Supplemental Information

Chimeric TK-NOG Mice: A Predictive Model for Cholestatic Human Liver Toxicity

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Characterization of the performance of the in situ liver perfusion system using control and humanized TK-NOG mice. To characterize the performance of the *in situ* perfusion system, we first measured the uptake of a fluorescent non-metabolizable dye (carboxydichlorofluorescein or **CDCF**), which is used as a measure of transport activity (Pratt et al., 2006; Zamek-Gliszczynski et al., 2003). CDCF transport into bile was saturable in both control and chimeric mice (**Fig. S2**). Consistent with the dependence of biliary CDCF transport is upon Mrp2 transporter activity (Zamek-Gliszczynski et al., 2003), an inhibitor of Mrp2 (probenecid) completely inhibited CDCF transport into bile (Fig. S2). Rifampin is an inhibitor of human organic anion transporting polypeptides (OATP) that are critical for hepatic drug uptake, and it has been shown to inhibit the hepatic uptake of drugs such as bosentan (Treiber et al., 2007; van Giersbergen et al., 2007). While repeated doses of rifampicin will induce the expression of drug metabolizing enzymes and transporters, its primary effect in a single dose study that is conducted over a very short time period is to inhibit drug transport. Addition of rifampin to the perfusate solution also substantially inhibited CDCF transport into bile in both control and humanized mice (Fig. S2). These results indicate that CDCF uptake into the liver and its transport into bile is saturable and carrier dependent in both humanized and control mice during an *in situ* liver perfusion experiment.

References

Pratt, S., Chen, V., Perry, W.I., 3rd, Starling, J.J., and Dantzig, A.H. (2006). Kinetic validation of the use of carboxydichlorofluorescein as a drug surrogate for MRP5-mediated transport. European journal of pharmaceutical sciences : official journal of the European Federation for Pharmaceutical Sciences *27*, 524-532.

Treiber, A., Schneiter, R., Hausler, S., and Stieger, B. (2007). Bosentan is a substrate of human OATP1B1 and OATP1B3: inhibition of hepatic uptake as the common mechanism of its interactions with cyclosporin A, rifampicin, and sildenafil. Drug Metab Dispos *35*, 1400-1407.

van Giersbergen, P.L., Treiber, A., Schneiter, R., Dietrich, H., and Dingemanse, J. (2007). Inhibitory and inductive effects of rifampin on the pharmacokinetics of bosentan in healthy subjects. Clin Pharmacol Ther *81*, 414-419.
Zamek-Gliszczynski, M.J., Xiong, H., Patel, N.J., Turncliff, R.Z., Pollack, G.M., and Brouwer, K.L. (2003). Pharmacokinetics of 5 (and 6)-carboxy-2',7'-dichlorofluorescein and its diacetate promoiety in the liver. J Pharmacol Exp Ther *304*, 801-809.

Table S1. A list of the chimeric TK-NOG mice used in this study. Donor 1 was a 3-year old female, donor 2 was a 2-year old female and donor 3 was a 1-year-old female.

Mouse #	Donor	Treatment	hAlb mg/ml
633	2	Cefmetazole 25	13.7
621	2	Cefmetazole 25	12.3
620	2	Cefmetazole 25	11.6
622	2	Cefmetazole 25	11.8
390	1	Cefmetazole 25	12.5
373	1	Cefmetazole 25	11
369	1	Cefmetazole 25	16.5
380	1	Cefmetazole 25	10.9
987	1	Bosentan 160	10.6
977	2	Bosentan 160	9.9
932	2	Bosentan 160	12.1
981	1	Bosentan 160	14.2
967	2	Bosentan 160	15.8
961	2	Bosentan 160	9.1
74	1	Bosentan 32	9.9
89	1	Bosentan 32	7.2
90	1	Bosentan 32	10.2
240	3	Bosentan 32	7.2
266	3	Bosentan 32	12.1
268	3	Bosentan 32	10.1
61	2	Bosentan 6	12.3
64	2	Bosentan 6	14.1
63	1	Bosentan 6	9.3
280	3	Bosentan 6	14.1
283	3	Bosentan 6	13.2
281	3	Bosentan 6	18.7
931	2	Veh	16.7
933	1	Veh	12.3
964	2	Veh	10.5
953	2	Veh	11.4
298	3	Veh	7.2
102	1	Veh	7.8
254	3	Veh	13.2
727	2	In situ	16.7
729	2	In situ	17.5
773	1	In situ	18.7

Table S2. The measured amounts of cefmetazole in feces and urine collected over the two indicated time periods (0 to 8 hr, and 8 to 24 hr) from 3 control and 3 chimeric TK-NOG mice after treatment with cefmetazole (25 mg/kg IP) are shown. Fold indicates the ratio of cefmetazole in urine or feces of control mice relative to humanized mice, and the P-value assesses the difference between the values measured in the groups of control and humanized mice.

