St. Jude Children's Research Hospital

P-PKSR
Preclinical
Pharmacokinetics

PRECLINICAL PHARMACOKINETIC REPORT

Developmental Biology and Solid Tumor Program

P-PKSR Study 833309 - 3179073

STUDY TITLE:

SCREENING PLASMA AND TUMOR PHARMACOKINETICS OF LURBINECTEDIN IN ATHYMIC NUDE MICE BEARING MAST 39 XENOGRAFT TUMORS AFTER A SINGLE ORAL DOSE

SHORT TITLE:	Lurbinectedin Screening Plasma and Tumor PK (SPTPK)
TEST ARTICLE:	Lurbinectedin (free base), PM01183, LY-01017
SECTION:	Nonclinical Pharmacokinetics (Non-GLP)
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SJCRH SRM2 O/R:	833309-3179073 Preclinical Pharmacokinetic Shared Resource
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Quality Statement

This non-GLP study was conducted using sound scientific principles and established techniques in accordance with the relevant guidelines and standard operating procedures (SOPs) of the Preclinical Pharmacokinetic Shared Resource (P-PKSR) and St. Jude Children's Research Hospital (SJCRH), Memphis, TN, USA. This report accurately reflects the data obtained during the course of this study.

These results represent part of an early phase preclinical pharmacology program. This study has been conducted to provide preliminary insights into the pharmacokinetic (PK) properties of the compound(s) in the indicated preclinical model(s). This study and its results are not intended to provide a comprehensive PK evaluation of the compound(s). The applied bioanalytical method was validated/qualified to support this specific study and discovery-style sample analyses.

Substantial study-to-study and inter-animal variability in preclinical PK exists. Such variability depends upon the in vivo scientists' experience, variations in compound purity and formulation, animal strains, sex and age, and other situational fixed effects (i.e. husbandry conditions, chow constituents, presence or absence of disease, concomitant drugs). As such, the actual PK, plasma or tissue compound concentrations, or equivalent dose in other studies or preclinical models may vary significantly from that reported herein.

1.0 METHODS

1.1 In Vivo Pharmacokinetic (PK) Study

The plasma and tumor pharmacokinetic (PK) profiles of lurbinectedin were evaluated in normal female Athymic nude mice (Charles River), approximately 8 to 12 weeks in age. Pharmacy grade lyophilized lurbinectedin powder was dissolved in sterile water to achieve the concentration at 0.5 mg/mL dosing stock. The dosing stock was further diluted to 0.018 mg/mL for a 0.18 mg/kg dose, using 10 mL/kg IV tail vein injection. Terminal samples were collected over a 36-hour post-dose period by cardiac puncture using a 1 mL syringe, and the blood placed in a Sarstedt Microvette K3EDTA 500 µL tube, and immediately processed to plasma. The carcass was then perfused with PBS, the tumor extracted, rinsed, and placed in a microcentrifuge tube. All samples were immediately stored on dry ice and transferred to -80 °C until analysis.

1.2 Bioanalysis

MAST39 tumor samples were weighed in 15 mL Lysing Matrix D tubes (MP Biomedicals, Santa Ana, CA), diluted with a 1:5 volume of ultra-pure water and homogenized using a FastPrep-24 system (MP Biomedicals, Santa Ana, CA) for three cycles of 1 min vibration at 6.5 M/S speed, with 5 min in ice bath between each cycle to prevent over-heating. The homogenates were then stored at -80 °C for 24 hours until analysis.

