Andaya et al.:

A Proof-of-Concept Rat Toxicity Study Highlights the Potential Utility and Challenges of Virtual Control Groups

Supplementary Data

Tab. S1: Study design of evaluated POC studies
A historical rat toxicity study was used as the primary study in the proof-of-concept (POC) of virtual control groups (VCG). This 7-day repeat-dose pilot study administered internal compound GXX to Sprague-Dawley rats. Three separate datasets were evaluated. In each dataset, the control group (VCG1, VCG2, a.k.a. the CCG, or VCG3) was designated as Group 1 to assess GXX-related changes.

Group	Test article	Dose level (mg/kg)	Route of administration	Dosing schedule	Animal numbers	Toxicity evaluation
1	Vehicle	0	Oral gavage	Daily x 7 days	6M	Bodyweights Clinical pathology Organ weights Macroscopic pathology Microscopic pathology
2	GXX	10	Oral gavage	Daily x 7 days	6M	
3	GXX	30	Oral gavage	Daily x 7 days	6M	
4	GXX	100	Oral gavage	Daily x 7 days	6M	

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Tab. S2: Raw data for hematology parameters analyzed in the POC studies showing mean, standard deviation (SD), and number of animals (n)

Animals at 100 mg/kg (high dose) were euthanized early (moribund) or found dead; sampling was conducted prior to euthanasia.

	Control groups			Test groups (GXX)		
	VCG1	VCG2 (CCG)	VCG3	10 mg/kg (low)	30 mg/kg (mid)	100 mg/kg (high)
Basophils Mean (10^9/L)	0.015	0.018	0.013	0.012	0.012	0.02
SD	0.008	0.016	0.008	0.004	0.004	0.0
Basophils/Total Cells Mean (%)	0.13	0.17	0.12	0.12	0.12	0.2
SD	0.052	0.121	0.041	0.041	0.041	0.0
Eosinophils Mean (10^9/L)	0.083	0.115	0.108	0.135	0.157	0.05
SD	0.046	0.038	0.047	0.033	0.066	0.0141
Eosinophils/Total Cells Mean (%)	0.75	1.1	0.88	1.35	1.42	0.60
SD	0.327	0.316	0.354	0.235	0.605	0.2121
Hematocrit Mean (%)	43.65	45.67	42.87	45.18	44.5	40.75
SD	1.454	1.799	2.32	1.45	1.826	2.334
Hemoglobin Mean (g/dL)	14.58	14.92	14.3	14.82	14.55	13.6
SD	0.64	0.564	0.844	0.64	0.547	0.7071
Immature Reticulocyte Fraction Mean (%)	59.23	58.98	60.9	59.05	58.48	43.9
SD	2.844	1.897	2.42	2.101	2.649	17.819
Lymphocytes Mean (10^9/L)	9.237	8.293	10.12	8.252	8.89	5.365
SD	2.575	1.171	2.552	2.274	1.042	1.8173
Lymphocytes/Total Cells Mean (%)	83.87	81.1	83.77	81.98	78.98	61.7
SD	4.25	3.735	5.215	2.733	2.549	17.112
Ery. Mean Corpuscular Hemoglobin Mean (pg)	20.42	19.67	20.05	20.48	19.9	19.3
SD	1.155	0.524	0.862	0.387	0.663	0.4243
Ery. Mean Corpuscular HGB Conc. Mean (g/dL)	33.4	32.68	33.35	32.78	32.73	33.4
SD	0.729	0.747	0.586	0.655	0.68	0.1414
Ery. Mean Corpuscular Volume Mean (fL)	61.15	60.13	60.13	62.48	60.87	57.75
SD	4.363	1.756	2.782	1.927	2.216	0.9192
Monocytes Mean (10^9/L)	0.345	0.347	0.515	0.388	0.42	0.345
SD	0.260	0.60	0.229	0.055	0.14	0.1344
Monocytes/Total Cells Mean (%)	3.03	3.45	4.2	4.02	3.72	3.95
SD	2.138	0.914	1.853	0.711	1.214	1.3435
Mean Platelet Volume Mean (fL)	7.72	7.83	7.66	7.82	7.72	8.25
SD	0.147	0.577	0.261	0.438	0.327	0.2121
Neutrophils, Segmented Mean (10^9/L)	1.362	1.462	1.297	1.252	1.77	2.82
SD	0.507	0.491	0.428	0.399	0.406	1.3859
Platelets Mean (10^9/L)	1235.5	1194.7	1215	1148.2	1322.8	827.5
SD	100.7	185.7	103.1	109.1	86.13	649.83
Erythrocytes Mean (10^{12}/L)	7.17	7.6	7.138	7.24	7.31	7.06
SD	0.571	0.427	0.472	0.383	0.206	0.5091
Erythrocytes Distribution Width Mean (%)	14.65	15.65	14.8	14.62	14.7	14.45
SD	1.308	1.084	1.198	0.935	1.367	0.2121
Reticulocytes Mean (10^9/L)	566.78	706.73	558.67	706.3	767.17	609.5
SD	69.05	68.85	85.485	86.735	64.64	57.134
Reticulocytes/Erythrocytes Mean (%)	7.955	9.303	7.873	9.758	10.502	8.685
SD	1.2372	0.7755	1.4035	1.1257	0.9401	1.435
Leukocytes Mean (10^9/L)	11.042	10.235	12.075	10.038	11.248	8.62
SD	3.112	1.443	2.596	2.643	1.212	0.5515