	Control			Humanized			Control/Human	
	CMZ Concentration	Sample Volume	CMZ 0-24h (mg)	CMZ concentration	Sample Volume	CMZ 0-24h (mg)	Fold	P-value
Feces 0-8h	0.93 ug/mg	161.3 mg	699.6	0.13 ug/mg	181 mg	60.6	11.5	0.006
Feces 8-24h	0.95 ug/mg	594.3 mg		0.09 ug/mg	337.9 mg			
Urine 0-8h	257.47 ug/ml	0.72 ml	167.2	1397.9 ug/ml	0.20 ml	279.6	0.6	0.03
Urine 8-24h	32.00 ug/ml	0.59 ml		24.57 ug/ml	0.39 ml			

Table S3. An *in situ* perfusion system was used to measure the rate of hepatic uptake (the hepatic extraction ratio or Eh), biliary clearance (CL_b) and affinity (Kp) of cefmetazole in 3 control and 3 humanized TK-NOG mice. The rate of hepatic uptake (Eh) of cefmetazole was low in control or humanized livers. However, the humanized liver had a 4-fold lower rate (p=0.007) of biliary cefmetazole clearance (CL_b= 0.02) relative to control mice (CL_b=0.08). The humanized liver also had a five-fold (p=0.003) higher affinity for cefmetazole (Kp=2.7) than control mouse liver (Kp=0.5). The p-values comparing the values measured for the livers of control and humanized TK-NOG mice are shown. The decreased rates of cefmetazole transport (CL_b, p=0.007) and increased liver retention (Kp, p=0.003) explain why biliary cefmetazole clearance is reduced in human liver.

	E_h	Cl_h	E_b	$CL_{b\ (ml/min)}$	K_p
Control 1	0.022	0.090	0.770	0.051	0.463
Control 2	0.110	0.440	1.390	0.096	0.452
Control 3	0.072	0.290	1.500	0.094	0.494
Average	**0.068**	**0.273**	**1.220**	**0.080**	**0.470**
Humanized 1	0.092	0.370	4.930	0.028	4.193
Humanized 2	0.006	0.020	1.800	0.021	1.808
Humanized 3	0.110	0.440	1.390	0.021	2.050
Average	**0.069**	**0.277**	**2.707**	**0.023**	**2.684**
P value	**0.756**	**0.724**	**0.197**	**0.007**	**0.003**

Figure Legends

Figure S1. Control (n=3) and humanized (n=3) TK-NOG mice were dosed with cefmetazole (25 mg/kg IP), and the amount of cefmetazole in plasma (**A**) and bile (**B**) was measured by LC/MS analysis at the indicated times after dosing. (**C**) The biliary cefmetazole concentrations in control and humanized TK-NOG mice at 0.5 hr after dosing, which is when the maximal cefmetazole concentration was observed, are shown. Each data point represents the average (± SEM) of the measured cefmetazole concentration at each time point in control or humanized TK-NOG mice.

Figure S2. Biliary transport measured using an *in situ* perfusion system is saturable and can be inhibited by agents that are transport inhibitors. The uptake of CDCF into bile in control (n=3) and humanized (n=3) TK-NOG mice was measured using an *in situ* perfusion system in the presence or absence of probenecid (20 uM) or rifampin (20 uM). Bile samples were collected every 2.5 min over a 1 hour period of perfusion and the amount of CDCF was measured by LC/MS analysis. In both control and humanized mice, probenecid completely inhibited CDCF excretion into bile, while rifampin partially inhibited CDCF excretion in to bile.

