Prediction of human pharmacokinetics of phosphorodiamidate morpholino oligonucleotides in Duchenne muscular dystrophy patients using viltolarsen

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Running title page

Running title: Predicting the human pharmacokinetics of PMOs

Number of text pages: 36
Number of tables: 6
Number of figures: 3
Number of references: 37
Number of words in Abstract: 230
Number of words in Introduction: 597
Number of words in Discussion: 1099
Abstract

Several modified antisense oligonucleotides (ASOs) have recently been approved for clinical use. Some are phosphorodiamidate morpholino oligomers (PMOs), which, unlike other nucleic acids, are not negatively charged. Thus, PMOs differ from other ASOs in their pharmacokinetic (PK) properties. Drugs with a PMO backbone have been administered to Duchenne muscular dystrophy pediatric patients; however, appropriate methodologies are not currently available to predict their human PK from nonclinical data. In this study, we used viltolarsen as a representative PMO to investigate the applicability of the allometric scaling approach to human PK prediction. We first summarized the nonclinical and clinical PK data for viltolarsen as showing high total clearance, low serum protein binding, metabolic resistance, and urinary excretion as the unchanged drug in both animals and humans. We then investigate the PK of viltolarsen in mice, rats, cynomolgus monkeys, and dogs and used the results, with body weight, to extrapolate to humans by several methods. The estimate of human total clearance obtained from cynomolgus monkeys was the best, and body weight may be the key factor in accurately predicting human total clearance. In contrast, all of the well known prediction methods for the volume of distribution at steady state gave underestimates. However, the human PK profiles predicted from the PK parameters in cynomolgus monkeys fit the observed human plasma concentrations well. These results are expected to contribute to the further development of PMOs.
Significance Statement

We investigated how to predict the human pharmacokinetics of phosphorodiamidate morpholino oligomers from nonclinical data. The estimates of human pharmacokinetic parameters and profiles determined from cynomolgus monkeys by an allometric scaling approach were the most suitable, and the cynomolgus monkey body weight may be the key factor in accurately predicting human total clearance.
Abbreviations

ASO, antisense oligonucleotide; CL_{tot}, total clearance; DMD, Duchenne muscular dystrophy;
DMPK, drug metabolism and pharmacokinetics; DNase I, deoxyribonuclease I; GFR, glomerular filtration rate; HPLC, high-performance liquid chromatography; IS, internal standard; LC-MS/MS, high-performance liquid chromatography with tandem mass spectrometry; PDE I, phosphodiesterase I; PMO, phosphorodiamidate morpholino oligomer; PK, pharmacokinetics; PS-ASO, phosphorothioate ASO; radio-HPLC, high-performance liquid chromatography with radio detector; V_{dss}, volume of distribution at steady state
Introduction

Many kinds of RNA therapeutic drugs have recently been developed, and antisense oligonucleotides (ASOs) account for the largest number of those that have been approved for clinical use (Curreri et al., 2022). ASOs developed as medicines are short single-stranded nucleic acids (18 to 30 mers) modified to enhance their nuclease stability and affinity for their targets. After cellular uptake by endocytosis, ASOs generally escape from the endosomes and specifically hybridize to their complementary RNA target in the cytoplasm or nucleus (Miller et al., 2018). Approved ASOs can be divided into two groups according to their mechanism of action: gapmer oligonucleotides mediated by RNase H and splicing-switching oligonucleotides that induce exon skipping or exon inclusion (Aupy et al., 2017). ASO research on Duchenne muscular dystrophy (DMD) has long been focused on exon skipping (Pramono et al., 1996), and many reports have demonstrated the efficacy of ASOs in DMD animal models (Lu et al., 2003). Research initiated by the National Center of Neurology and Psychiatry and conducted with the collaboration of Nippon Shinyaku Co., Ltd, focused on phosphorodiamidate morpholino oligomers (PMOs) as DMD therapeutic ASOs, which are mainly administered to male pediatric patients because it is almost exclusively males who are affected by DMD and they generally lose the ability to walk by the age of 12. Following sequence optimization, the PMO viltolarsen (NS-065/NCNP-01) was chosen and developed as an exon 53 skipping therapeutic product (Watanabe et al., 2018). It was successfully
approved and launched in Japan and the US in 2020 under the trade name Viltepso® (Komaki et al., 2018; Komaki et al., 2020; Clemens et al., 2020).

Currently approved ASOs fall into two major categories, PMOs and phosphorothioate ASOs (PS-ASOs), according to the nature of their backbone (Shadid et al., 2021; Takakusa et al., 2023). PMOs and PS-ASOs differ in their physical properties. For example, unlike other nucleic acids, PMOs do not have a negative charge because the furanose units have been replaced with morpholine rings; PMOs have improved cellular uptake due to the lack of electrostatic repulsion by the negatively charged cell membrane (Bege and Borbás, 2022). In addition, PMOs have better hybridization properties and higher specificity to the target than PS-ASOs (Summerton and Weller, 1997). Regarding metabolism and excretion, gapmer PS-ASOs cannot be fully modified because the administered oligonucleotide, which binds to a target complementary mRNA, has to be recognized by RNase H to degrade the mRNA (Aupy et al., 2017). As a result, there are generally a lot of metabolites in vivo, including those excreted in the urine, after administration of gapmer PS-ASOs (Geary et al., 2003; Yu et al., 2007; Post et al., 2019). Regarding the prediction of the human pharmacokinetics (PK) of gapmer PS-ASOs, PK parameters have been successfully extrapolated by allometric scaling in several studies (Boxenbaum, 1984; Boxenbaum and D’Souza, 1990; Chappell and Mordenti, 1991); this is a well established prediction tool that is important for effective drug development. In contrast, only limited information is available on the prediction of the human
PK of PMOs. Therefore, there is still a need to investigate how to accurately predict human exposure and plasma concentration profiles for PMOs.

