Supplemental Information

Article Title: Translational PK/PD modeling of tumor growth inhibition and target inhibition to support dose range selection of the LMP7 inhibitor M3258 in relapsed/refractory multiple myeloma

Authors: Floriane Lignet, Christina Esdar, Gina Walter-Bausch, Manja Friese-Hamim, Sofia Stinchi, Elise Drouin, Samer El Bawab, Andreas D. Becker, Claude Gimmi, Michael P. Sanderson, Felix Rohdich

Supplemental Methods

Plasma protein binding

Human plasma was prepared from whole blood collected from one female volunteer and transferred to tubes containing EDTA as anticoagulant. Plasma from dogs (Marshall Beagle®, female), rats (Wistar Han IGS,Crl :WI(Han), female), and mice (CD-I , Crl :CDI (ICR) female) was prepared from whole blood provided by Nuvisan GmbH, Grafing, Germany and collected into tubes containing EDTA as anticoagulant. Monkey plasma (Cynomolgus, female) containing EDTA as an anticoagulant was purchased from Bioculture (Mauritius) Ltd.

All incubations were performed in triplicates in a Dianorm system. After equilibrium dialysis, the contents of both compartments of each dialysis cell containing M3258 and [^{14}C]-testosterone were transferred into brown Eppendorf tubes and LSC vials, respectively. The samples containing M3258 were acidified by dilution with 22% formic acid (FA), (Sample/FA (22%), 90:10, VIV), leading to a final sample with 2.2% FA. 20 μL aliquots were then precipitated by the

addition of an internal standard solution. Samples were incubated on a shaker at 1000 rpm for 10 min, incubated for 20 min at -20°C, and re-shaken at 1000 rpm for 10 min before centrifugation at 2180 × g (4000 rpm) for 10 min. Aliquots of the supernatant from incubation samples were diluted with Milli Q water before injection into the UPLC-MS/MS system. For [^{14}C]-testosterone, incubation sample aliquots of 300 µL of the plasma or buffer compartments were mixed thoroughly with 10 mL of Ultima GoldTM XR scintillator and were cooled prior to analysis by liquid scintillation counting (LSC). The recoveries of M3258 and [^{14}C]-testosterone in all incubations ranged from 84.1 to 102% and 96.2 to 97.1%, respectively, indicating that no relevant loss of either test item or reference compound (positive control) occurred during the experiments.

Blood to plasma ratio

Whole blood samples from all species were collected into tubes containing EDTA on the day of the study (human, mouse, rat and dog) or up to three days in advance (monkey). All blood matrices were supplemented with 5% phosphate buffer (70 nM, pH 7.4) and stored at 4°C until start of the incubations. Aliquots (237.5 µL) of human, dog, monkey, rat, and mouse whole blood, as well as control plasma (separated from the same blood samples by centrifugation), were spiked with M3258 at final concentrations of 0.5, 5, and 50 µM and incubated for 30 min at 37°C (n=3) in a light restricted laboratory. Whole blood samples were centrifuged for 2 min at ~3340 *x g* to obtain plasma. Aliquots of the plasma supernatant, as well as control plasma, were transferred into light-protected Eppendorf tubes and stabilized with 22% FA (9:1, V/V). Acidified plasma sample aliquots of 20 µL were transferred into a 96-well plate (on ice) and diluted with 130 µL of an ice-cold internal standard solution in order to precipitate the proteins. The wells were sealed and shaken for 10 min under light protection and incubated for 30 min at -20°C. Thereafter, samples were shaken again for 10 min under light protection and centrifuged at 4°C for 10 min at 2180×g. A 20 µL aliquot of the supernatant was mixed with 280 µL 0.14% FA and injected onto the UPLC-MS/MS system.

