chain reaction (qPCR) analysis using a PCR instrument (Thermo Fisher Scientific, Waltham, MA).

The primers and probes used for qRT–PCR were purchased from Integrated DNA Technologies (Coralville, IA) for Malat1 and Thermo Fisher Scientific for ACTB. The Malat1 RNA level was normalized to ACTB mRNA levels.

Model analysis

PK/PD model analysis was performed using NONMEM7 (ICON Development Solutions, Ellicott City, MD) using the first-order conditional estimation method with interaction (FOCE-I). The convergence criterion was set at three significant digits. As reported in the previous study (Nagata et al., 2021), Chol-HDOs enter the brain in an intact duplex form and separate inside the brain capillarity endothelial cells and neurons in the cerebral cortex. After releasing from the double strand Chol-HDO, the single strand ASO in the cells induces RNA knockdown. In the present study,
the total concentration of parent ASO of intact double-stranded Chol-HDO and ASOs separated from Chol-HDO was measured. In this analysis, all measured ASOs in tissues were assumed as an effective ASO for Malat1 RNA knockdown. The structure of the PK/PD model is illustrated in Fig. 1.

For the PK model, a basic two-compartment model with specific tissue compartments (brain, liver, and kidney) was constructed. To explain the initial high drug concentration in tissue samples, blood concentration in tissue vessels was included. The total concentration in tissue vessels and parenchyma compartments was calculated as the tissue concentration in each case. The redistribution from each tissue to blood was estimated as very small values at the initial modeling step; therefore, we assumed redistribution as minimal and removed it from the model. All PK data was fitted by developed model simultaneously. The ASO used in the present study enhanced the ribonuclease H (RNase H)-dependent degradation of Malat1 RNA. In this model, ASO enhanced the elimination of Malat1 RNA. The transit compartment to the effective site in the brain tissue was also investigated to aid the description of the observed data (see the Results and Discussion section).

The differential equations for this PK model were as follows:

\[
\frac{dA_{\text{plasma}}}{dt} = -(K_{12} + K_{13} + K_{14} + K_{15} + K_{16} + K_{17} + K_{18}) \times A_{\text{plasma}} + K_{21} \times A_{\text{peripheral}} - K_{e1} \times A_{\text{plasma}}
\]  
(1)

\[
\frac{dA_{\text{peripheral}}}{dt} = K_{12} \times A_{\text{plasma}} - K_{21} \times A_{\text{peripheral}}
\]  
(2)

\[
\frac{dA_{\text{liver}}}{dt} = K_{13} \times A_{\text{plasma}} - K_{e\text{liver}} \times A_{\text{liver}}
\]  
(3)
\[ \frac{dA_{\text{kidney}}}{dt} = K_{14} \times A_{\text{plasma}} - K_{\text{el,kidney}} \times A_{\text{kidney}} \]  \quad (4)

\[ \frac{dA_{\text{cortex}}}{dt} = K_{15} \times A_{\text{plasma}} - \frac{v_{\text{max,trans}} \times c_{\text{cortex}}}{K_{\text{m,trans}} + c_{\text{cortex}}} \]  \quad (5)

\[ \frac{dA_{\text{bp,cortex}}}{dt} = \frac{v_{\text{max,trans}} \times c_{\text{cortex}}}{K_{\text{m,trans}} + c_{\text{cortex}}} - K_{\text{el,cortex}} \times A_{\text{bp,cortex}} \]  \quad (6)

where $K_{12}$, $K_{21}$, $K_{13}$, $K_{14}$, and $K_{15}$ are the distribution rate constants from plasma to peripheral, peripheral to plasma, plasma to the liver, plasma to kidney, and plasma to the cortex, respectively. $K_{\text{el-[compartment]}}$ is the elimination rate constant for a given compartment.