In order to explore feasible prediction methodologies, we first summarized the PK, distribution, metabolism, and excretion (drug metabolism and pharmacokinetics; DMPK) characteristics of viltolarsen, as a representative PMO, in nonclinical and clinical studies. We then used allometric scaling from up to four species, namely, mice, rats, cynomolgus monkeys, and dogs, and assessed the accuracy of estimates of human total clearance (CL_{tot}) and volume of distribution at steady state (V_{dss}) by comparing the predicted and observed values.
Materials and methods

Compounds and biological reagents

Viltolarsen and $^{14}$C-viltolarsen were synthesized at Nippon Shinyaku Co., Ltd. Viltolarsen is a 21-base ASO with a PMO backbone (5’-CCTCCGGTTCTGAAGGTGTTC-3’; molecular weight, 6924.82). The radiolabeled position of $^{14}$C-viltolarsen is the 3’ end. Propranolol hydrochloride used as an internal standard (IS) was obtained from Fujifilm Wako Pure Chemical Corporation (Osaka, Japan) or Tokyo Chemical Industry (Tokyo, Japan). A natural (phosphodiester) oligonucleotide with the same sequence as viltolarsen was synthesized as a positive control for nuclease stability assays at Hokkaido System Science (Hokkaido, Japan). Human deoxyribonuclease I (DNase I) and phosphodiesterase I (PDE I) were purchased from Worthington Biochemical Corp. (NJ, USA). Liver microsomes and S9 fractions from male mice (CD-1), male rats (Sprague–Dawley), male cynomolgus monkeys, and mixed-gender humans were obtained from Sekisui XenoTech (KS, USA). The blank plasma used in the determination of human plasma concentrations was obtained from Veritas Corporation (CA, USA), who imported it from Tennessee Blood Services (TN, USA).

Animal studies and handling of blood in all species
Since DMD occurs almost exclusively in males, male animals were used in this study. CD-1 mice (7 weeks old), Sprague–Dawley rats (7 weeks old), cynomolgus monkeys (4 years old), and beagle dogs (5 years old) underwent a quarantine and acclimation period at three facilities (Nippon Shinyaku Co., Ltd, Shin Nippon Biomedical Laboratories, Ltd., and Huntingdon Life Sciences Ltd. [now Labcorp Drug Development Inc.]). The animals were housed at an appropriate temperature and humidity with regular ventilation and a 12-h light/dark cycle with artificial lighting. The mice and rats were given pellet chow and tap water ad libitum, the cynomolgus monkeys 100 to 200 g of standard diet per day and the dogs 250 g of pellet chow per day. The cynomolgus monkeys and dogs had free access to tap water at all times. Doses of viltolarsen in animal studies were set based on repeated-dose GLP toxicity studies (data not shown) to avoid side effects such as nephrotoxicity. All animal experimental procedures were performed in accordance with the regulations on animal experiments at each facility. Blood samples were taken from healthy volunteers in compliance with the version of the Ethical Guidelines for Medical and Health Research Involving Human Subjects that was valid when the study was conducted. The sera used for protein binding and stability assays in vitro were prepared from mice, rats, cynomolgus monkeys, and healthy volunteers at Nippon Shinyaku Co., Ltd. In the in vivo animal studies, after administration of viltolarsen and/or 14C-viltolarsen to mice, rats, cynomolgus monkeys, or dogs, blood samples were taken and plasma or serum was obtained by centrifugation. The radioactivity in plasma
following administration of $^{14}$C-viltolarsen was measured by liquid scintillation counting in a Tri-Carb 3100TR / Tri-Carb 3110TR or a Wallac 1409 scintillation counter (PerkinElmer, MA, USA) and plasma concentrations of viltolarsen were determined by high-performance liquid chromatography with tandem mass spectrometry (LC-MS/MS; LC = 10A, 20A, UFLC [Nexera, Shimadzu, Kyoto, Japan]; MS/MS = API3200, API4000, TQ4500 [Sciex, MA, USA]).

**Clinical trials**

The plasma concentrations, PK parameters, and body weights were obtained from a phase I/II study in Japan (JAPIC CTI-163291 (Komaki et al., 2020)) and a phase II study in the US (NCT02740972 (Clemens et al., 2020)) after intravenous infusion (1 h) once weekly at 40 or 80 mg/kg for 24 weeks to male pediatric DMD patients who are amenable to exon 53 skipping therapy. Both clinical trials were performed in accordance with the principles of the Declaration of Helsinki and Good Clinical Practice regulations.

**Bioanalytical methods for viltolarsen with LC-MS/MS**

Plasma concentrations of viltolarsen were determined by a different bioanalytical method for each animal species and humans based on LC-MS/MS equipped with a Unison UK-Amino column (3-μm particle size; 2.0 × 75 mm or 3.0 × 150 mm; Imtakt, Kyoto, Japan).
The lower limits of quantification were 0.5 µg/mL (mouse), 0.04 µg/mL (cynomolgus monkey), 0.02 µg/mL (dog), and 0.0191 µg/mL (human), and the linearity of the calibration curves was confirmed for all methods. The mobile phases 0.1% formic acid and acetonitrile were used with appropriate elution gradients. The column was operated at 0.5 or 0.6 mL/min and 25°C or 40°C and the autosampler temperature was set at 20°C or 23°C. Compounds were quantified in positive-ion multiple-reaction-monitoring mode by applying the parent-to-product transitions viltolarsen m/z 1155 → 112 and propranolol m/z 260 → 116. All plasma samples with internal standard or standard solutions underwent solid-phase extraction on Oasis HLB µElution Plates (Waters, MA, USA), and the eluates (1 to 10 µL) were subjected to LC-MS/MS analysis.

Serum protein binding in vitro

14C-Viltolarsen (1 and 10 µg/mL) was added to each serum sample from mice, rats, cynomolgus monkeys, and humans. A 0.5-mL aliquot of each serum sample was transferred to an ultracentrifuge tube and centrifuged at 436,000 × g for 180 min at 15°C, upon which the samples separated into three layers according to density. The radioactivity in a 0.1-mL aliquot of each middle layer (unbound fraction; Cf) and an uncentrifuged serum sample (total; Ct) was determined by liquid scintillation counting. The degree of protein binding of
$^{14}$C-viltolarsen (%) was calculated as $(1 - \frac{C_f}{C_t}) \times 100$, where $C_t$ is the total radioactivity (dpm) and $C_f$ is the radioactivity in the unbound fraction (dpm).