Metabolic stability

Cryopreserved hepatocytes were thawed in a water bath at 37°C, transferred to a large volume of thawing medium and centrifuged for 5 min at 100 *x g*. After careful removal of the supernatant, the cell pellet was suspended in Krebs-Henseleit buffer in order to obtain the desired cell count. Suspensions of 2 x 10^6 cells/mL without serum were incubated with M3258 at 0.25 µM in triplicate for 5 h at 37°C. Samples aliquots (18 µL) were taken at time 0 and after 0.08. 0.25, 0.5, 1, 2,3, 3.3, 3.7, 4 and 5 h. The aliquots were transferred into a 96-well-plate (on ice) and diluted with 2 µL of 22% FA and 180 µL of an ice-cold internal standard solution to lyse the hepatocytes and precipitate proteins. The wells were sealed and shaken for 10 min under light protection and incubated for 20 min at -20°C. Thereafter, samples were shaken again for 10 min under light protection and centrifuged at 4°C for 10 min at 2180 *x g*. A 40 µL aliquot of the supernatant was mixed with 60 µL Milli Q® water and injected onto the UPLC-MS/MS system. The *in vitro* intrinsic clearance was calculated from the rate of compound disappearance, and corrected for hepatocyte protein binding calculated from the compound logP (Kilford et al., 2008).
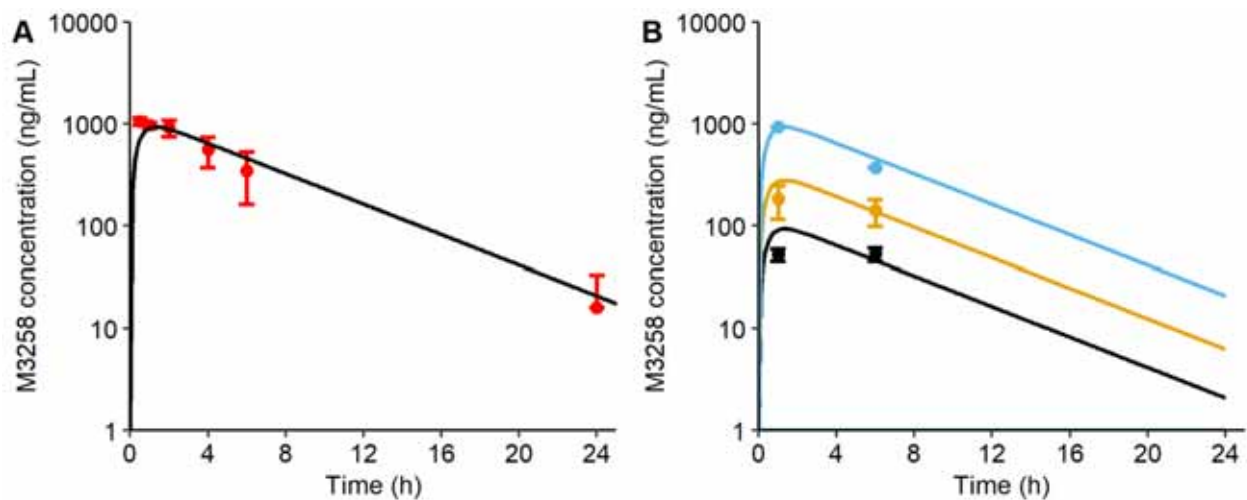
Supplemental Figures

Fig. S1 Simulated and observed mouse M3258 PK profiles. **A** Observed (points) and model-fitted (lines) M3258 plasma concentrations in mice after single oral M3258 application at 1 mg/kg. **B** Mean observed (points and error bars) and model-fitted (lines) M3258 plasma concentrations in mice after repeated oral M3258 application at 0.1 mg/kg (black), 0.3 mg/kg (yellow) or 1 mg/kg (blue). Data are expressed as mean ± standard deviation
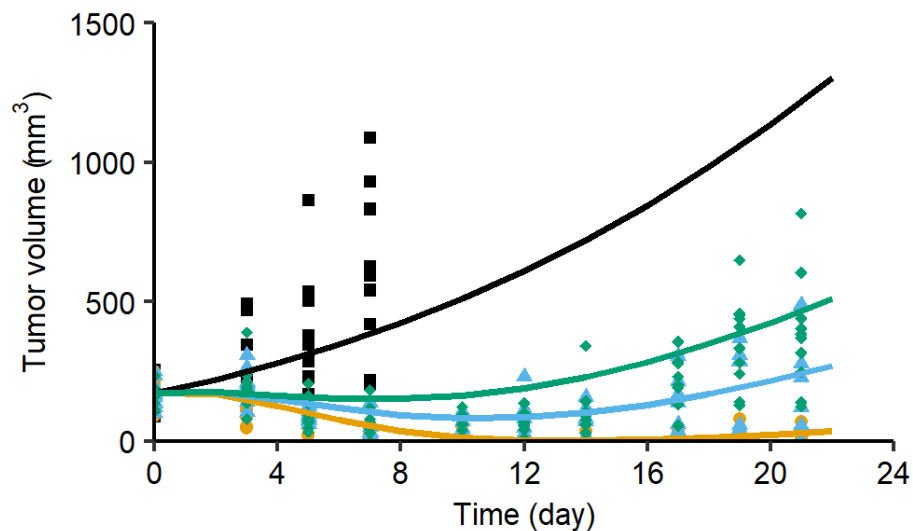