$v_{\text{max,trans}}$ and $K_{\text{m,trans}}$ are the maximum velocity and concentration that show half the maximum velocity for the brain depo to the effective site, respectively. For the striatum, hippocampus, and cerebellum, the same equation as the cortex (Eqs. 5 and 6) was applied to each region-specific parameter ($K_{16}$ for striatum, $K_{17}$ for hippocampus, $K_{18}$ for cerebellum). This model did not specify brain distribution pathway. $K_{15}$, $K_{16}$, $K_{17}$, and $K_{18}$ may include the distribution via BBB penetration, blood-CSF barrier (BCSFB) crossing, and CSF flow pathway.

\[ C_{\text{plasma}} = \frac{A_{\text{plasma}}}{V_{\text{plasma}}} \]  \quad (7)

where $A_{\text{plasma}}$ and $C_{\text{plasma}}$ denote the amount and concentration of drug in plasma compartment, respectively. $V_{\text{plasma}}$ is distribution volume in plasma compartment. Concentration in tissues were calculated as below,

\[ C_{\text{brain}} = \left( A_{\text{brain}} + A_{\text{bp,brain}} \right) / V_{\text{brain}} + C_{\text{plasma}} \times R_{\text{vessel,brain}} \]  \quad (8)
\[ C_{\text{tissue}} = A_{\text{tissue}} / V_{\text{tissue}} + C_{\text{plasma}} \times R_{\text{vessel.tissue}} \] (9)

\[ C_{\text{kidney}} = A_{\text{kidney}} / V_{\text{kidney}} + C_{\text{plasma}} \times R_{\text{vessel.kidney}} \] (10)

where \( C_{\text{tissue}} \) and \( A_{\text{tissue}} \) are the drug concentration and amount in each tissue, respectively. \( A_{\text{tissue.bp}} \) is the amount of drug in each brain region biophase compartment, respectively. \( V_{\text{tissue}} \) and \( R_{\text{vessel}} \) are distribution volume of tissue compartment and volume ratio of microvessel in each tissue, respectively. Here, subscript “tissue” represents liver, kidney, cortex, striatum, hippocampus, or cerebellum. Although physiological blood volume in each brain region is not identical (Chugh et al., 2009), \( R_{\text{vessel}} \) values of striatum, hippocampus, and cerebellum were supposedly assumed as same value with that of cortex in this analysis. The assumption is based on confirmation difficulty of consistency with PK data due to the lack of data in striatum, hippocampus, and cerebellum at early time points. \( R_{\text{vessel}} \) value significantly affected on apparent PK profile at early time point but not at later time points. Therefore, this assumption minimally contributes to PK/PD analysis in striatum, hippocampus, and cerebellum evaluated in this study. An alternative to \( R_{\text{vessel}} \) is to include the mass-balance equation of the brain capillary compartment, which is connected to the plasma compartment via blood rate. Unless the extraction ratio of Chol-HDO is significant, the two assumptions have no effect on model dependencies in the optimized parameters.

The PD model equation for the brain was as follows:
where \( K_{in} \) and \( K_{out} \) represent the production and elimination (turnover) rates of \( Malat1 \) RNA, respectively, and \( KE \) is the coefficient of efficiency. The initial conditions for each PK compartment were 0. The dose was applied to the plasma compartment at the timing of administration. The initial condition of the \( Malat1 \) RNA compartment was 100 (%).

To simulate an appropriate dosing regimen, we incorporated the PK results after both single and repeated dosing. Berkeley Madonna (University of California, Berkeley, CA) was used for the simulation and sensitivity analysis. Sensitivity analysis was conducted on the \( Malat1 \) RNA knockdown time profile by changing \( K_{out} \) (turnover rate), \( K_{el} \) (PK elimination rate from cortex), \( K_{15} \) (distribution rate to cortex), \( KE \) (knockdown efficiency), and the dose frequency. This was done to evaluate the effects of changing each parameter (or the dose frequency) on temporal variations in PK/PD.
Results

PK in a mouse model

Acceptable accuracy and precision of QC samples at three different concentration levels were confirmed at every bioanalytical assay. Following single (Study No. 1 and 2 in Table 1) and repeated (Study No. 3 in Table 1) IV injections of Chol-HDO, a high tissue concentration was observed immediately after dosing (Fig. 2, symbols). Significant ASO levels were observed in all brain regions (cortex, striatum, cerebellum, and hippocampus), even after systemic injection. Furthermore, the concentration-time profiles differed among the brain regions. Up to 500 h, the concentration was highest in the striatum, followed by the hippocampus, cortex, and cerebellum. However, the cerebellum had the longest apparent terminal half-life (49 days) among the four brain regions (cortex, 27 days; striatum, 17 days; hippocampus, 17 days). These data indicate that the distribution or elimination of Chol-HDO was inhomogeneous across these brain regions.