Figure S3. Control (non-humanized) or chimeric TK-NOG mice were treated with bosentan 160 mg/kg/day PO or vehicle for 28 days, and the plasma protein and cholesterol levels were measured after 4 weeks of dosing. Each symbol represents the value measured in a control or humanized TK-NOG mouse, and the short dashed line shows the average for each group.

Figure S4. H&E stained sections of liver regions containing human hepatocytes obtained from chimeric mice (indicated by number) that received bosentan 160 mg/kg/day or vehicle for 28 days. A few fat vacuoles were noted in the hepatocytes in both treatment groups. Arrows indicate representative fat vacuoles. The original magnification in each panel is 400x.

Figure S5. Control (non-humanized) (n=6) or chimeric (n=6) TK-NOG mice were treated with 32 or 6 mg/kg/day PO bosentan or vehicle for 14 days, and their weights were measured on a daily basis.

Fig S1

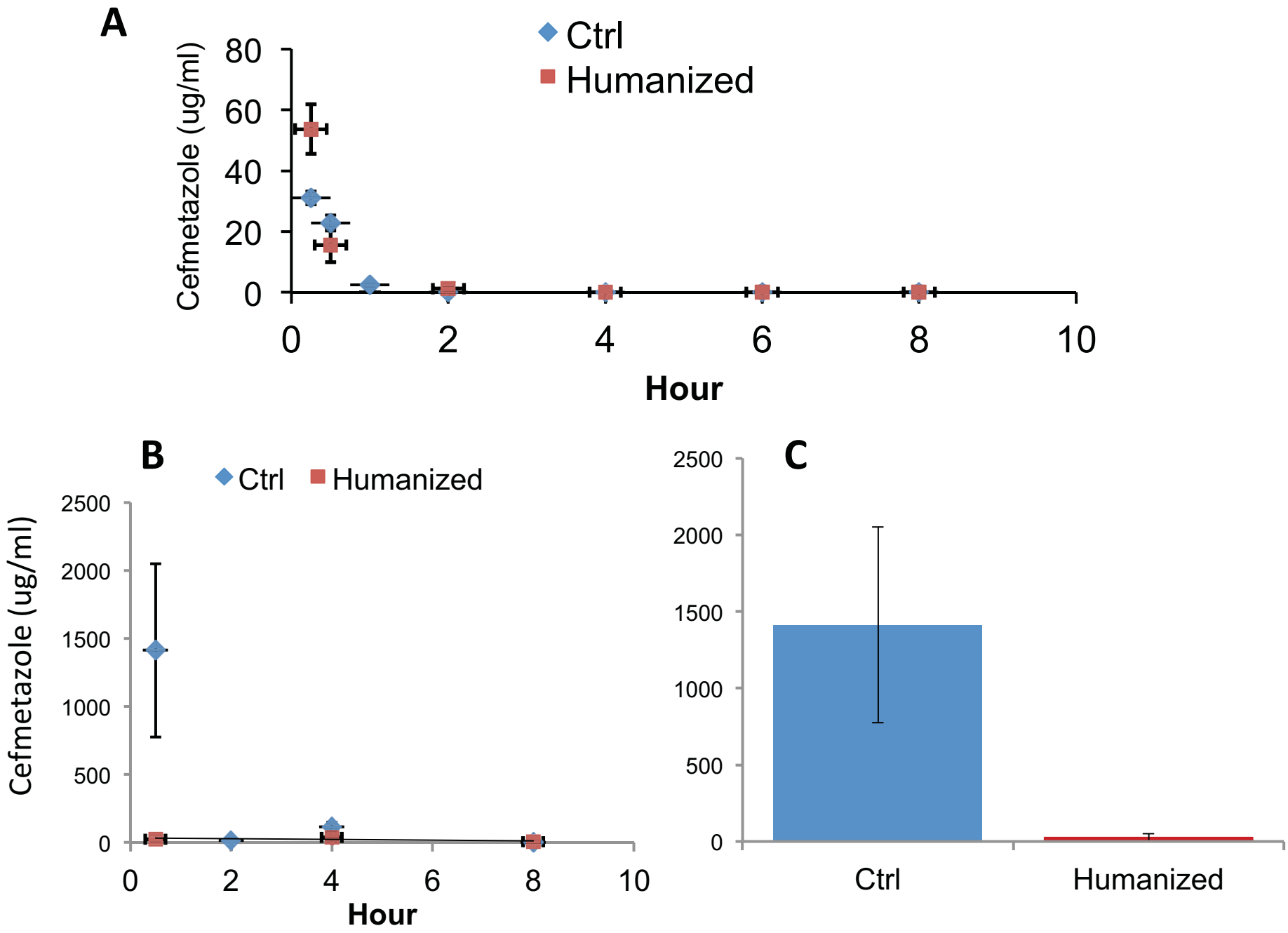

A
Ctrl
Humanized
Cefmetazole (ug/ml)
80
60
40
20
0
0
2
4
6
8
10
Hour
B
Ctrl
Humanized
Cefmetazole (ug/ml)
2500
2000
1500
1000
500
0
0
2
4
6
8
10
Hour
C
2500
2000
1500
1000
500
0
Ctrl
Humanized