Plasma and tumor homogenate samples were analyzed for lurbinectedin (Medkoo Bisciences, CAT# 581065, LOT# T24D08P26, 98% Purity) using a qualified liquid chromatography – tandem mass spectrometry (LC-MS/MS) assay. Plasma calibrators and quality controls were spiked with solutions, corrected for salt content and purity as necessary, prepared in dimethyl sulfoxide: 10mM ammonium acetate (1:1, v/v) (pH 3.1). Plasma and tumor homogenate samples, 100 µL each, plus 5 µL of 200 ng/mL of trabectedin (MedChemExpress, CAT# HY-15605, LOT# 255518, 98.12% Purity) as an internal standard in of 0.1% formic acid in acetonitrile/water (1:1, v/v) was extracted with 1.4 mL of methyl-tert-butyl-ether. After 5 min vortex and 15 min centrifuge at 4400 rpm at 5°C, 1 mL of top layer was evaporated to dryness using CentriVap system (LABCONCO, Kansas City, MO) at 55°C. The dried extracts were reconstituted with 80 µL of 0.1% formic acid in acetonitrile/water (1:1, v/v) and vortexed for 1 min. A 6 µL aliquot of the reconstitute was injected onto a SCIEX ExionLC high performance liquid chromatography system via a SCIEX ExionLC autosampler. The LC separation was performed using a Waters XBridge C18 (2.5 µm, 75 mm x 2.1 mm) column maintained at 45 °C with gradient elution at a flow rate of 0.40 mL/min. The binary mobile phase consisted of water- ammonium hydroxide (100:0.1 v/v) in reservoir A and acetonitrile- ammonium hydroxide (100:0.1 v/v) in reservoir B. The initial mobile phase consisted of 10% B for 0.5 min with a linear increase to 98% B in 0.75 min. The column was then rinsed for 1.25 min at 98% B and returned to initial mobile phase conditions in 0.5 mins, then the column was equilibrated for another 1.5 min for a total run time of 4.5 min. Under these conditions, lurbinectedin and trabectedin (IS) were eluted at 2.57 and 2.42 min, respectively.

Analyte and IS were detected with tandem mass spectrometry SCIEX Triple Quad™ 3500 LC-MS/MS system in the positive ESI mode and the following mass transitions were monitored: Lurbinectedin 767.4 → 272.9, trabectedin 744.2 → 495.3. The method qualification and bioanalytical runs all passed acceptance criteria for non-GLP assay performance. A linear model ($1/X^2$ weighting) fit the calibrators across the 0.1 to 50 ng/mL range, with a correlation coefficient (R) of ≥ 0.9986. The lower limit of quantitation (LLOQ), defined as a peak area signal-to-noise ratio of 5 or greater verses a matrix blank with IS, was 0.1 ng/mL. Sample dilution integrity was confirmed. The intra-run precision and accuracy was ≤ 7.29% CV and 94.0% to 99.57%, respectively.

1.3 Pharmacokinetic (PK) Analysis

Plasma concentration-time (Ct) data for lurbinectedin was grouped by matrix and nominal time point. Manual imputation of data below the lower limit of quantitation (BLOQ) was as follows: IF at any time point ≥ 2/3rds of the Ct results were above the LLOQ, the BLOQ data were replaced with a value of ½

LLOQ, ELSE the entire time point's data were treated as missing. Summary statistics were calculated, and the arithmetic mean Ct values were subjected to noncompartmental analysis (NCA) using Phoenix WinNonlin 8.1 (Certara USA, Inc., Princeton, NJ). The IV bolus model was applied, and area under the Ct curve (AUC) values were estimated using the "linear up log down" method. The terminal phase was defined as at least three time points at the end of the Ct profile, and the elimination rate constant (Kel) was estimated using an unweighted log-linear regression of the terminal phase. The terminal elimination half-life (T1/2) was estimated as 0.693/Kel, and the AUC from time 0 to infinity (AUCinf) was estimated as the AUC to the last time point (AUClast) + Clast (predicted)/Kel. Other parameters estimated included observed maximum concentration (Cmax), time of Cmax (Tmax), concentration at the last observed time point (Clast), time of Clast (Tlast), back extrapolated initial concentration (C0), clearance (CL = Dose/AUCinf), volume of distribution at steady state (Vss), and the terminal volume of distribution (Vz).The plasma-to -tumor partition coefficient (Kp,inf) was estimated as the ratio of the AUCinf in brain to AUCinf plasma, whereas Kp,last was similarly estimated using AUClast values.