Fig. S2 Observed and model-fitted tumor volumes in U266B1 xenograft tumor-bearing mice treated with M3258 (p.o.) administered at 1 mg/kg daily (orange line and circles), once every two days (blue line and triangles) or twice weekly (green line and diamonds) or with vehicle only (black line and squares). Lines represent model fitted tumor volumes and points indicate observed tumor volume data
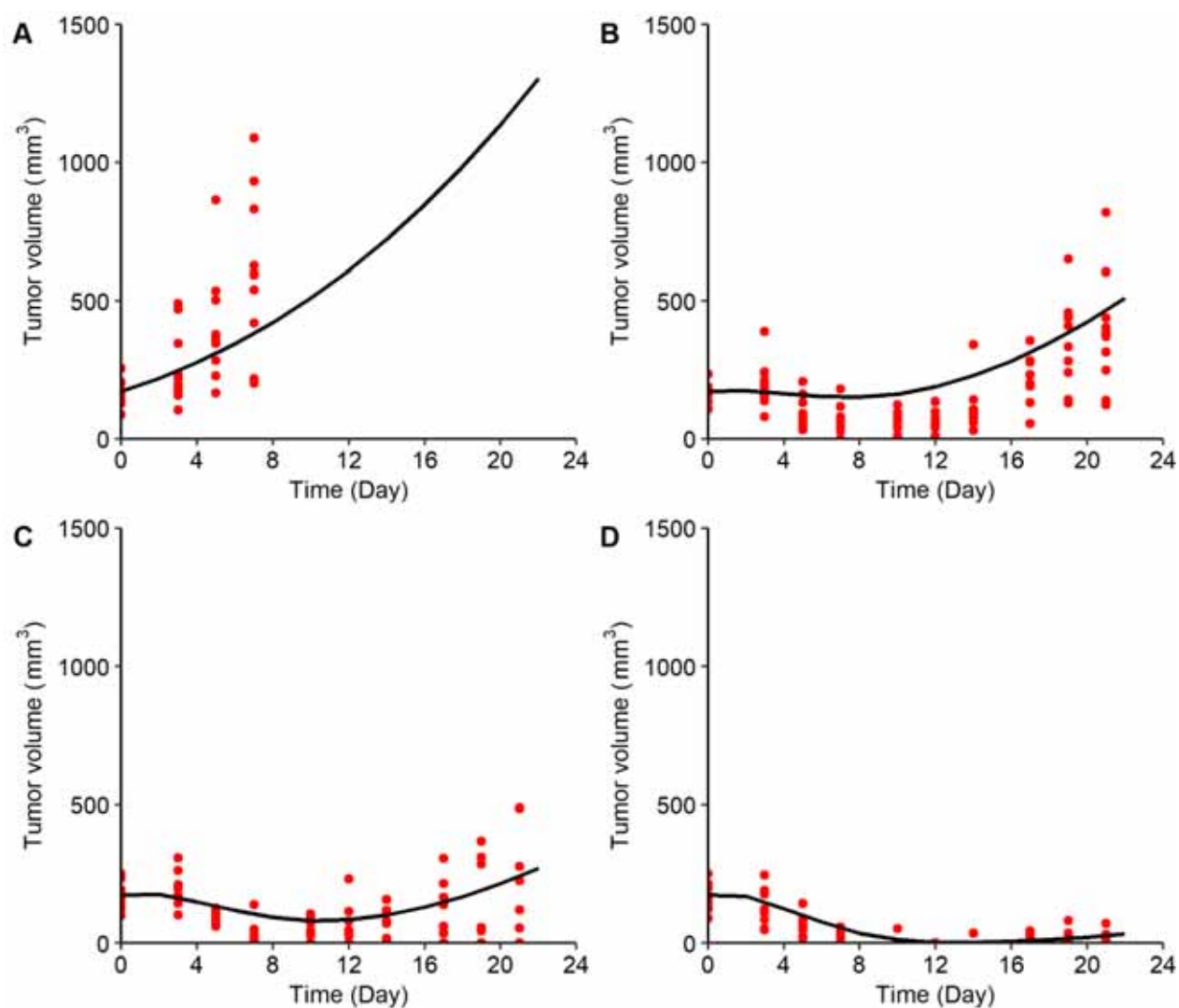