Tab. S3: Raw data for clinical chemistry endpoints analyzed in the POC studies showing mean, standard deviation (SD), and number of animals (n)

Animals at 100 mg/kg (high dose) were euthanized early (moribund) or found dead; sampling was conducted prior to euthanasia.

	Control groups			Test groups (GXX)		
	VCG1	VCG2 (CCG)	VCG3	10 mg/kg (low)	30 mg/kg (mid)	100 mg/kg (high)
Albumin Mean (g/dL) SD	3.15 0.105	3.58 0.098	3.22 0.117	3.58 0.098	3.47 0.103	3.1 0.1414
Alkaline Phosphatase Mean SD	225.8 65.99	240.2 54.47	248.2 48.4	235.7 56.93	235.5 68.68	412.5 118.09
Alanine Aminotransferase Mean (IU/L) SD	40.3 8.43	55.8 12.54	49.5 8.19	61. 16.15	79.7 13.84	139.5 16.264
Aspartate Aminotransferase Mean (IU/L) SD	96 12.66	107.8 10.19	99.2 17.23	117.8 14.91	149.3 29.12	326.0 195.16
Bilirubin Mean (mg/dL) SD	0.1 0	0.1 0	0.1 0	0.1 0	0.12 0.041	0.1 0
Calcium Mean (mg/dL) SD	10.07 0.197	10.03 0.266	10.35 0.266	9.98 0.147	10.02 0.214	11.15 0.7778
Cholesterol Mean (mg/dL) SD	54.5 14.28	67.8 12.97	63 14.2	85.3 13.28	89.7 17.14	160.5 45.96
Creatine Kinase Mean (U/L) SD	972.5 504.57	494 50.71	378.3 148.2	475.2 156.06	366.2 170.02	645 137.18
Chloride Mean (mEq/L) SD	101.5 1.76	105 2.53	102.3 1.63	103.7 1.03	104.2 1.47	99.0 2.83
Creatinine Mean (mg/dL) SD	0.203 0.0294	0.242 0.0194	0.195 0.0217	0.248 0.016	0.25 0.0126	0.39 0.0707
Glutamate Dehydrogenase Mean (U/L) SD	7.2 1.17	10.3 4.97	7.3 0.52	10.7 2.73	14.2 3.66	43.5 6.36
Globulin Mean (g/dL) SD	-	-	-	2.32 0.075	2.45 0.055	2.8 0.42
Glucose Mean (mg/dL) SD	136.5 62.45	109.5 23.99	114.2 25.24	103.2 8.89	103.5 11.74	169 14.11
Potassium Mean (mEq/L) SD	4.85 0.152	5.38 0.16	5.05 0.281	5.38 0.325	5.58 0.183	4.30 0.141
Phosphate Mean (mg/dL) SD	8.55 0.446	7.2 0.718	8.35 0.841	7.03 0.299	7.4 0.354	7.95 0.636
Protein Mean (g/dL) SD	5.5 0.2	5.75 0.176	5.68 0.279	5.9 0.063	5.92 0.147	6.35 0.282
Sodium Mean (mEq/L) SD	143 1.41	145.8 1.33	144 0.89	146.2 2.04	147 2.1	140 2.83
Triglycerides Mean (mg/dL) SD	79.7 34.98	64.2 12.09	97.5 48.85	65 18.63	49 18.06	42 24.04
Urea Nitrogen Mean (mg/dL) SD	14.3 1.63	15.5 3.08	16.2 1.6	15.8 2.56	19.3 3.56	17.5 3.54