Lurbinectedin fraction unbound in mouse plasma (Fu,p,m) was determined using a HTD-96B equilibrium dialysis device (12-14K MWCO, HTDialysis LLC, Gales Ferry, CT). Briefly, blank plasma was spiked with compound at a final concentration of 5 µM. The spiked plasma was placed in one side of the membrane on the HTD-96B plate in sextuplicates, dialyzed against equal volume of the 100 mM PBS (pH 7.4), and permitted to equilibrate for 6 hours at 37 °C with 5% CO2. Compound concentrations were assayed on both side of the dialysis membrane using the qualified LC-MS/MS assay, with the fraction unbound calculated as the ratio of concentration in receiver to donor, adjusted for any dilution if necessary [1].

A clinically relevant dose (CRD) for mice was estimated from unbound plasma PK and exposure. The CRD was defined as the mouse dose achieving a predicted mean steady state unbound plasma AUC (AUCu) similar to humans at the single agent maximum tolerated dose (MTD), recommended Phase II dose (RP2D), or FDA-approved dose. Dose proportional, linear, and time-invariant PK across species was assumed. Human and mouse plasma protein binding were assumed similar when data were not available or were inconclusive. This is similar to the clinical relevance approach proposed by Spilker [2] which uses unbound plasma average steady state concentrations. Some latitude in dose rounding was permitted in the CRD recommendation, and an unbound exposure within 2-fold of the clinical target was considered acceptable. Additional considerations influenced the final recommended mouse dose, including mouse dosing regimens prevalent in the literature and the tolerability of the compound in mice.

2.0 RESULTS

Since MAST39 tumor samples concentrations were BLOQ by analyte peaks for all time points, NCA parameters could not be estimated. There were issues with inconsistent and variable responses of the internal standard (IS) trabectedin in tumor matrix during the analysis, which may have negatively impacted the accuracy of lurbinectedin quantitation. Another reason could be the instability of the compound in tumor tissue, since the samples were stored for a long period, approximately three months until the analysis. Even without IS normalization, concentrations of lurbinectedin by analyte peak area alone were observed as BLOQ, and are unreliable. Nonetheless, the tumor concentration estimates by analyte peak area can be found in **Table 4.5**. Both lurbinectedin and trabectedin covalently bind to the N2 of guanine in the minor groove of DNA in a slowly reversable fashion [3–5]. We speculate that these issues may stem from this binding – both agents may have low and variable detection in tissue matrix due to destructive covalent binding to DNA.

Lurbinectedin plasma Ct data demonstrated low variability between mice, with coefficients of variation ranging from 1.62% to 48.7%. After Cmax, occurring at the first sample time point of 0.125 hr post dose, plasma concentrations diminished in a bi-exponential manner with two observations at 24-hour (M14 and M15) and all observations at 36-hour time point being BLOQ. The apparent plasma terminal half-life of lurbinectedin was 5.26 hours. The plasma clearance (CL) of lurbinectedin was very high at 139 mL/min/kg, or approximately 154% of murine hepatic blood flow. The apparent plasma terminal volume of distribution (Vss) for lurbinectedin was high at 28.6 L/kg, in far excess of total body water.

In our equilibrium dialysis plasma protein binding study, mice demonstrated a very low fraction unbound value of 0.000729 ± 0.000145. However, regulatory documents claim that the fraction unbound across species is ~0.05 [6], and specifically in humans that it's less than 0.01 (range: 0.00151 to 0.00668) [6,7] depending on the corresponding alpha acid glycoprotein (AAG) concentration. Therefore, it is assumed that lurbinectedin is similarly and highly protein bound in both human and mouse plasma, with the caveat that inter-species differences in the drug's affinity to AAG and/or the expression of AAG may influence lurbinectedin PK.