**Metabolic stability of viltolarsen in vitro and in vivo**

The *in vitro* metabolic stability of viltolarsen was confirmed in the presence of serum, liver S9 (2 mg protein/mL) or microsomes (2 mg protein/mL) from mice, rats, cynomolgus monkeys, and humans. NADPH (0.88 mg/mL) was included in the liver and microsome reaction mixtures. After the addition of $^{14}$C-viltolarsen (ca. 0.1 mg/mL), the serum and reaction mixtures were incubated at 37°C for 60 or 120 min. To human DNase I (0.1 mg/mL) or PDE I (0.02 mg/mL) were added $^{14}$C-viltolarsen (ca. 0.1 mg/mL) and positive-control DNA (ca. 0.1 mg/mL), and the reaction mixtures were incubated at 37°C for 30 min. (All concentrations given above are final concentrations.) Reaction mixtures without biological reagents were also prepared as negative controls, and all reactions were terminated by appropriate procedures. The serum and reaction mixtures were deproteinized and centrifuged, and the supernatants were subjected to high-performance liquid chromatography with a radio detector (radio-HPLC; Radiomatic D-515TR; PerkinElmer). For the *in vivo* metabolic stability tests in cynomolgus monkeys, the plasma obtained at 0.083, 0.25, 0.5, and 1 h post-dose and the pooled urine collected from 6 to 24 h post-dose after deproteinization were analyzed by radio-HPLC.
Excretion studies

$^{14}$C-Viltolarsen was administered at 20 mg/kg to rats and cynomolgus monkeys and the urine, feces, expired air (rats only), cage washings, cage debris (cynomolgus monkeys only) and kidneys (rats only) were collected. The collecting time periods for urine were 0 to 6 h, 6 to 24 h, and every 24-h period thereafter until 168 h post-dose. The feces, cage washings (cynomolgus monkeys only), and cage debris (cynomolgus monkeys only) were collected every 24 h until 168 h post-dose. The expired air, cage washings, and kidneys of rats were collected at 24, 168, and 168 h, respectively. The radioactivity of all biological samples obtained was measured by liquid scintillation counting.

Interspecies scaling from animals to humans for prediction of CL_{tot} and Vd_{ss}

Prediction of CL_{tot} and Vd_{ss} in humans was conducted by various allometric scalings using up to four species (mice, rats, cynomolgus monkeys, and dogs).

1-species scaling

Human CL_{tot} was predicted as reported by Tang et al. (2007) (eqs. 1 to 3) and Nanavati et al. (2021) (eq. 4).

\[ CL_{\text{tot, human/kg}} = 0.152 \times (CL_{\text{rat/kg}}) \] (1)

\[ CL_{\text{tot, human/kg}} = 0.407 \times (CL_{\text{monkey/kg}}) \] (2)
\[ \text{CL}_{\text{tot, human/kg}} = 0.410 \times (\text{CL}_{\text{dog/kg}}) \quad (3) \]

\[ \text{CL}_{\text{tot, human}} = \text{CL}_{\text{tot, animal}} \times \left( \frac{\text{human weight}}{\text{animal weight}} \right)^b \quad (4) \]

where \( b \) is the exponent of the allometric equation, which was set to 0.75 or 1.

For prediction of human \( V_{ds} \), eq. 5 with a fixed exponent of 1 was used.

\[ V_{ds, \text{human}} = V_{ds, \text{animal}} \times \left( \frac{\text{human weight}}{\text{animal weight}} \right) \quad (5) \]

**2-species scaling**

Human \( \text{CL}_{\text{tot}} \) was predicted from eqs. 6 and 7 from 2 species, rat-monkey and rat-dog, as reported by Tang et al. (2007).

\[ \text{CL}_{\text{tot, human}} = a_{\text{rat-monkey}} \times (\text{human weight})^{0.650} \quad (6) \]

\[ \text{CL}_{\text{tot, human}} = a_{\text{rat-dog}} \times (\text{human weight})^{0.628} \quad (7) \]

In both equations, \( a \) is the coefficient from 2-species scaling.

To predict human \( V_{ds} \) from two species, the usual equation, eq. 8, was adopted.

\[ V_{ds, \text{human}} = a \times (\text{human weight})^b \quad (8) \]

where \( a \) and \( b \) were determined from the relationship between body weight and \( V_{ds} \) in each animal used for 2-species scaling.

**3- or 4-species scaling**

Scaling of \( \text{CL}_{\text{tot}} \) from 3 or 4 species was based on eq. 9.

\[ \text{CL}_{\text{tot, human}} = a \times (\text{human weight})^b \quad (9) \]
For $V_{dss}$, eq. 8 was also used for comparison with other estimated values. In both predictions, $a$ and $b$ were determined from the relationship between body weight and $V_{dss}$ in each animal used for scaling.

**Assessment of prediction accuracy**

The accuracy of predictions was determined for each estimate from eq. 10:

\[
\text{Ratio} = \frac{\text{predicted value}}{\text{observed value}} \quad (10)
\]

**Comparison of observed and predicted plasma concentration profiles from pharmacokinetic parameters obtained in cynomolgus monkey**

On the basis of the plasma concentrations obtained from cynomolgus monkeys, PK profiles in humans were simulated, and the estimated and observed profiles were compared under the same administration conditions in humans. To describe the PK profiles in cynomolgus monkeys, a two-compartment PK model was built, and the PK parameters of the model were estimated. The plasma concentration of viltolarsen in humans after a 1-h intravenous infusion (40 or 80 mg/kg) was simulated from the PK parameters of the two-compartment model obtained from cynomolgus monkeys. Simulated plasma time-concentration profiles were compared to those obtained in clinical trials.
Analysis software