PK modeling

A two-compartment model with a tissue compartment was constructed to describe the ASO concentration-time profile in the plasma, brain, liver, and kidney (Figs. 1 and 2). The simulation of plasma concentration at the terminal phase after single administration overestimated the observed concentration, but terminal elimination decline was comparable between simulation and observed
data. Additionally, after repeated dosing, the simulation was slightly lower than observed data. The difference on plasma concentration between two studies is unknown. However, it was considered that Chol-HDO or ASO distribution to tissue was almost completed by 72 h after administration. This indicated slight overestimation or underestimation at the terminal phase in plasma minimally affects the tissue distribution and subsequent Malat1 RNA knockdown. Therefore, overestimation and underestimation at the terminal phase in plasma was accepted in this analysis. The initial high concentration in tissues was modeled by consideration of concentration in tissue vessels. The estimated volume ratio of the vessel to tissue (R_vessel) values (0.327 for the liver and kidney, 0.0165 for the brain) were comparable to the physiological fraction of the blood volume in each tissue sample (Brown et al., 1997). For brain modeling, different distributions and elimination rates for each brain region were used to describe differences in the concentration-time profiles of these brain regions. Accumulations of ASO were observed in the brain, liver, and kidney after repeated dosing (Fig. 2). These accumulations could be explained by the PK model, using the same PK parameters between single-dose and repeated-dose data. The estimated PK parameters are presented in Table 2.

**PD in a mouse model**

Efficient Malat1 RNA knockdown was observed in each brain region after a single IV dose of Chol-HDO (Fig. 3); these results are consistent with previously reported data (Nagata et al., 2021).
The Malat1 RNA knockdown level in the cerebellum was weaker than those in the other regions, including the cortex, hippocampus, and striatum. The Malat1 RNA knockdown level was saturated at approximately 60% against the ASO concentration in each brain region 72 h after single dosing (Fig. 4). However, the Malat1 RNA knockdown levels in each brain region were enhanced by repeated dosing (Fig. 3).

**PD modeling**

The time profile of Malat1 RNA in each brain region was modeled using the PK/PD model shown in Fig. 1. To describe the saturation (60% knockdown) at 72 h after single dosing and the enhancement of knockdown by repeated dosing, the transit compartment to the effective site in the brain was incorporated into the PD model. The PK/PD model successfully described a unique Malat1 RNA time profile after both single and repeated doses of Chol-HDO; the estimated PD parameters are listed in Table 2. The goodness-of-fit plot is shown in Supp. Fig. 2.

**Model-based simulation**

The results of the sensitivity analysis are shown in Fig. 5. Changing $K_{out}$ slightly altered the time at which maximum knockdown was reached, but not the recovery rate (Fig. 5A). On the contrary, changing the PK elimination rate ($K_{el}$) altered the Malat1 RNA recovery (Fig. 5B). Moreover,
increased brain distribution (K15) and knockdown efficiency (KE) both indicated strong Malat1 RNA knockdown (Figs. 5C and D). Less frequent dosing rates (biweekly and monthly) reached knockdown efficiencies of 80% and 60%, respectively, at a steady state (Fig. 5E).
Discussion

Here, a PK model was established to reveal the unique PK profile of Chol-HDO following IV administration in mice. In the present study, the concentration of Chol-HDO was found to be highest in the striatum, which is a deep brain area, followed by the hippocampus, cortex, and cerebellum. Following the IT injection of single-strand ASO into rats and monkeys, lower ASO concentrations and inevitably weaker Malat1 RNA knockdown effects have been reported in the deep brain regions, compared with those in the cortex (Jafar-Nejad et al., 2021). The deep brain areas were heretofore undruggable sites after IT administration of single-strand ASO. The ability of Chol-HDO to address the deep brain regions is a profitable characteristic to achieve knockdown of target RNA after systemic administration, compared with the IT administration of single-strand ASO. It is crucial to quantitatively interpret the unique PK profile of Chol-HDO in each brain region using the PK/PD model. We assumed the unique structure of the PK/PD model for the brain, which has different distribution rates (K15–18), elimination rates (Kel), and Vmax for each brain region (Table 2). The established PK model could successfully describe the time-concentration profile in each brain region (Fig. 2).