In a population PK analysis across a series of lurbinectedin clinical studies, the mean value for total plasma clearance (CL) in the typical patient was 11.2 L/h, with a terminal half-life of 46 hours [8]. The FDA approved dose for lurbinectedin is 3.2 mg/m2 IV every 21 days [9]. Therefore, assuming similar plasma protein binding between humans and mice, an AUC-based CRD would be ~4.4 mg/kg. This far exceeds the reported MTD in mice of 0.18 mg/kg IV once weekly for 3 weeks [6].

The plasma exposure in our current PK study is lower than anticipated by allometric scaling across species using standard exponents of either 0.67 or 0.75 (see **Appendix 6.1**). Unfortunately, the mouse PK of lurbinectedin has not been reported in either regulatory documents or the literature for a more direct comparison. Our high systemic blood clearance of lurbinectedin in mice (207 mL/min/kg), when considering the reported blood-to-plasma partition ratio of 0.67 [6,8], exceeds reported murine hepatic blood flow values (90 to 120 mL/min/kg [10,11]). This suggests 1) extrahepatic metabolism or elimination of lurbinectedin occurs in mice, 2) lurbinectedin was unstable in mouse plasma at -80 °C during 3 months of storage, and/or 3) an IV dose of less than 0.18 mg/kg IV was administered in this study.

The metabolism and elimination of lurbinectedin was characterized both in vitro and in vivo in rats, monkeys, and humans [12]. Across the species, the IV clearance of lurbinectedin was low-to-moderate in proportion to hepatic blood flow. The majority of the clearance in all species was metabolic, and driven by CYP3A4/5 isoforms, but to a lesser extent in rats. Across the species, approximately 75-90% of the drug-related radioactivity was excreted in bile and feces as a mix of predominantly metabolites and unchanged drug. Only about 3-5% of total radioactivity was excreted in urine. The consistency of ADME processes in rodent and non-rodent species here suggests a low likelihood for mice having a different profile or exhibiting extrahepatic metabolism and elimination.

Plasma stability of lurbinectedin was studied most extensively for human, but also preliminarily for mouse, rat, dog, monkey, and mini-pig. Pernice et al. found that lurbinectedin was not stable in any species' plasma at room temperature after 2 or 4 hours, with losses of 26% and 37% respectively [13]. However, all plasma was found to be stable at 4 °C for 2 hours on the bench top, and extracted samples were stable for a 24-hour evaluation period. Conversely in a separate study, human plasma samples were found stable under all tested conditions, including room temperature for 16.5 hours and at -80 °C for 853 days [7]. While lurbinectedin instability in mouse plasma is a possibility, particularly around the experimental blood and plasma collection, these findings suggest a high likelihood of storage stability. Regarding a lurbinectedin dose less than 0.18 mg/kg being administered, there's the possibility that formulation was unstable, mis-diluted, or that the IV injection extravasated resulting in an effectively lower dose reaching circulation.

In conclusion, the current study suffers from multiple shortcomings making PK and CRD assessments for lurbinectedin inconclusive. Tumor penetration, while confounded by analytical issues, was apparently low and incongruent with lurbinectedin's large volume of distribution. Lurbinectedin's apparent plasma clearance was unexpectedly and unphysiologically high, and not in line with allometry or the known cross-species ADME profile for the drug. It is suggested that another tumor bearing PK study of lurbinectedin in mice be carefully redesigned and conducted.