Fig. S3 Simulated and observed tumors volumes in for alternate M3258 dosing schedules. Observed (points) and model-fitted (lines) tumor volumes in U266B1 xenograft tumor-bearing mice treated orally with vehicle (**A**) or M3258 twice weekly (BIW) at 1 mg/kg (**B**), once every two days (Q2D) at 1 mg/kg (**C**) or daily (QD) at 1 mg/kg (**D**)
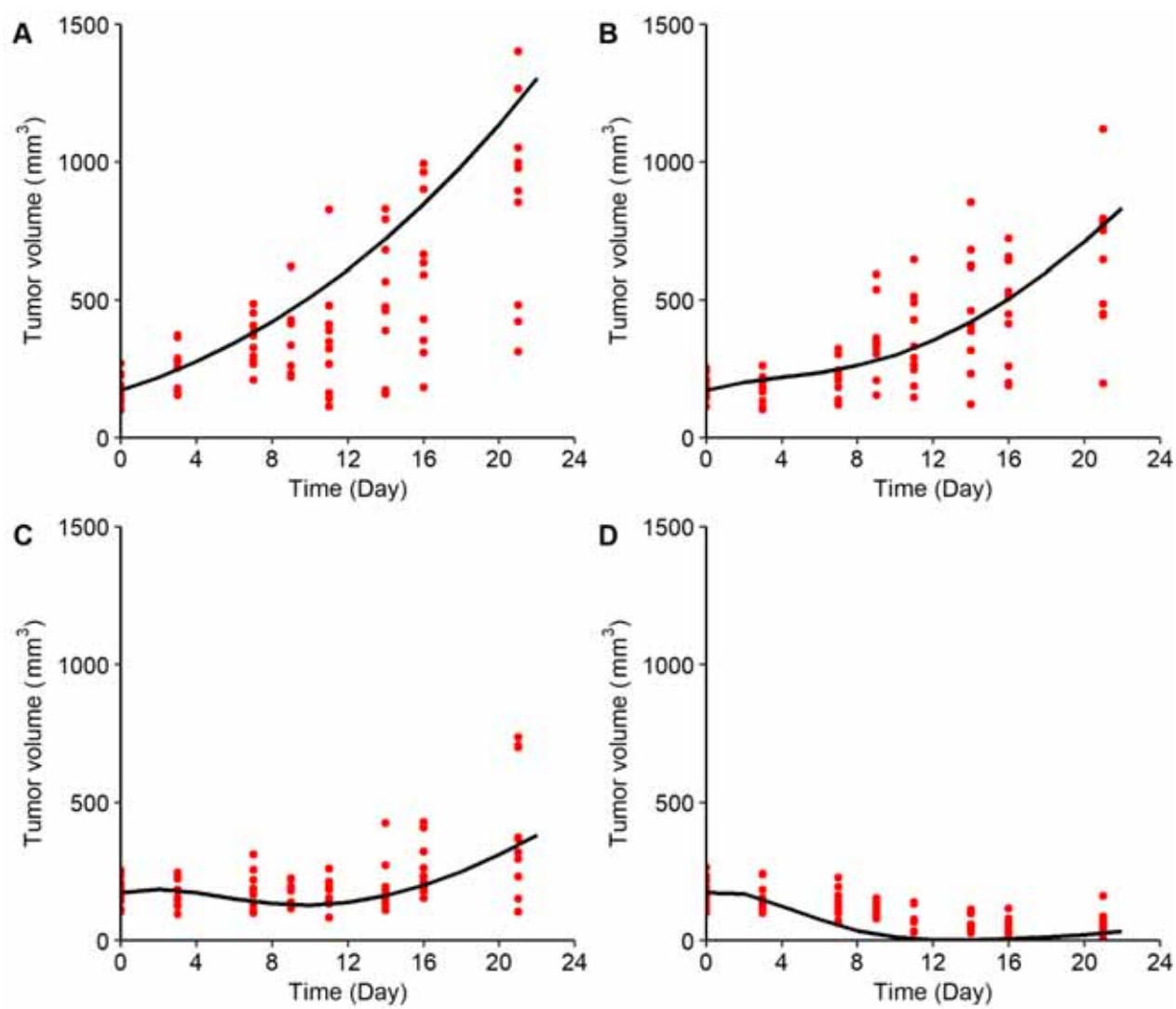
Fig. S4 Simulated and observed tumors volumes in M3258 dose range finding study. Observed (points) and model-fitted (lines) tumor volumes in U266B1 xenograft tumor-bearing mice treated orally once per day with vehicle (**A**) or M3258 at 0.1 mg/kg (**B**), 0.3 mg/kg (**C**), or 1 mg/kg (**D**)
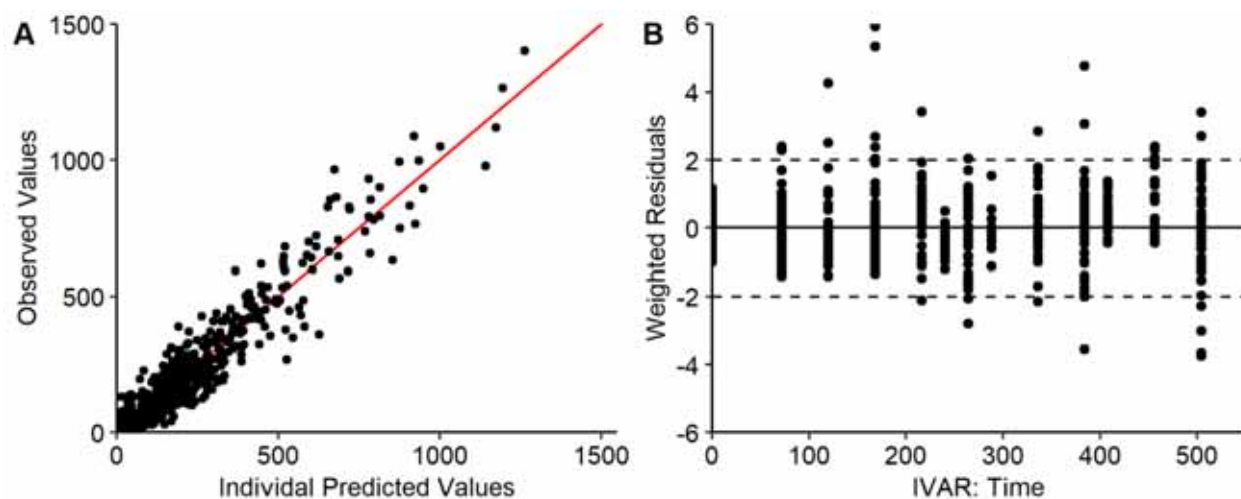
Fig. S5 Diagnostic plots for the tumor growth inhibition model. **A** Observed vs. individual predicted values (black points), overlaid with the unity line (red line). **B** Weighted residuals vs. time
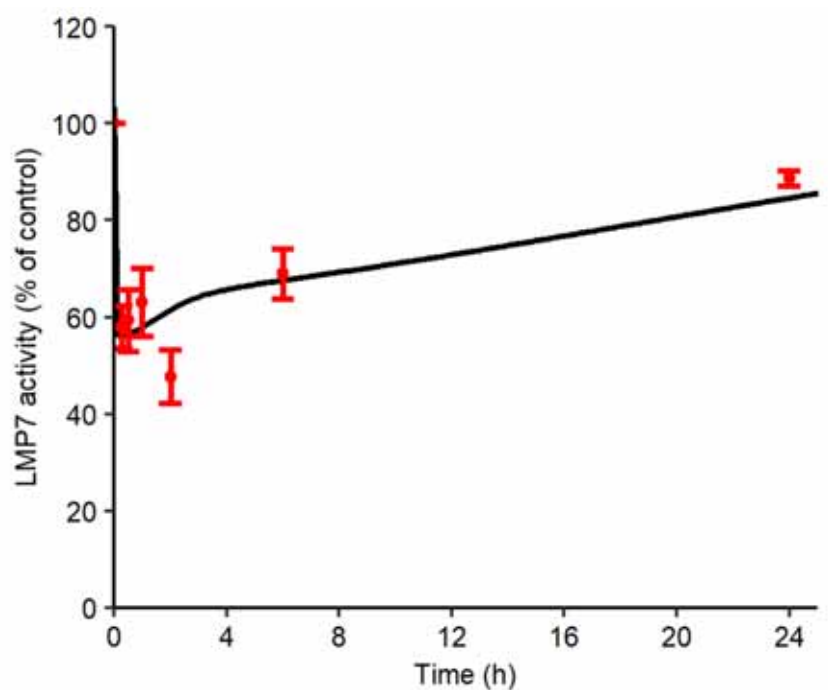