The cause of the observed regional differences in the distribution of Chol-HDO is important for better interpretation; however, they remained to be elucidated. The difference in the BBB surface area per unit tissue volume among brain regions could cause regional differences in the distribution
if the distribution efficiency per unit surface area is identical. However, the cerebral blood volume in the striatum (a region with high distribution) is rather lower than that in the cerebellum (a region with low distribution) (Chugh et al., 2009), indicating no correlation between the BBB surface area/tissue volume and observed regional distribution difference. The blood-CSF barrier (BCSFB) has also been suggested as a penetration site for Chol-HDO (Nagata et al., 2021). Therefore, diffusion efficiency of Chol-HDO from the CSF through the ependymal surface to each brain region could be other causes of the observed differences in the distribution process. One potential reason for the observed differences in elimination rates is regional variability in nuclease expression or its activity. However, the authors are not aware of any relevant studies in this regard. One further possibility is that there are regional differences in the excretion rate to the systemic circulation via lymphatic clearance (Simon and Iliff, 2016). Further investigations are needed to identify the actual cause of these differences in the distribution and elimination mechanism of Chol-HDO and to improve the PK model to more physiologic one.

As the ASO elimination rate is slow (its half-life is in the range of weeks to months; (Biliouris et al., 2018; Nagata et al., 2021), administering multiple systemic doses of Chol-HDO would be useful for achieving and maintaining target concentrations for Malat1 RNA knockdown. Accordingly, the knockdown effect was cumulative during repeated administration (Fig. 3).

Furthermore, PD compartments were incorporated into the PK model to account for the PK/PD...
relationship. The key finding was that 72 h after a single dose of Chol-HDO, the saturation of
Malat1 RNA knockdown was observed at approximately 60% (Fig. 4); nevertheless, a stronger
effect (over 90% knockdown) could be achieved after repeated dosing (Fig. 3). To describe the
observed PD data, a simple indirect response model that represents the saturable inhibition of Malat1
RNA production was tested. However, the model could not describe the saturation after 72 h of
administration and subsequent knockdown after repeated dosing appropriately (data not shown).
Therefore, we assumed an effective compartment named the brain biophase (Fig. 1), which connects
with the transit compartment to limit the transition rate. In detail, several potential steps could delay
the knockdown effect. Immunohistochemical analysis of the phosphorothioate backbone revealed
the initial accumulation of released ASO or Chol-HDO in brain endothelial cells and choroid plexus,
followed by its distribution to neurons (Nagata et al., 2021). The transition to brain parenchyma cells
across the BBB/BCSFB from blood circulation is a major obstacle for macromolecules. Thus, it
could be the rate-limiting step for Chol-HDO distribution. Additionally, the intracellular distribution
step of parenchyma cells represents a potential further limiting step. ASO is commonly taken up via
receptor-mediated endocytosis, and only a small portion of the uptaken ASO can escape from
endosomes or lysosomes (Crooke et al., 2017). Therefore, the endosomal/lysosomal escape and
subsequent transition to nuclei are key steps to achieving sufficient knockdown effects. The
established PK/PD model described the saturation of the knockdown effect at 72 h after dosing and a
Here, *Malat1* RNA knockdown levels were found to differ among different brain regions, with the cerebellum showing a slightly weaker knockdown than those of other regions. To investigate the cause of regional differences in the knockdown effect, model-estimated PD parameters were compared among the brain regions. Estimated PD parameters, such as $EC_{50\text{PD}}$ (efficiency) and $V_{\text{max,trans}}$ (transition to the effective site), in the cerebellum, were comparable with those in other regions (Table 2), which suggested that the weak knockdown effect in the cerebellum was caused mainly by the low ASO concentration. Furthermore, these results also indicated that the exposure level was a key factor in achieving sufficient therapeutic efficacy. Additionally, the cell (e.g., neuron, oligodendrocyte, microglia) population and density in the cerebellum are different from those in other brain regions (Herculano-Houzel and Lent, 2005; Azevedo et al., 2009; Ero et al., 2018). These anatomical differences might cause an inhomogeneous distribution followed by a regional difference in knockdown. To understand dose-dependency, time-dependency, and regional differences more clearly, the interpretation of cellular ASO/HDO distribution would be helpful.