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4.0 TABLES, LISTINGS, AND FIGURES (TLFS)

Figure 4.1: Mean (SD) Ct Profiles by Analyte and Group

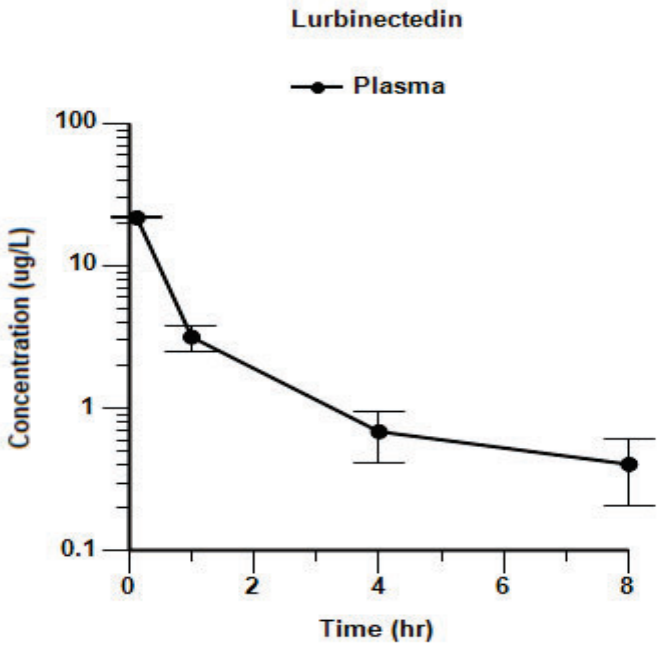


Table 4.1: NCA Parameter Estimates by Analyte and Group

		Analyte
		Lurbinectedin
		Group
		Plasma
Parameter	**Units**	**Estimate**
C0	ug/L	28.7
Cmax	ug/L	21.8
Tmax	hr	0.125
AUClast	hr*ug/L	18.6
AUCinf	hr*ug/L	21.7
Kel	1/hr	0.132
T1/2	hr	5.26
CL	L/hr/kg	8.31
Vss	L/kg	28.6
Clast	ug/L	0.405
Tlast	hr	8.00

Table 4.2: Full Summary Statistics of Ct Data by Analyte and Group

Lurbinectedin Screening Plasma and Tumor PK (SPTPK)

		Analyte
		Lurbinectedin
		Group
		Plasma
Time (hr)		Concentration (ug/L)
0.125	N	3
	Mean	21.8
	SD	0.353
	Min	21.4
	Median	22.0
	Max	22.0
	CV%	1.62
	Geometric Mean	21.8
	CV% Geometric Mean	1.63
1.00	N	3
	Mean	3.17
	SD	0.667
	Min	2.42
	Median	3.39
	Max	3.69
	CV%	21.1
	Geometric Mean	3.12
	CV% Geometric Mean	22.7
4.00	N	3
	Mean	0.686
	SD	0.269
	Min	0.496
	Median	0.568
	Max	0.995
	CV%	39.3
	Geometric Mean	0.654
	CV% Geometric Mean	38.2
8.00	N	3
	Mean	0.405
	SD	0.197
	Min	0.177
	Median	0.517
	Max	0.521
	CV%	48.7
	Geometric Mean	0.363
	CV% Geometric Mean	68.5
24.0	N	
	Mean	
	SD	
	Min	
	Median	

		Analyte
		Lurbinectedin
		Group
		Plasma
Time (hr)		Concentration (ug/L)
	Max	
	CV%	
	Geometric Mean	
	CV% Geometric Mean	
36.0	N	
	Mean	
	SD	
	Min	
	Median	
	Max	
	CV%	
	Geometric Mean	
	CV% Geometric Mean	