Fig S2

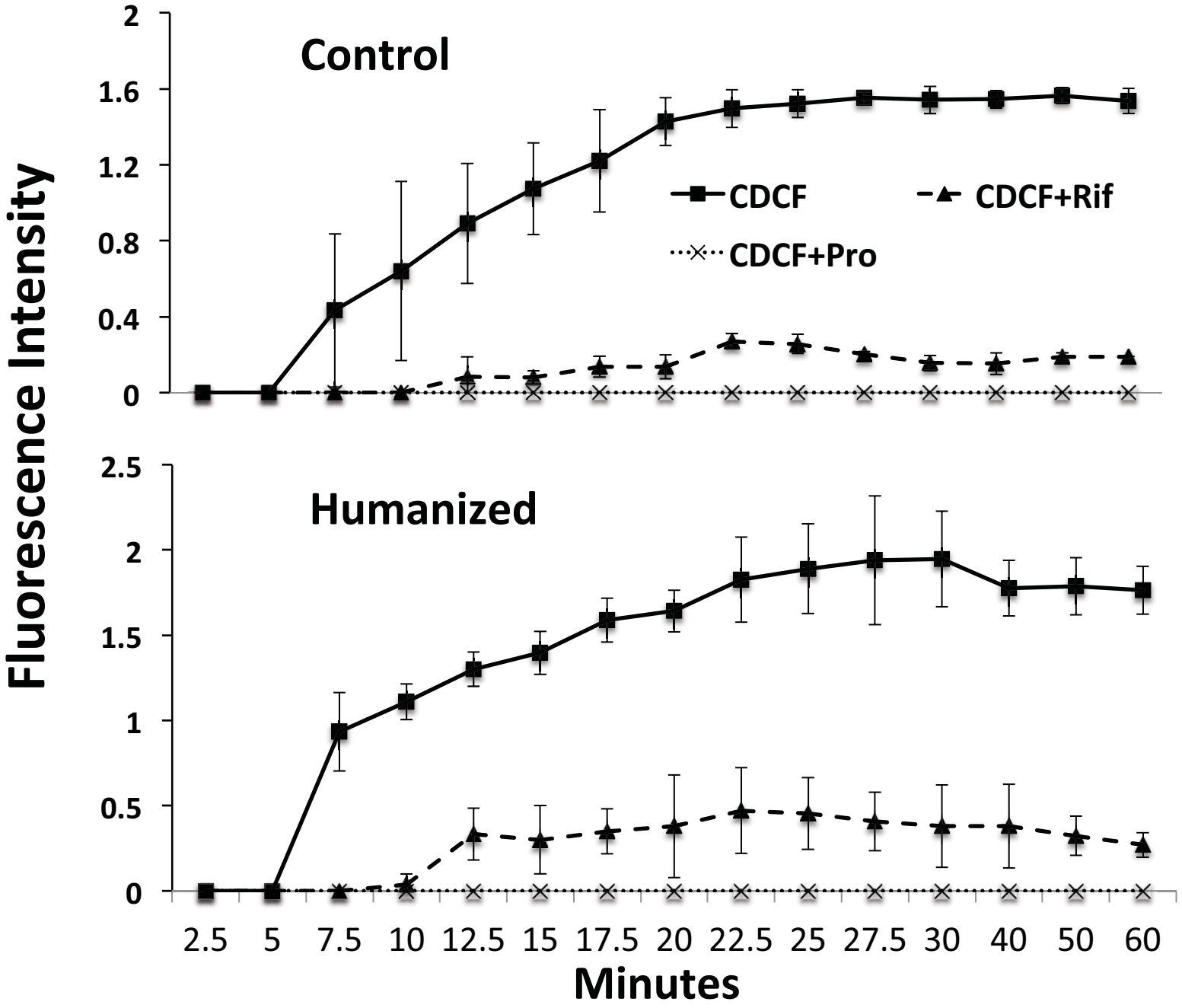

Control
Humanized
CDCF
CDCF+Rif
CDCF+Pro
Fluorescence Intensity
Minutes
2
1.6
1.2
0.8
0.4
0
2.5
1.5
1
0.5
2.5 5 7.5 10 12.5 15 17.5 20 22.5 25 27.5 30 40 50 60

Fig S3