Fig. S6 Simulated and observed rat PD profile. Observed (points) and model-fitted (lines) LMP7 activity in spleens of rats treated once orally with the vehicle (black) or M3258 at 10 mg/kg. Data are expressed as mean ± standard deviation
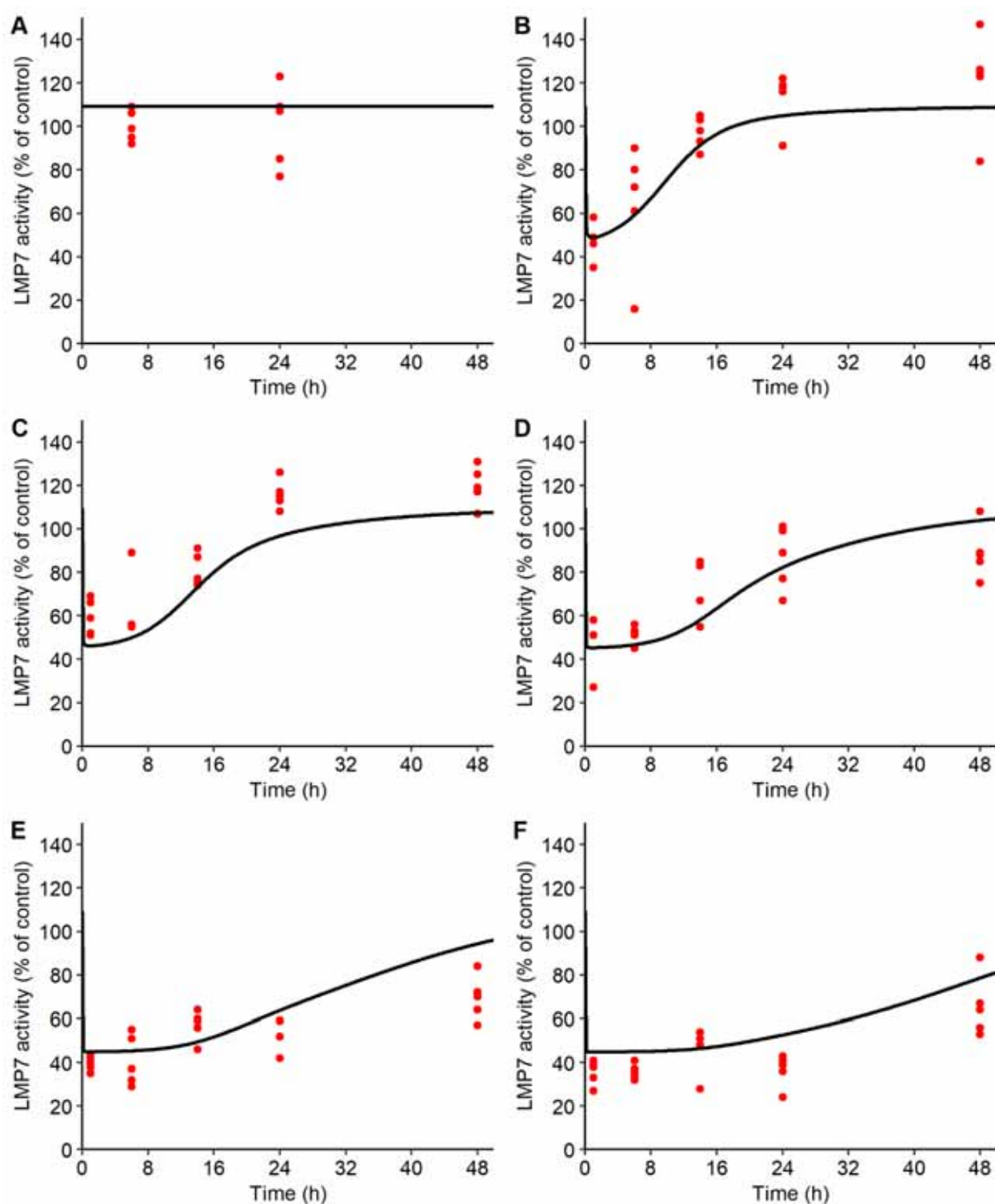
Fig. S7 Simulated and observed mouse M3258 PD profiles. Observed (points) and model fitted (lines) LMP7 activity in U266B1 xenograft tumors in mice treated orally with vehicle (**A**) or M3258 at 0.3 mg/kg (**B**), 1 mg/kg (**C**), 3 mg/kg (**D**), 10 mg/kg (**E**) or 30 mg/kg (**F**)