Tab. S4: Coefficients of variation (%) for the clinical pathology endpoints to assess any differences in variability between the VCGs and CCG

	Coefficient of variation (%)		
	VCG1	VCG2 (CCG)	VCG3
Albumin (g/dL)	3%	3%	4%
Alkaline phosphatase (IU/L)	29%	23%	20%
Alanine aminotransferase (IU/L)	21%	22%	17%
Aspartate aminotransferase (IU/L)	13%	9%	17%
Bilirubin (mg/dL)	0%	0%	0%
Calcium (mg/dL)	2%	3%	3%
Cholesterol (mg/dL)	26%	19%	23%
Creatine kinase (IU/L)	52%	10%	39%
Chloride (mEq/L)	2%	2%	2%
Creatinine (mg/dL)	14%	8%	11%
Glutamate dehydrogenase (U/L)	16%	48%	7%
Glucose (mg/dL)	46%	22%	22%
Potassium (mEq/L)	3%	3%	6%
Phosphate (mg/dL)	5%	10%	10%
Protein (g/dL)	4%	3%	5%
Sodium (mEq/L)	1%	1%	1%
Triglyceride (mg/dL)	44%	19%	50%
Urea nitrogen (mg/dL)	11%	20%	10%
Basophils (10^9/L)	56%	87%	61%
Basophils/total cells (%)	39%	73%	35%
Eosinophils (10^9/L)	56%	33%	43%
Eosinophils/total cells (%)	44%	29%	40%
Hematocrit (%)	3%	4%	5%
Hemoglobin (g/dL)	4%	4%	6%
Immature reticulocyte fraction (%)	5%	3%	4%
Lymphocytes (10^9/L)	28%	14%	25%
Lymphocytes/total cells (%)	5%	5%	6%
Ery. mean corpuscular hemoglobin (pg)	6%	3%	4%
Ery. mean corpuscular HGB concentration (g/dL)	2%	2%	2%
Ery. mean corpuscular volume (fL)	7%	3%	5%
Monocytes (10^9/L)	76%	17%	44%
Monocytes/total cells (%)	70%	26%	44%
Mean platelets volume (fL)	2%	7%	3%
Neutrophils, segmented (10^9/L)	37%	34%	33%
Platelets (10^9/L)	8%	16%	8%
Erythrocytes (10^{12}/L)	8%	6%	7%
Erythrocytes distribution width (%)	9%	7%	8%
Reticulocytes (10^9/L)	12%	10%	15%
Reticulocytes/erythrocytes (%)	16%	8%	18%
Leukocytes (10^9/L)	28%	14%	22%