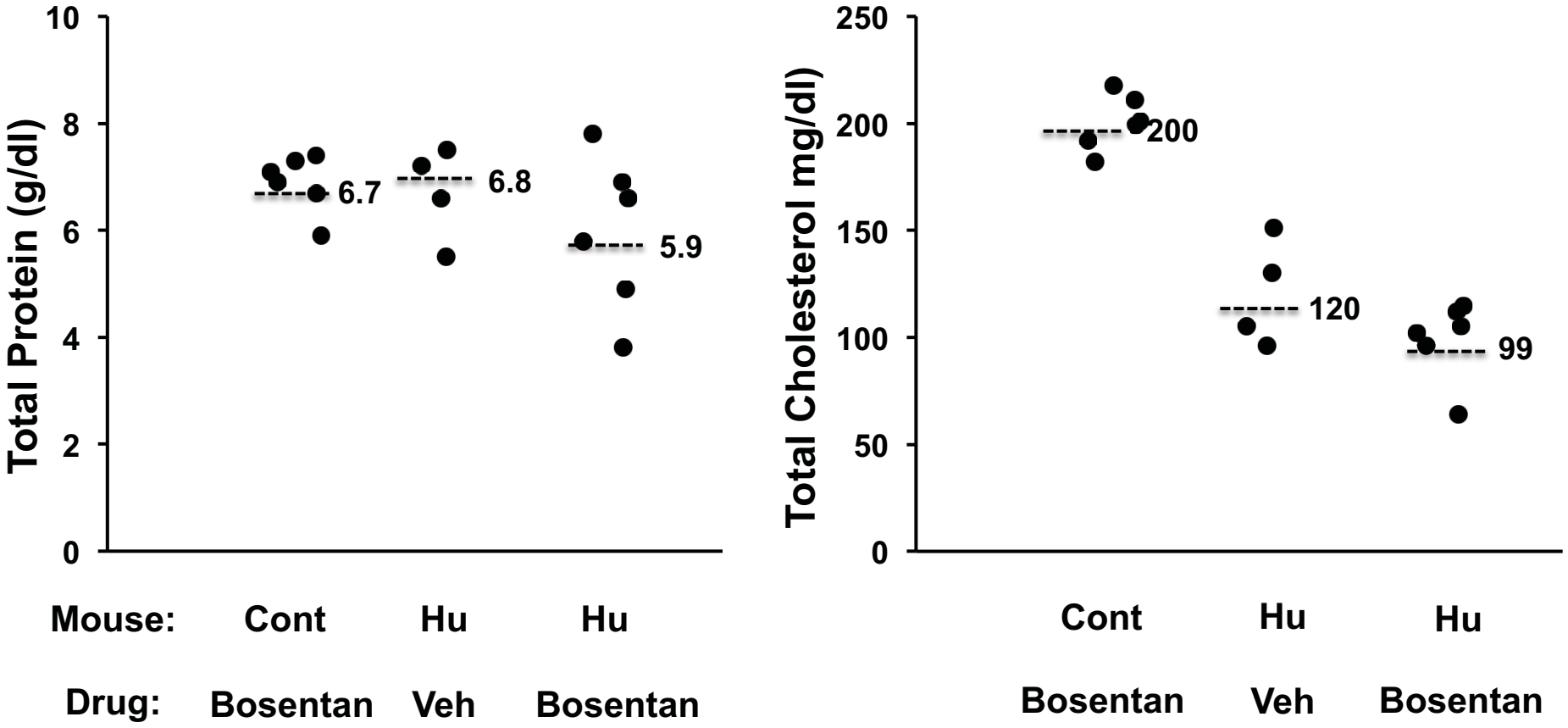

Total Protein (g/dl)
10
8
6
4
2
0
6.7
6.8
5.9
Mouse:
Cont
Hu
Hu
Drug:
Bosentan
Veh
Bosentan
Total Cholesterol mg/dl)
250
200
150
100
50
0
200
120
99
Cont
Hu
Hu
Bosentan
Veh
Bosentan