PK parameters calculated by non-compartmental PK analysis in all species and simulated plasma concentration profiles obtained from cynomolgus monkey data were determined with Phoenix WinNonlin™ (Certara, NJ, US).
Results

Plasma pharmacokinetics

Irrespective of the administration procedure in the in vivo experiments, the plasma concentrations decreased rapidly in all species after intravenous administration (Fig. 1 and Table 1). In a previous report, the values of AUC₀-t for viltolarsen in humans after the first infusion at 1.25, 5, and 20 mg/kg were 8.41 ± 1.31, 28.7 ± 3.9, and 98.9 ± 54.1 µg·h/mL, respectively (Komaki et al., 2018), and linearity was confirmed up to 80 mg/kg in a phase I/II (P1/2) study (Komaki et al., 2020) and a phase II (201) study (Clemens et al., 2020). Thus, systemic exposure to viltolarsen in humans increased in a dose-dependent manner from 1.25 to 80 mg/kg. In rats and cynomolgus monkeys, exposure to viltolarsen also increased in a dose-dependent manner in the dose range of 6 to 60 mg/kg. CL₉₀ in humans was lower than in rodents and higher than in dogs; however, it was almost the same in cynomolgus monkeys and humans. In contrast, Vd₉₀ in humans was a little higher than in all the other species.

Protein binding

Serum protein binding assays were conducted by ultracentrifugation, and the results are shown in Table 2. The serum protein binding ratios did not depend on the concentration of ¹⁴C-viltolarsen (1000 and 10000 ng/mL). The mean serum binding ratios of ¹⁴C-viltolarsen in mice, rats, cynomolgus monkeys, and humans were 24.4%, 31.0%, 36.2%, and 39.9%,
respectively; thus, relatively low serum protein binding was observed in all species, with no great differences among the species.

**Metabolic stability of viltolarsen**

The metabolism data are shown in Table 3 (in vitro) and Table 4 (in vivo). After incubation of $^{14}$C-viltolarsen with liver microsomes or S9 fractions from animals and humans in the presence of NADPH, close to 100% of the viltolarsen remained unchanged in all reactions, indicating no apparent metabolism by cytochrome P450 in any species. After incubation of $^{14}$C-viltolarsen with human DNase I or PDE I, viltolarsen was similarly unchanged in spite of the virtually complete digestion by both nucleases of positive-control DNA with the same sequence as viltolarsen. In addition, close to 100% of the viltolarsen remained unchanged in serum from all species tested. Thus, viltolarsen was stable to all biological reagents tested, and there were no interspecies differences in vitro.

To demonstrate stability in vivo, the plasma and urine obtained after intravenous administration of 20 mg/kg $^{14}$C-viltolarsen to cynomolgus monkeys were analyzed. The predominant peak on the chromatograms by radio-HPLC was unchanged viltolarsen, and the peak ratios of viltolarsen to total radioactivity were 92.1% or greater in all plasma and urine samples, indicating that viltolarsen was stable and excreted in the urine as the unchanged drug in cynomolgus monkeys. Thus, there was no clear evidence of metabolism of viltolarsen.
either *in vitro* (cytochrome P450 system and serum in all species, and human nucleases) or *in vivo* (cynomolgus monkeys).

**Excretion**

After administration of $^{14}\text{C}$-viltolarsen to rats (20 mg/kg) or cynomolgus monkeys (20 mg/kg), renal excretion was the major elimination route of radioactivity, with less than 10% of the administered dose being recovered in the feces in both species (Table 5). In the cynomolgus monkeys, the low proportion of the dose present in the feces indicates that the radioactivity present in the cage washings and cage debris was likely to have been associated predominantly with urinary excreted material (e.g., urine-soaked food residues). No radioactivity was detected in the expired air from the rats. Most of the radioactivity was excreted within the first 24 h. However, small but measurable amounts continued to be excreted in the urine and feces throughout the 7-day collection period. In rats, 1.20% of the administered dose was present in the kidneys at 168 h post-dose.

**Prediction of human CL$_{\text{tot}}$ and Vd$_{ss}$ using 1- to 4-species allometric scaling approaches**

Table 6 shows the predicted/observed ratios for CL$_{\text{tot}}$ and Vd$_{ss}$ of viltolarsen in humans, and Fig. 2 shows the linearity of these ratios with body weight in 3- and 4-species allometries and the individual and average observed values in humans. Table 1 shows the plasma
pharmacokinetic parameters obtained at 15 mg/kg for mice and 20 mg/kg for rats, cynomolgus monkeys, and dogs, and the body weights were used for allometric scaling.

Nominal body weights were used for mice (0.02 kg) and rats (0.25 kg), while the mean actual body weights were used for cynomolgus monkeys (5.71 kg; range, 4.19 to 6.67 kg) and dogs (13.6 kg; range, 12 to 15 kg). In humans, the recorded individual body weights were used to obtain CL\textsubscript{tot} and V\textsubscript{dss}. For both CL\textsubscript{tot} and V\textsubscript{dss}, the human measurements can be accurately extrapolated from the straight line for mouse, rat, and cynomolgus monkey (Fig. 2II, α panels), whereas in the other 3- and 4-species predictions that include the dog data, the human measurements cannot be accurately extrapolated (Fig. 2 and Table 6). The predicted human CL\textsubscript{tot} values indicate that 1-species prediction from cynomolgus monkeys with the allometry exponent (b) set to 1 was the closest to the observed value, with a ratio of 0.854. The mean values of the predicted human V\textsubscript{dss} determined by all equations were lower than the observed ones, although 1-species prediction from rats or cynomolgus monkeys and 3-species prediction from mice, rats, and cynomolgus monkeys, at ratios of close to 0.7, were not too far from 1.