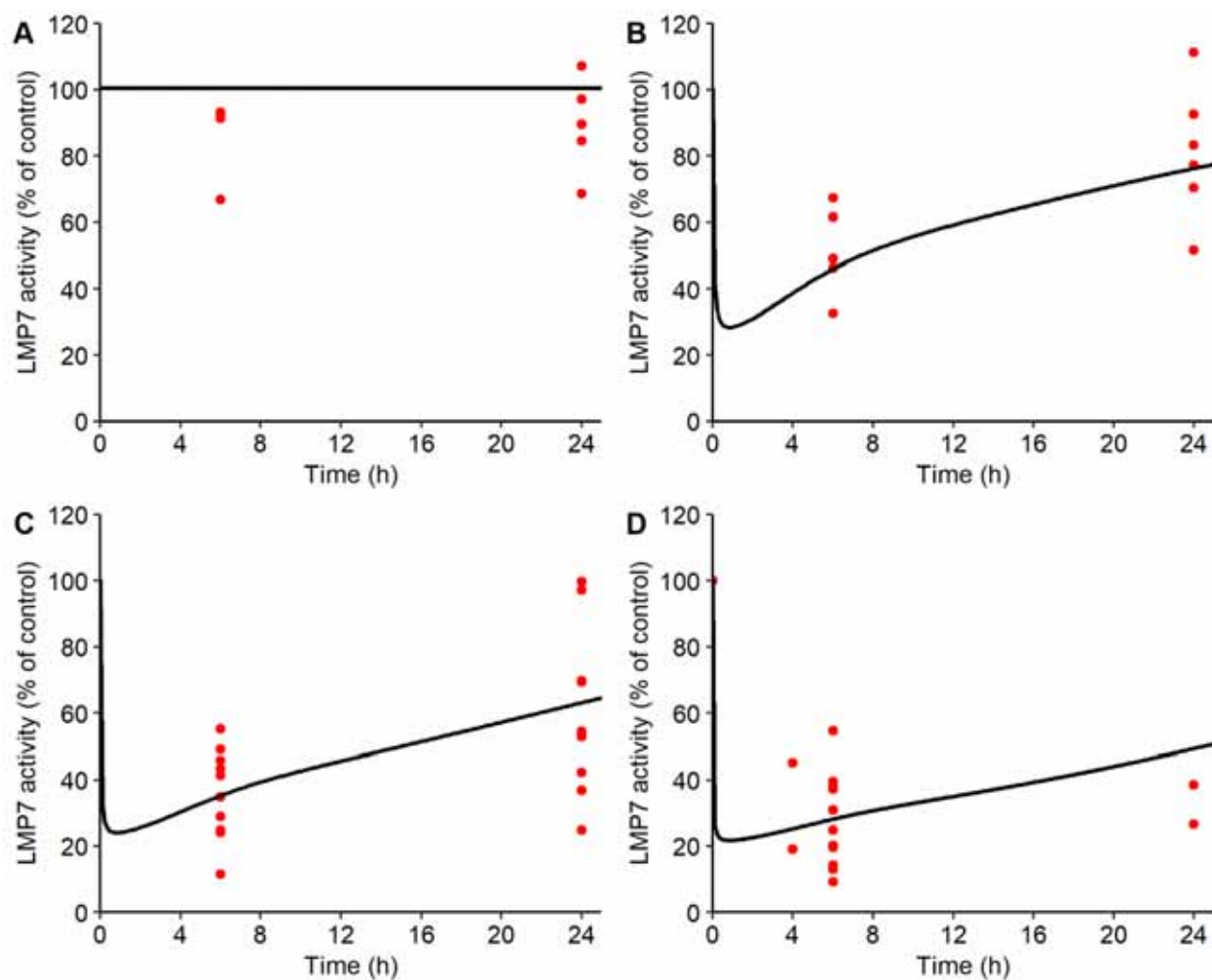
Fig. S8 Simulated and observed dog M3258 PD profiles. Observed (points) and model fitted (lines) LMP7 activity in PBMCs from dogs treated orally with vehicle (**A**) or M3258 at 0.75 mg/kg (**B**), 1.5 mg/kg (**C**) or 3 mg/kg (**D**)
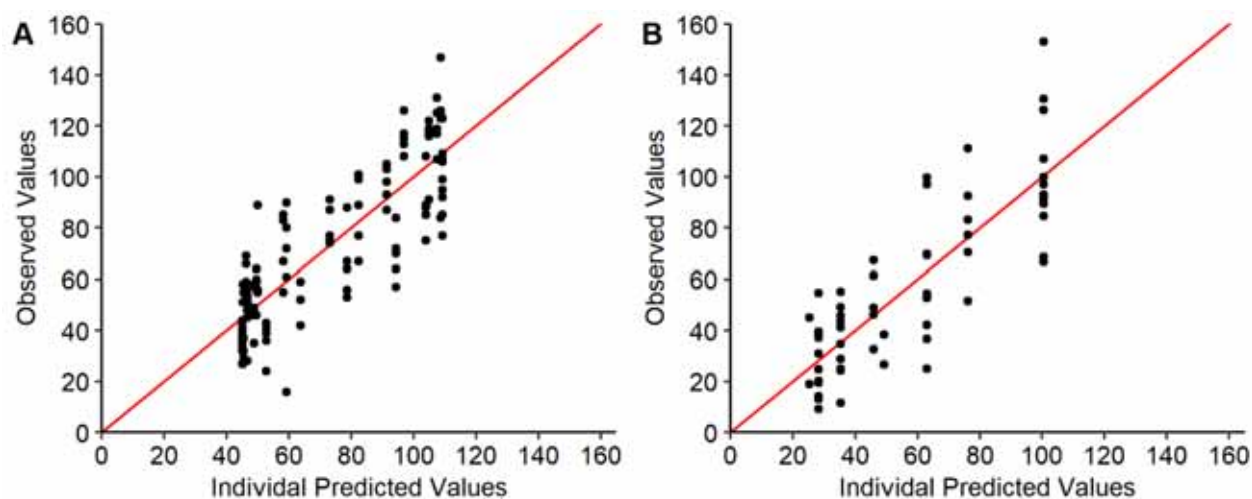
Fig. S9 Observed PD vs. PD predicted using the M3258 PK/PD models in mouse (**A**) and dog (**B**) black dots, overlaid with the unity line (red line)
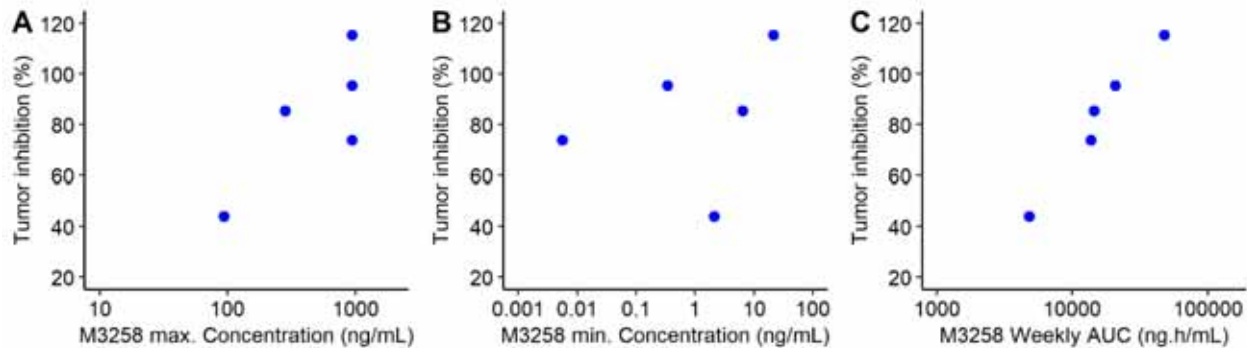