Table 4.3: Ct Data Listings by Subject, Analyte, Group, and Time

Subject	Analyte	Group	Time (hr)	Concentration (ug/L)
M1	Lurbinectedin	Plasma	0.125	21.99
M2	Lurbinectedin	Plasma	0.125	22.03
M3	Lurbinectedin	Plasma	0.125	21.40
M4	Lurbinectedin	Plasma	1.00	3.69
M5	Lurbinectedin	Plasma	1.00	3.39
M6	Lurbinectedin	Plasma	1.00	2.42
M7	Lurbinectedin	Plasma	4.00	0.50
M8	Lurbinectedin	Plasma	4.00	0.99
M9	Lurbinectedin	Plasma	4.00	0.57
M10	Lurbinectedin	Plasma	8.00	0.18
M11	Lurbinectedin	Plasma	8.00	0.52
M12	Lurbinectedin	Plasma	8.00	0.52
M13	Lurbinectedin	Plasma	24.0	0.45
M14	Lurbinectedin	Plasma	24.0	BLOQ
M15	Lurbinectedin	Plasma	24.0	BLOQ
M16	Lurbinectedin	Plasma	36.0	BLOQ
M17	Lurbinectedin	Plasma	36.0	BLOQ
M18	Lurbinectedin	Plasma	36.0	BLOQ

Table 4.4: Ct Summary (Mean, SD, N) by Analyte and Group

Analyte	Group	Time (hr)	Mean (ug/L)	SD (ug/L)	N
Lurbinectedin	Plasma	0.125	21.8	0.353	3
Lurbinectedin	Plasma	1.00	3.17	0.667	3
Lurbinectedin	Plasma	4.00	0.686	0.269	3
Lurbinectedin	Plasma	8.00	0.405	0.197	3
Lurbinectedin	Plasma	24.0			0
Lurbinectedin	Plasma	36.0			0

Table 4.5: Tumor Concentration Estimates of Lurbinectedin Without Internal Standard (IS) Normalization

Subject	Analyte	Group	Time (hr)	Concentration (ug/L)*
M1	Lurbinectedin	Tumor	0.125	1.5793
M2	Lurbinectedin	Tumor	0.125	2.7445
M3	Lurbinectedin	Tumor	0.125	1.3699
M4	Lurbinectedin	Tumor	1.00	1.8060
M5	Lurbinectedin	Tumor	1.00	0.58604
M6	Lurbinectedin	Tumor	1.00	0.92116
M7	Lurbinectedin	Tumor	4.00	0.79388
M8	Lurbinectedin	Tumor	4.00	1.0654
M9	Lurbinectedin	Tumor	4.00	1.2883
M10	Lurbinectedin	Tumor	8.00	0.68277
M11	Lurbinectedin	Tumor	8.00	0.32024
M12	Lurbinectedin	Tumor	8.00	0.66586
M13	Lurbinectedin	Tumor	24.0	0.65092
M14	Lurbinectedin	Tumor	24.0	0.53051
M15	Lurbinectedin	Tumor	24.0	0.42014
M16	Lurbinectedin	Tumor	36.0	1.2373
M17	Lurbinectedin	Tumor	36.0	0.78376
M18	Lurbinectedin	Tumor	36.0	0.017179

* NOTE: The lower limit of quantitation for tumor was 6 ng/mL, and all tumor concentrations are BLOQ.

5.0 ATTACHED FILES

Attached File 5.1 Lurbinectedin Screening Plasma and Tumor PK V1.0.docx – *Final in vivo study plan as executed*

Attached File 5.2 Lurbinectedin SPTPK TLFs.docx – *Report TLFs as a Word document for manipulation, plotting, and further presentation*

Attached File 5.3 Lurbinectedin TBPKStudySheet.pdf - *data collection form for the PK study from in vivo scientists.*