**Plasma concentration profiles determined from cynomolgus monkey plasma concentrations**
As described above, the prediction of human \( \text{CL}_{\text{tot}} \) only from cynomolgus monkeys provided the most accurate estimate of human \( \text{CL}_{\text{tot}} \), and there was little difference in the prediction of \( \text{Vd}_{\text{ss}} \) between 1-species prediction from cynomolgus monkeys and other predictions (1-species prediction from rats and 3-species prediction from mice, rats, and cynomolgus monkeys). Therefore, human PK profiles were predicted from a two-compartment model by allometric scaling from cynomolgus monkeys, and the results are shown in Fig. 3. The human PK profiles predicted from cynomolgus monkeys fit the observed human plasma concentrations well.
Discussion

Many DMPK studies have shown that PMOs have low protein binding in serum/plasma, are highly resistant to nucleases, and are rapidly eliminated from the blood circulation, mainly by urinary excretion (Amantana and Iversen, 2005; Amantana et al., 2007; Dirin and Winkler, 2013; Takakusa et al., 2023). However, there are few reports summarizing the DMPK properties of a specific compound such as a gapmer in non-clinical studies (Yu et al., 2007; Post et al., 2019) or evaluating metabolism and excretion in vivo in animals. In addition, for drug development to proceed smoothly in terms of human PK prediction, it is important to understand the DMPK characteristics of drug candidates in animals.

In this paper, we present novel data that are informative for human PK prediction. The metabolic stability of nucleic acid analogues shows little interspecies difference. Thus, similar metabolites of the PS-ASO ISIS104838 were observed in mouse, cynomolgus monkey, and human urine (Geary et al., 2003). Komaki et al. (2020) reported a high urinary excretion ratio of the unchanged drug after administration of viltolarsen to humans, similar to our findings that most of the drug was excreted unchanged in the urine in cynomolgus monkeys, and that there were no across-species differences, including in the in vitro metabolic stability data. Furthermore, the nuclease resistance of PMOs greatly increases their renal clearance, which is related to renal injury, a characteristic toxicological finding of PMOs (Sazani et al., 2011), and their excretion ratio is higher than that of PS-ASOs due to the low protein binding of
PMOs in the serum/plasma (Takakusa et al., 2023). Thus, CL$_{tot}$ could be considered to be approximately equal to the renal clearance, and the CL$_{tot}$ values in mice, rats, cynomolgus monkeys, and dogs are similar to the respective glomerular filtration rates (GFR) (De Vries et al., 1997; Laroute et al., 1999; Luippold et al., 2002; Iwama et al., 2014). These reviews provide a comprehensive understanding of the DMPK of PMOs in general and viltolarsen in particular.

Historically, the PK of PS-ASOs in humans have been predicted through allometric scaling based on non-clinical animal data (Geary et al., 2003; Yu et al., 2007; Mahmood, 2011). Several recent studies have suggested that single-species allometric scaling from monkeys is the superior way to predict the human PK of PS-ASOs; there are big differences in human predictions between rodents and monkeys, with monkeys providing the more accurate predictions Yu et al., 2015; Wang et al., 2019; Nanavati et al., 2021). On the other hand, there have been no reports of human PK prediction for PMOs; therefore, in the present study we aimed to explore methods to predict the human PK of PMOs and we investigated several allometric scaling approaches using up to four species. We obtained the most accurate prediction of human CL$_{tot}$ by 1-species allometry from cynomolgus monkey with an exponent of 1 (ratio obtained, 0.854) rather than the exponent of 0.75 that is usually used in small-molecule PK prediction (ratio obtained, 0.584; Table 6); this result is the same as the prediction for PS-ASOs (Geary, 2009; Wang et al., 2019; Nanavati et al., 2021). However,
even though monkey with an exponent of 1 gave the best estimate, the range of the ratios (0.579 to 1.52) was extremely broad. One reason for this could be that body weight and GFR were not correlated; such a lack of correlation has a particularly large impact on PK prediction in pediatric patients, who are easy to distinguish from adolescent or adult patients on this basis (Cristea et al., 2020). In fact, in this study the body weights of patients treated with viltolarsen ranged from 14.9 to 52.1 kg, and there was a tendency to a negative correlation between body weight and CL_\text{tot} (Supplemental Fig. 1). A similar relationship between body weight and GFR in dogs has been reported (McKenna et al., 2020). Thus, the CL_\text{tot} and V_{\text{dss}} outliers we obtained for dogs in the 4-species allometric scaling approach may be related to body weight because the average weight of the beagle dogs we used, 13.6 kg, was greater than the reported weight of 11.1 kg (El-Wahab et al., 2023). In addition, in the present study CL_\text{tot} for the two heavier cynomolgus monkeys, corrected for body weight, was lower than that of the lightest one. Therefore, it is important to take body weight into consideration to achieve accurate prediction of CL_\text{tot} and systemic exposure.

The V_{\text{dss}} predictions were no better than the CL_\text{tot} predictions by any of the methods tried. Eteplirsen, which also has a PMO backbone, showed the same tendency as viltolarsen, the cynomolgus monkey values being lower than the human values at a dose near the clinical dose (Highlights of prescribing information / Exondys 51 (eteplirsen) injection, Revised: 1/2022; Sazani et al., 2011). In DMD patients, the lack of dystrophin in the muscle causes
myofiber death, fibrotic infiltration, and mitochondrial dysfunction, resulting in muscle weakness, associated motor delays, loss of ambulation, respiratory impairment, and cardiomyopathy (Reid and Alexander, 2021); accordingly, it is considered that the tissue distribution of ASOs in DMD patients would be enhanced by an ability to penetrate muscle tissue. In the present study, we administered viltolarsen to \textit{mdx} mice, which are often used as a DMD model, and wild-type mice, and obtained the \textit{Vd}_{ss} data; however, unexpectedly, the \textit{Vd}_{ss} values in the \textit{mdx} mice were lower than in the wild-type mice (Supplemental Table 1). Therefore, another way will need to be found to improve \textit{Vd}_{ss} prediction in humans.