Fig S4

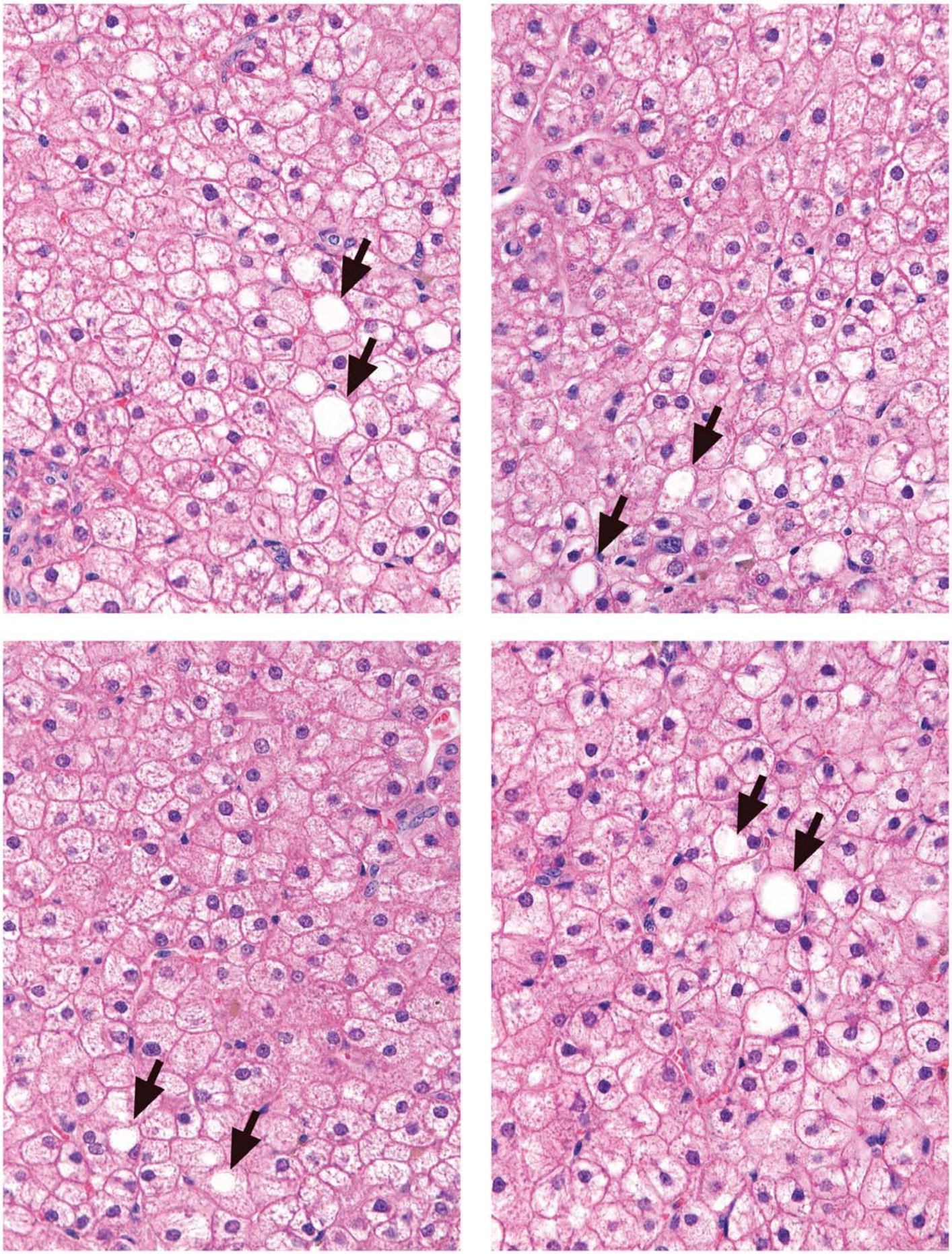
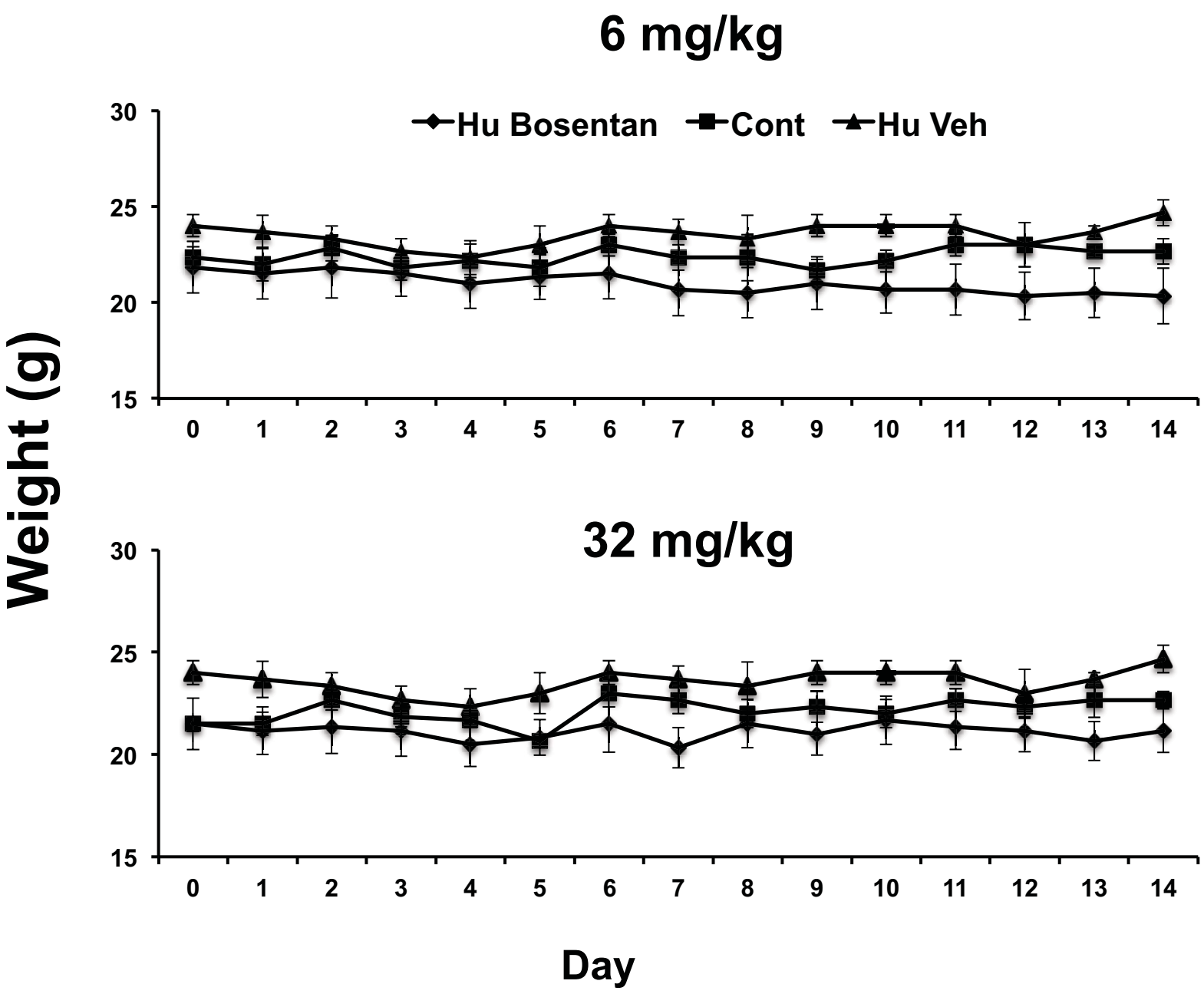


Figure S5


6 mg/kg
Hu Bosentan
Cont
Hu Veh
Weight (g)
30
25
20
15
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
32 mg/kg
Day