Fig. S10 Correlation between tumor growth inhibition of U266B1 xenografts in mice and C_{max} (**A**), C_{min} (**B**) and weekly AUC (**C**) of M3258 plasma exposure estimated by non-compartmental analysis.

Supplemental Tables

Table S1 Prediction of M3258 human clearance using different methods

Clearance	IVIVE	Allometric Scaling				Mean ± SD
	Regression method[a]	SAfu[b]	TME[c]	SAfu/ MLP[d]	NASfu[e]	
(L/h/kg)	<0.021[f]	0.030	0.058	0.021	0.018	0.033 ± 0.016

a: Poulin method (Poulin et al., 2012)
b: Simple allometric scaling normalized by fu (Ring et al., 2011)
c: Tang-Mayersohn-Equation (Tang and Mayersohn, 2005)
d: Simple allometric scaling normalized by fu and maximum life span (Mahmood and Balian, 1996)
e: Normalized simple allometry corrected by Clint und fu (Lave et al., 1999)
f: Clint value was below the LLOQ (0.6 µL/min/10^6 cells)

Table S2 Prediction of M3258 human volume of distribution using different methods

Volume of Distribution	SA$_{fu}$ [a] (4-species)	Human-Dog proportionality	Øie-Tozer Method [b]	In Silico (PBPK)	Mean ± SD
(L/kg)	0.273	0.406	0.288	0.150	0.28 ± 0.10

a: Simple allometric scaling normalized by fu (Jones et al., 2011)
b: Described in Obach et al. (Obach et al., 1997)

References

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