Finally, using the constructed model, sensitivity analysis was conducted to identify the key factors of PK/PD output and to estimate PK/PD under various conditions. Changes in $K_{\text{out}}$ (*Malat1* RNA turnover rate) did not alter the apparent recovery rate of *Malat1* RNA (Figs. 5A). However, the recovery rate of *Malat1* RNA was found to be sensitive to changes in the PK elimination rate in the
brain, with slow PK elimination maintaining Malat1 RNA knockdown and fast PK elimination leading to rapid recovery (Fig. 5B). These simulations indicate that the recovery of Malat1 RNA was determined by PK persistence, and not by the Malat1 RNA turnover rate. In other words, both the exposure level and persistency of ASO play critical roles in the strong and sustained Malat1 RNA knockdown effect by Chol-HDO treatment. In addition to Malat1 RNA turnover and PK elimination, several important factors contribute to strong Malat1 RNA knockdown effects, such as the distribution rate to the brain and the efficiency of knockdown (Figs. 5C and D). Although the mechanism of efficient distribution in the brain is unknown, a faster distribution rate would result in a stronger knockdown effect. Regarding the translation of these findings to human PK/PD, future studies must clarify the distribution mechanism and conduct mechanism-based simulation with an aspect of species differences. Moreover, the distribution to the target cell type is also important, as there are regional differences in the expression of therapeutic target Malat1 RNAs, such as HTT, SOD1, ATXN2, SCA2, MAPT, and C9ORF72 (Uhlen et al., 2005; Sjostedt et al., 2020). A previous study proposed the existence of a non-productive pathway (Geary et al., 2009). The interpretation of such a non-productive pathway could also aid further mechanistic analysis while helping to reveal the translation of the distribution of Chol-HDO. Here, the knockdown efficiency of ASO was also found to be an important factor for achieving strong efficacy in vivo (Fig. 5D). Mechanism-based analysis has previously been reported using in vitro assays for RNase H-dependent target
knockdown (Pedersen et al., 2014); the absolute target RNA level, RNase H protein level, and
ASO-binding kinetics were all identified as critical factors for knockdown efficiency. More
translatable and complex *in vitro* assay systems would be valuable in filling the current deficiencies
of mechanism-based analyses. The Chol-HDO used here achieved sustained 80% and 60%
knockdown effects under biweekly and monthly dosing, respectively (Fig. 5E). If this phenomenon
is reproducible for actual HDO products when targeting the genes responsible for neurogenic
disorders, then IV Chol-HDO injection may represent a more valuable therapeutic option than IT
ASO injection (as it has a less invasive dosing route and a lower sufficient dosing interval, thereby
improving patient quality of life).

In conclusion, the relationship between the PK profile and target RNA levels after Chol-HDO IV
injection can be characterized by quantitative PK/PD model analysis. Based on the model analysis
presented here, Chol-HDO appears to be an efficient drug delivery platform for the systemic
treatment of neurogenic disorders. Furthermore, sensitivity analysis identified key factors for
efficient and sustained target RNA knockdown, such as PK exposure/persistency, knockdown
potency, and dosing frequency. Moreover, current limitations regarding the prediction of the human
PK/PD relationship were identified, providing useful information for further studies on distribution
mechanisms and species differences. The present study provides new insights into drug discovery
and development strategies for HDO, which constitutes a promising therapeutic option for patients
with CNS diseases.

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Authorship Contributions

Participated in the research design: Goto, Yamamoto, Igari, Matsumoto, Chisaki, Nakayama, Uchida, Miyata, Nishikawa, Nagata, Kusuhara, Yokota, and Hirabayashi.

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Performed data analysis: Goto, Yamamoto, Igari, Matsumoto, Chisaki, Uchida, Miyata, Nishikawa, Nagata, Kusuhara, Yokota, and Hirabayashi.