This study presents several useful additions to our current knowledge. Firstly, from the non-clinical DMPK data for viltolarsen we can better understand the PK characteristics of PMOs, including species similarities and differences. Secondly, predictions of human \textit{CL}_{tot} and \textit{Vd}_{ss} conducted using several allometric scaling approaches suggest that 1-species allometry from cynomolgus monkey with an exponent of 1 is the best way to predict human \textit{CL}_{tot}. The simulated human PK profiles obtained from plasma concentration in cynomolgus monkey suggest that the \textit{Vd}_{ss} value estimated by 1-species allometry from cynomolgus monkey is acceptable because there were only negligible differences between the predicted and observed values in humans. In addition, it is important to consider the body weight of cynomolgus monkeys to accurately predict systemic exposure in pediatric patients.
The results presented here are expected to be beneficial to the efficient and effective
development of PMOs, specifically suggesting that 1-species allometry from cynomolgus
monkey combined with minimal animal experiments is potentially the best way to accurately
predict the human PK of PMOs. These findings are also expected to contribute to the
development of other uncharged new modalities such as methylphosphonates and
methoxypropylphosphonates, especially in translational research.
Acknowledgements

We thank Dr. Gerald E. Smyth, Discovery Research Laboratories in Tsukuba, Nippon Shinyaku Co., Ltd., and Dr. Hiromu Nakajima, chief medical officer, Nippon Shinyaku Co., Ltd., for their help with the manuscript.
Data Availability Statement

The authors declare that all the data supporting the findings of this study are contained within the paper.
Authorship Contributions

Contributed to study conception and design: Imai, Suda, Mori, Sasaki, Yamada, Kusano.

Conducted animal experiments and PK analysis: Imai, Suda, Mori, Sasaki.

Conducted allometric scaling: Imai, Suda.

Wrote or contributed to the writing of the manuscript: Imai, Suda, Mori, Sasaki, Yamada, Kusano.
Declaration of interest

The authors declare no competing financial interest. This study was supported by Nippon Shinyaku Co., Ltd.
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Figure Legends

**Fig. 1** Plasma concentration of viltolarsen or $^{14}$C-viltolarsen following a single intravenous administration of each compound to mouse, rat, cynomolgus monkey, and dog at a dose level of 15 or 20 mg/kg. Mouse, closed triangles; rat, closed squares; cynomolgus monkey, closed circles; dog, closed triangles. Values are presented as the mean ± standard deviation (mouse [n = 3], rat [n = 3], cynomolgus monkey [n = 3], dog [n = 5]). Lower limits of quantification: 0.5 µg/mL (mouse), 0.00317 µg eq./mL (rat), 0.04 µg/mL (cynomolgus monkey), and 0.02 µg/mL (dog).

**Fig. 2** Linearity of 3- and 4-species allometries in mouse, rat, cynomolgus monkey, and dog, and observed values in humans (Japanese and non-Japanese). Animals, closed squares; human (individual), open circles; human (n = 27; mean), gray triangles. Body weights: mouse, 0.02 kg (nominal); rat, 0.25 kg (nominal); cynomolgus monkey, 5.71 kg (actual); dog, 13.6 kg (actual).

**Fig. 3** Plasma concentration of viltolarsen predicted from cynomolgus monkey and observed in Japanese and non-Japanese DMD patients after intravenous infusion (1 h) at (a) 40 mg/kg and (b) 80 mg/kg. Simulated plasma concentrations, open gray circles merging into solid gray line; observed plasma concentrations in Japanese patients on Day 1, closed triangles; observed plasma concentrations in non-Japanese patients on Day 1,
open squares. Values are presented as the mean ± standard deviation (n = 5–8). Lower limit of quantification: 0.0191 µg/mL.
Table 1 Plasma pharmacokinetic parameters of viltolarsen in mouse, rat, cynomolgus monkey, dog, and human following intravenous administration on Day 1 (single dose)

<table>
<thead>
<tr>
<th>Species</th>
<th>Test article</th>
<th>Dose (mg/kg)</th>
<th>Route</th>
<th>AUC0-24h (µg・h/mL)</th>
<th>AUC0-last (µg・h/mL)</th>
<th>AUC0-inf (µg・h/mL)</th>
<th>t1/2 (h)</th>
<th>CLtot (mL/h/kg)</th>
<th>Vdss (mL/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td>Viltolarsen</td>
<td>15</td>
<td>Slow bolus</td>
<td>24.9</td>
<td>NA</td>
<td>NA</td>
<td>0.242</td>
<td>705</td>
<td>168</td>
</tr>
<tr>
<td></td>
<td></td>
<td>60</td>
<td></td>
<td>93.6</td>
<td>NA</td>
<td>NA</td>
<td>0.213</td>
<td>725</td>
<td>149</td>
</tr>
<tr>
<td>Rat</td>
<td>14C-Viltolarsen</td>
<td>6</td>
<td>Bolus</td>
<td>NA</td>
<td>NA</td>
<td>17.5 ± 4.0</td>
<td>1.19 ± 0.74</td>
<td>358 ± 95</td>
<td>201 ± 61</td>
</tr>
<tr>
<td></td>
<td></td>
<td>20</td>
<td></td>
<td>NA</td>
<td>NA</td>
<td>61.4 ± 6.4</td>
<td>1.19 ± 0.13</td>
<td>328 ± 33</td>
<td>184 ± 1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>60</td>
<td></td>
<td>NA</td>
<td>NA</td>
<td>207 ± 44</td>
<td>10.5 ± 3.0</td>
<td>298 ± 60</td>
<td>217 ± 54</td>
</tr>
<tr>
<td>Cynomolgus monkey</td>
<td>14C-Viltolarsen</td>
<td>20</td>
<td>Bolus</td>
<td>NA</td>
<td>161 ± 39</td>
<td>163 ± 38</td>
<td>1.63 ± 0.06</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>Viltolarsen</td>
<td>6</td>
<td>Bolus</td>
<td>NA</td>
<td>38.1 ± 6.7</td>
<td>38.3 ± 6.8</td>
<td>2.10 ± 0.17</td>
<td>160 ± 32</td>
<td>185 ± 44</td>
</tr>
<tr>
<td></td>
<td></td>
<td>20</td>
<td></td>
<td>NA</td>
<td>123 ± 19</td>
<td>124 ± 19</td>
<td>2.00 ± 0.26</td>
<td>164 ± 28</td>
<td>179 ± 39</td>
</tr>
<tr>
<td></td>
<td></td>
<td>60</td>
<td></td>
<td>NA</td>
<td>437 ± 143</td>
<td>430</td>
<td>3.4</td>
<td>157</td>
<td>196</td>
</tr>
<tr>
<td>Dog</td>
<td>Viltolarsen</td>
<td>20</td>
<td>Bolus</td>
<td>NA</td>
<td>289 ± 87</td>
<td>290 ± 87</td>
<td>1.38 ± 0.64</td>
<td>75.8 ± 29.3</td>
<td>103 ± 36</td>
</tr>
<tr>
<td>Human (P1/2)</td>
<td>Viltolarsen</td>
<td>40</td>
<td>Infusion (1 h)</td>
<td>NA</td>
<td>235 ± 60</td>
<td>235 ± 60</td>
<td>2.43 ± 0.527</td>
<td>179 ± 37.7</td>
<td>238 ± 39.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>80</td>
<td></td>
<td>NA</td>
<td>490 ± 125</td>
<td>491 ± 125</td>
<td>2.49 ± 0.163</td>
<td>164 ± 51.5</td>
<td>223 ± 69.4</td>
</tr>
<tr>
<td>Human (201)</td>
<td>Viltolarsen</td>
<td>40</td>
<td>Infusion (1 h)</td>
<td>176 ± 30.1</td>
<td>175 ± 31.4</td>
<td>175 ± 31.1</td>
<td>1.89 ± 0.726</td>
<td>234 ± 41.4</td>
<td>297 ± 18.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>80</td>
<td></td>
<td>437 ± 134</td>
<td>436 ± 136</td>
<td>436 ± 135</td>
<td>2.21 ± 0.497</td>
<td>196 ± 50.2</td>
<td>283 ± 41.6</td>
</tr>
</tbody>
</table>