6.0 APPENDICES

Appendix 6.1 Allometric Scaling Analysis of Lurbinectedin Across Species

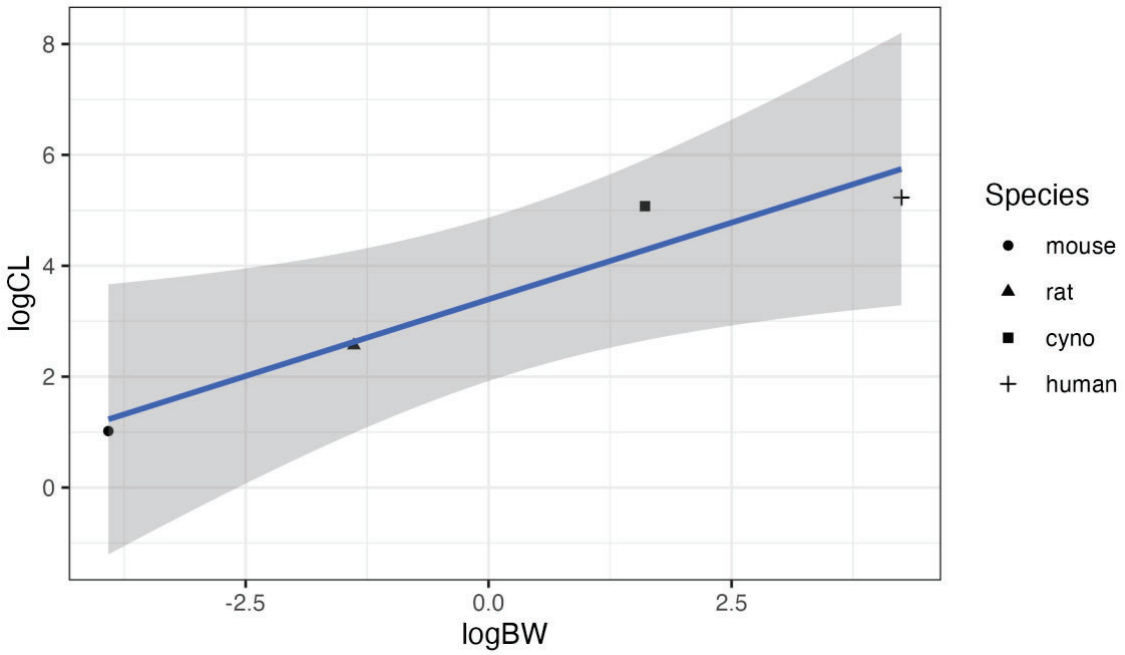
Total plasma clearance (CL) values after IV administration to multiple species (human, cynomolgus monkey, rat) were obtained or calculated as Dose/AUC from the FDA review and clinical population PK. The CL value for mouse was obtained from the current PK study (139 mL/min/kg, 2.77 mL/min/20g).

Clearances were then converted from weight normalized (mL/min/kg) to absolute (mL/min) values using standard body weights (BW) for each species – human, 70kg; cyno, 5kg ; rat, 0.25kg; mouse, 0.02kg. Both the body weights and absolute CL values were natural log transformed, with logCL regressed on logBW using R software.

The following is a table of the data analyzed:

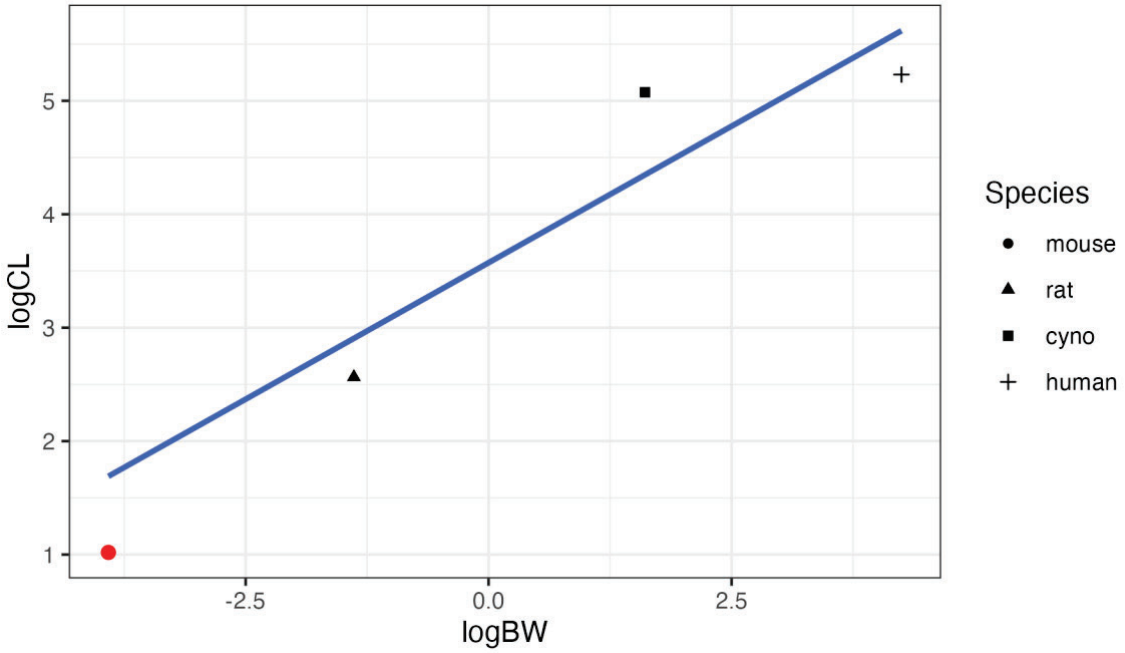
Species	BW (kg)	CL (mL/min)	logBW	logCL
mouse	0.02	2.77	-3.912023	1.01884732
rat	0.25	13	-1.3862944	2.56494936
cyno	5	160	1.60943791	5.07517382
human	70	187	4.24849524	5.23110862