Wrote or contributed to the writing of the manuscript: Goto, Yamamoto, Igari, Matsumoto, Chisaki, Iida, Nakayama, Oda, Kakoi, Uchida, Miyata, Nishikawa, Nagata, Kusuhara, Yokota, and Hirabayashi.
References


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Willmann S, Marostica E, Snelder N, Solms A, Jensen M, Lobmeyer M, Lensing AWA, Bethune C,

Footnote

A.G., S.Y., T.I., S.M., I.C., K.I., Mi. N., A.O., Y.K., A.U., K.M., and H.H. are employees of Takeda Pharmaceutical Company Limited. This is the result of collaborative research with Takeda Pharmaceutical Company Limited, Tokyo Medical and Dental University, Tokyo University of Science and The University of Tokyo. T.Y. and T.N. are inventors on patents on BBB-HDO filed by Tokyo Medical and Dental University and have benefits from licensing of the associated intellectual property. This work received no external funding. There is no other conflict of interest.
**Figure legends**

**Fig. 1.** Scheme of PK/PD model. $K_{12}$, $K_{21}$, $K_{13}$, $K_{14}$, and $K_{15}$ are distribution rate constants from plasma to peripheral, peripheral to plasma, plasma to the liver, plasma to kidney, and plasma to the brain, respectively. $K_{el}$ is the elimination rate constant of each compartment. $V_{max_{trans}}$ and $K_{m_{trans}}$ are maximum velocity and concentration corresponding to half of the maximum velocity for brain depo to the effective site, respectively. $K_{in}$ and $K_{out}$ represent production and elimination (turnover) rates of Malat1 RNA, respectively. KE is the coefficient of efficiency.

**Fig. 2.** PK data and model-based simulation after single (left and middle) and repeated (right) IV administration of Chol-HDO at 50 mg·kg$^{-1}$ in plasma, liver, kidney, cortex, striatum, hippocampus, and cerebellum. Symbols show observed data, lines show model-based simulations.

**Fig. 3.** PD data and model-based simulation after single (upper) and repeated (bottom) IV administration of Chol-HDO at 50 mg·kg$^{-1}$ in the cortex, striatum, cerebellum, and hippocampus. Symbols show observed data, lines show model-based simulations.

**Fig. 4.** Relationship between Malat1 RNA knockdown and brain PK at 72 h after single administration. Symbols show observed data, lines show model-based simulations.
Fig. 5. Sensitivity analysis on Malat1 RNA knockdown in the cortex after IV administration at 50 mg·kg⁻¹. A: Malat1 RNA turnover rate (K_{out}), B: PK elimination rate from the cortex (K_{el}), C: Brain distribution rate (K_{d1}), D: knockdown efficiency (KE), E: dose frequency.
### TABLE 1. Overview of experimental design.

<table>
<thead>
<tr>
<th>Study No.</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brief description</td>
<td>Single dosing, long term</td>
<td>Short term PK</td>
<td>Weekly dosing</td>
<td>Dose dependency on day 3</td>
</tr>
<tr>
<td>Dose</td>
<td>50 mg·kg⁻¹</td>
<td>50 mg·kg⁻¹</td>
<td>50 mg·kg⁻¹·week⁻¹</td>
<td>5, 10, 20, 50, 75, 100 mg·kg⁻¹</td>
</tr>
<tr>
<td>Time point</td>
<td>3, 7, 14, 21, 28, 42, and 56 d</td>
<td>5, 10, 15, and 30 min; 1, 2, 4, 8, and 24 h</td>
<td>3 d after 1ˢᵗ, 2ⁿᵈ, and 4ᵗʰ dosing</td>
<td>3 d</td>
</tr>
<tr>
<td>Measurement</td>
<td>PK and PD</td>
<td>PK</td>
<td>PK and PD</td>
<td>PK and PD</td>
</tr>
<tr>
<td>Analyzed tissues</td>
<td>Plasma (PK only), Cortex, Striatum, Hippocampus, Cerebellum, Liver, Kidney (PK only)</td>
<td>Plasma, Cortex, Liver,</td>
<td>Plasma (PK only), Cortex, Striatum, Hippocampus, Cerebellum, Liver (PK only), Kidney (PK only)</td>
<td>Plasma (PK only), Cortex, Striatum, Hippocampus, Cerebellum, Liver (PK only), Kidney (PK only)</td>
</tr>
</tbody>
</table>

The dose amount of Chol-HDO is expressed as the amount of single strand ASO (e.g., 50 mg·kg⁻¹ is equivalent to 105.5 mg·kg⁻¹ as Chol-HDO)
TABLE 2. Estimated PK/PD parameters.