Values are presented as the mean (mouse [n = 3] and cynomolgus monkey [n = 2; 60 mg/kg]) or mean ± standard deviation (rat [n = 3], cynomolgus monkey [n = 3; except for 60 mg/kg], dog [n = 5], and human [n = 5–8]). Viltolarsen plasma concentrations were determined by four different bioanalytical methods with LC-MS/MS and 14C-viltolarsen plasma concentrations were measured by LSC. It took about 2 to 3 min to administer to mice, so the route is described as 'slow bolus'. When the values of AUC0-24h were calculated, plasma concentrations below the LLOQ were treated as 0 µg/mL. NA: not applicable.
<table>
<thead>
<tr>
<th>Concentration</th>
<th>Species</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mouse</td>
<td>Rat</td>
<td>Cynomolgus monkey</td>
<td>Human</td>
<td></td>
</tr>
<tr>
<td>1 µg/mL</td>
<td>23.0 ± 0.8</td>
<td>29.7 ± 3.1</td>
<td>36.1 ± 5.3</td>
<td>39.4 ± 0.7</td>
<td></td>
</tr>
<tr>
<td>10 µg/mL</td>
<td>25.7 ± 1.9</td>
<td>32.2 ± 2.1</td>
<td>36.2 ± 2.1</td>
<td>40.3 ± 2.6</td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>24.4</td>
<td>31.0</td>
<td>36.2</td>
<td>39.9</td>
<td></td>
</tr>
</tbody>
</table>

Values are presented as the mean ± standard deviation (n = 3).
<table>
<thead>
<tr>
<th>Species</th>
<th>Biological reagents</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Serum</td>
</tr>
<tr>
<td>Mouse</td>
<td>97.5</td>
</tr>
<tr>
<td>Rat</td>
<td>104</td>
</tr>
<tr>
<td>Cynomolgus monkey</td>
<td>101</td>
</tr>
<tr>
<td>Human</td>
<td>102</td>
</tr>
</tbody>
</table>

Values are expressed as the percentage of $^{14}$C-viltolarsen remaining after incubation. NA: not applicable.
**Table 4** Ratio of peak area (%) of $^{14}$C-viltolarsen to that of total radioactivity in cynomolgus monkey plasma and urine after a single intravenous administration at 20 mg/kg

<table>
<thead>
<tr>
<th>Sample</th>
<th>Sampling times (h)</th>
<th>Peak area ratio (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma</td>
<td>0.083</td>
<td>92.1 ± 1.0</td>
</tr>
<tr>
<td></td>
<td>0.25</td>
<td>98.3 ± 1.8</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>96.4 ± 6.3</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>98.4 ± 2.7</td>
</tr>
<tr>
<td>Urine</td>
<td>6-24</td>
<td>94.3 ± 2.7</td>
</tr>
</tbody>
</table>