Lurbinectedin allometry across 4 species
logCL = 0.553*logBW + 3.40, Adj R2 = 0.888



Cyno had higher CL than would be predicted from mouse, rat, and human, with the highest residual, about 2.2-fold higher than expected. CL was approximately proportional to (BW)^0.5. The mouse predicted CL was 3.42 mL/min (171 mL/min/kg – still more than HBF), with the actual observed being 2.77 mL/min; a difference less than 2-fold. This allometric exponent is atypically low compared with other compounds, and might be explained by differences in PPB or metabolism/elimination.

Lurbinectedin mouse CL is relatively well-predicted from 3-species allometry
logCL = 0.481*logBW + 3.57, Adj R2 = 0.645



The predicted CL value of 5.41 mL/min for mice is just within 2-fold of the observed value of 2.77 mL/min (red dot), but still overpredicts CL and is unphysiologically high.

Lurbinectedin mouse CL is poorly predicted from single species allometry, rat or human, using fixed exponents of 0.67 or 0.75

From human, 0.67 and 0.75 exponents:

$$CL_{mouse} = CL_{human} * \left(\frac{BW_{mouse}}{BW_{human}}\right)^{0.67}$$

$$CL_{mouse} = 187\frac{mL}{min} * \left(\frac{0.02kg}{70kg}\right)^{0.67} = 0.789\frac{mL}{min} = 39.5\frac{mL}{min}/kg$$

$$CL_{mouse} = CL_{human} * \left(\frac{BW_{mouse}}{BW_{human}}\right)^{0.75}$$

$$CL_{mouse} = 187\frac{mL}{min} * \left(\frac{0.02kg}{70kg}\right)^{0.75} = 0.411\frac{mL}{min} = 20.5\frac{mL}{min}/kg$$

From rat, 0.67 and 0.75 exponents:

$$CL_{mouse} = CL_{rat} * \left(\frac{BW_{mouse}}{BW_{rat}}\right)^{0.67}$$

$$CL_{mouse} = 13\frac{mL}{min} * \left(\frac{0.02kg}{0.25kg}\right)^{0.67} = 2.39\frac{mL}{min} = 52\frac{mL}{min}/kg$$

$$CL_{mouse} = CL_{rat} * \left(\frac{BW_{mouse}}{BW_{rat}}\right)^{0.75}$$

$$CL_{mouse} = 13\frac{mL}{min} * \left(\frac{0.02kg}{0.25kg}\right)^{0.75} = 1.96\frac{mL}{min} = 97.8\frac{mL}{min}/kg$$