<table>
<thead>
<tr>
<th></th>
<th>Plasma</th>
<th>Cortex</th>
<th>Striatum</th>
<th>Hippocampus</th>
<th>Cerebellum</th>
<th>Liver</th>
<th>Kidney</th>
</tr>
</thead>
<tbody>
<tr>
<td>Volume (L·kg⁻¹)</td>
<td>0.125</td>
<td>0.0310</td>
<td>0.0310</td>
<td>0.0310</td>
<td>0.0310</td>
<td>0.0987</td>
<td>0.0180</td>
</tr>
<tr>
<td>(1.76%)</td>
<td>(1.76%)</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
<td>(12.0%)</td>
<td>(16.9%)</td>
</tr>
<tr>
<td>Rate constant for</td>
<td>0.00723</td>
<td>0.0000833</td>
<td>0.000124</td>
<td>0.0000989</td>
<td>0.0000672</td>
<td>0.146</td>
<td>0.0178</td>
</tr>
<tr>
<td>distribution (1·h⁻¹)</td>
<td>(47.9%)</td>
<td>(10.6%)</td>
<td>(14.9%)</td>
<td>(6.5%)</td>
<td>(18.7%)</td>
<td>(5.47%)</td>
<td>(24.4%)</td>
</tr>
<tr>
<td>Kel from tissue</td>
<td>0.00191</td>
<td>0.00131</td>
<td>0.00181</td>
<td>0.00150</td>
<td>0.000718</td>
<td>0.00287</td>
<td>0.00256</td>
</tr>
<tr>
<td>(1·h⁻¹)</td>
<td>(56.7%)</td>
<td>(4.28%)</td>
<td>(0.441%)</td>
<td>(3.79%)</td>
<td>(0.182%)</td>
<td>(4.32%)</td>
<td>(6.29%)</td>
</tr>
<tr>
<td>Rvessel</td>
<td>NA</td>
<td>0.0165</td>
<td>0.0165</td>
<td>0.0165</td>
<td>0.0165</td>
<td>0.327</td>
<td>0.327</td>
</tr>
<tr>
<td></td>
<td>(1.68%)</td>
<td>(1.68%)</td>
<td>(1.68%)</td>
<td>(1.68%)</td>
<td>(1.68%)</td>
<td>(2.36%)</td>
<td>(2.36%)</td>
</tr>
<tr>
<td>V_{max_{trans}} (mg·h^{-1}·kg^{-1})</td>
<td>NA</td>
<td>0.000264 (0.661%)</td>
<td>0.000654 (8.99%)</td>
<td>0.000266 (4.14%)</td>
<td>0.000714 (-)</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Km_{trans} (μg·g^{-1})</td>
<td>NA</td>
<td>0.000926 (0.143)</td>
<td>0.000926 (-)</td>
<td>0.000926 (-)</td>
<td>0.000926 (-)</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>KE (g·µg^{-1})</td>
<td>NA</td>
<td>2.86 (4.81%)</td>
<td>0.905 (12.1%)</td>
<td>2.83 (3.51%)</td>
<td>1.32 (12.4%)</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>K_{out} (h^{-1})</td>
<td>NA</td>
<td>0.0487 (-)</td>
<td>0.0487 (-)</td>
<td>0.0487 (-)</td>
<td>0.0487 (-)</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

Values in parentheses show the coefficient of variance (CV).

NA: Not applicable.

Rate constant for distribution: K_{12}, plasma; K_{13}, liver; K_{14}, kidney; K_{15}, cortex, K_{16}, striatum; K_{17}, hippocampus; K_{18}, cerebellum

K_{21}: 0.00191 (1·h^{-1}) (CV: 56.7%)
\( K_{in} \text{ (%·h}^{-1}\text{)} \) is calculated by \( K_{out} \text{ (h}^{-1}\text{)} \times 100 \) (%)}
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5