Values are presented as the mean ± standard deviation (n = 3).
<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Urine</th>
<th>Feces</th>
<th>Cage washings</th>
<th>Total</th>
<th>Urine</th>
<th>Feces</th>
<th>Cage washings</th>
<th>Cage debris</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>0–6</td>
<td>71.9 ±</td>
<td>23.2</td>
<td>NA</td>
<td>71.9 ±</td>
<td>23.2</td>
<td>8.74 ±</td>
<td>10.18</td>
<td>NA</td>
<td>NC</td>
</tr>
<tr>
<td>0–24</td>
<td>87.5 ±</td>
<td>9.1</td>
<td>5.75 ± 4.29</td>
<td>NA</td>
<td>93.2 ±</td>
<td>6.1</td>
<td>23.5 ± 15.2</td>
<td>0.05 ± 0.04</td>
<td>20.8 ±</td>
</tr>
<tr>
<td>0–48</td>
<td>88.6 ±</td>
<td>8.8</td>
<td>7.01 ± 5.01</td>
<td>NA</td>
<td>95.6 ±</td>
<td>5.3</td>
<td>25.1 ± 14.9</td>
<td>0.28 ± 0.40</td>
<td>23.2 ±</td>
</tr>
<tr>
<td>0–72</td>
<td>89.0 ±</td>
<td>8.8</td>
<td>7.37 ± 5.20</td>
<td>NA</td>
<td>96.4 ±</td>
<td>5.2</td>
<td>26.4 ± 14.7</td>
<td>0.54 ± 0.64</td>
<td>24.1 ±</td>
</tr>
<tr>
<td>0–96</td>
<td>89.2 ±</td>
<td>8.8</td>
<td>7.49 ± 5.25</td>
<td>NA</td>
<td>96.7 ±</td>
<td>5.2</td>
<td>27.2 ± 14.4</td>
<td>0.74 ± 0.89</td>
<td>24.7 ±</td>
</tr>
<tr>
<td>0–120</td>
<td>89.4 ±</td>
<td>8.8</td>
<td>7.58 ± 5.29</td>
<td>NA</td>
<td>96.9 ±</td>
<td>5.2</td>
<td>27.8 ± 14.3</td>
<td>0.92 ± 1.06</td>
<td>26.2 ±</td>
</tr>
<tr>
<td>0–144</td>
<td>89.5 ±</td>
<td>8.8</td>
<td>7.66 ± 5.34</td>
<td>NA</td>
<td>97.2 ±</td>
<td>5.3</td>
<td>28.2 ± 14.2</td>
<td>1.09 ± 1.28</td>
<td>26.7 ±</td>
</tr>
<tr>
<td>0–168</td>
<td>89.6 ±</td>
<td>8.8</td>
<td>7.74 ± 5.37</td>
<td>0.48 ±</td>
<td>0.40</td>
<td>99.0 ±</td>
<td>5.0*</td>
<td>28.4 ± 14.0</td>
<td>1.20 ±</td>
</tr>
</tbody>
</table>

Additional information: In the rat study, expired air was also collected for the first 48 h, and there was no detectable radioactivity in the expired air.

*a The residual radioactivity in the rat kidney (1.20 ± 0.23% of the dose) was included for the total recovery.

NA, not applicable; NC, not calculated
Table 6 Predicted/observed ratios for $\text{CL}_{\text{tot}}$ and $\text{Vd}_{\text{ss}}$ of viltolarsen in humans

a) $\text{CL}_{\text{tot}}$

<table>
<thead>
<tr>
<th>Equation</th>
<th>Species</th>
<th>Ratio (mean)</th>
<th>Ratio (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1)</td>
<td>rat</td>
<td>0.260</td>
<td>0.176 - 0.462</td>
</tr>
<tr>
<td>(2)</td>
<td>monkey</td>
<td>0.348</td>
<td>0.236 - 0.619</td>
</tr>
<tr>
<td>(3)</td>
<td>dog</td>
<td>0.162</td>
<td>0.110 - 0.288</td>
</tr>
</tbody>
</table>

1-species

| (4) | mouse (b = 0.75) | 0.612 | 0.438 - 0.923 |
|     | rat (b = 0.75)   | 0.535 | 0.383 - 0.808 |
|     | monkey (b = 0.75)| 0.584 | 0.418 - 0.882 |
|     | dog (b = 0.75)   | 0.336 | 0.240 - 0.507 |
|     | mouse (b = 1)    | 3.67  | 2.49 - 6.54   |
|     | rat (b = 1)      | 1.71  | 1.16 - 3.04   |
|     | monkey (b = 1)   | 0.854 | 0.579 - 1.52  |
|     | dog (b = 1)      | 0.395 | 0.268 - 0.703 |

2-species

| (6) | rat, monkey      | 0.402 | 0.294 - 0.579 |
| (7) | rat, dog         | 0.306 | 0.225 - 0.436 |

3-species

| (9) | mouse, rat, monkey | 0.560 | 0.401 - 0.843 |
|     | mouse, rat, dog    | 0.324 | 0.236 - 0.468 |
|     | mouse, monkey, dog | 0.407 | 0.295 - 0.597 |
|     | rat, monkey, dog   | 0.395 | 0.288 - 0.575 |

4-species

| (9) | all               | 0.407 | 0.295 - 0.596 |
### b) Vd_{ss}

<table>
<thead>
<tr>
<th>Equation</th>
<th>Species</th>
<th>Ratio (mean)</th>
<th>Ratio (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>1-species</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(5)</td>
<td>mouse</td>
<td>0.652</td>
<td>0.514 - 1.15</td>
</tr>
<tr>
<td></td>
<td>rat</td>
<td>0.715</td>
<td>0.563 - 1.26</td>
</tr>
<tr>
<td></td>
<td>monkey</td>
<td>0.695</td>
<td>0.547 - 1.23</td>
</tr>
<tr>
<td></td>
<td>dog</td>
<td>0.400</td>
<td>0.315 - 0.706</td>
</tr>
<tr>
<td><strong>2-species</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(8)</td>
<td>rat, monkey</td>
<td>0.685</td>
<td>0.542 - 1.20</td>
</tr>
<tr>
<td></td>
<td>rat, dog</td>
<td>0.364</td>
<td>0.294 - 0.580</td>
</tr>
<tr>
<td><strong>3-species</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>(8)</td>
<td>mouse, rat, monkey</td>
<td>0.719</td>
<td>0.563 - 1.28</td>
</tr>
<tr>
<td></td>
<td>mouse, rat, dog</td>
<td>0.407</td>
<td>0.332 - 0.679</td>
</tr>
<tr>
<td></td>
<td>mouse, monkey, dog</td>
<td>0.494</td>
<td>0.398 - 0.844</td>
</tr>
<tr>
<td></td>
<td>rat, monkey, dog</td>
<td>0.456</td>
<td>0.370 - 0.745</td>
</tr>
<tr>
<td><strong>4-species</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>(8)</td>
<td>all</td>
<td>0.502</td>
<td>0.406 - 0.854</td>
</tr>
</tbody>
</table>
Fig. 1

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Fig. 3
(a) 40 mg/kg
(b) 80 mg/kg
Plasma concentration (μg/mL) vs. Time after administration (h)