Tab. S5: Comparison of microscopic findings in select tissues by group
Three separate evaluations of GXX toxicity were conducted using the concurrent control group (CCG) from the original study (a.k.a. the primary study in the proof-of-concept), and two unique virtual control groups (VCG) assembled from individual animal data in the Historical Control Database. Microscopic findings for select tissues of interest are tabulated, along with the frequency of findings per group. The concurrent control group (CCG) was identified as VCG2 to maintain a masked interpretation of GXX-related findings. Findings in the high-dose group were interpreted as GXX-related in each POC study, independent of the identity of the control group. Findings in the low- and mid-dose groups were interpreted as incidental background findings unrelated to GXX. Additional tissues were collected, and their tabulated findings were interpreted (data not shown).

		Control groups			Test groups (GXX)		
Original findings/ results	**Total**	**VCG1**	**VCG2 (CCG)**	**VCG3**	**10 mg/kg**	**30 mg/kg**	**100 mg/kg**
BONE MARROW, STERNUM: Normal	32	6	5	6	6	6	3
BONE MARROW, STERNUM: Hyperplasia; erythroid, diffuse, minimal	2		1				1
BONE MARROW, STERNUM: Immature; myeloid, diffuse, minimal	2						2
BONE MARROW, STERNUM: Fibrosis; focal, mild	1						1
BONE MARROW, STERNUM Examined:	**36**	**6**	**6**	**6**	**6**	**6**	**6**
HEART: Normal	26	4	6	3	6	6	1
HEART: INFILTRATION, HISTIOCYTIC; Ventricle; Right, Focal, Minimal	1	1					
HEART: INFILTRATION, MONONUCLEAR CELL; Multifocal, Minimal	2			2			
HEART: INFILTRATION, MONONUCLEAR CELL; Focal, Mild	1	1					
HEART: NECROSIS; Ventricle; Focal, Minimal: with histiocytic inflammation	1			1			
HEART: Atrophy; adipocyte; epicardium; diffuse, minimal	1						1
HEART: Atrophy; adipocyte; epicardium; diffuse, moderate	2						2
HEART: Atrophy; adipocyte; epicardium; diffuse, marked	1						1
HEART: Degeneration; atrioventricular valve; myxomatous, diffuse, minimal	1						1
HEART: Degeneration; myocardium; multifocal, minimal	3						3
HEART: Degeneration; myocardium; multifocal, mild	1						1
HEART: Hemorrhage; myocardium; multifocal, mild	1						1
HEART: Infiltration; myocardium; histiocytic, multifocal, minimal	3						3
HEART: Infiltration; myocardium; histiocytic, multifocal, mild	1						1
HEART: Infiltration; myocardium; lymphoplasmacytic, multifocal, minimal	3						3
HEART: Infiltration; myocardium; neutrophilic, multifocal, minimal	1						1
HEART: Necrosis; myocardium; multifocal, minimal	1						1
HEART Examined:	**36**	**6**	**6**	**6**	**6**	**6**	**6**
KIDNEYS: Normal	15	2	2		5	4	2
KIDNEYS: Basophilia; tubule; multifocal, minimal	7		4		1	2	
KIDNEY: BASOPHILIA; Cortical, Focal, Minimal	1	1					
KIDNEYS: BASOPHILIA; Tubule; Focal, Minimal	1			1			
KIDNEYS: CAST; Proteinacious, Focal, Minimal	1			1			
KIDNEY: CHRONIC PROGRESSIVE NEPHROPATHY; Minimal	4	1		3			
KIDNEYS: Cyst; tubule; focal	1					1	
KIDNEY: FIBROSIS; Cortical, Focal,	1			1			

Minimal							
KIDNEYS: FIBROSIS; Interstitial, Focal, Minimal	1	1					
KIDNEYS: FIBROSIS; Subcapsular, Focal, Minimal	1			1			
KIDNEYS: Infiltration; interstitium; mononuclear cell; focal, minimal	2		1		1		
KIDNEYS: INFILTRATION, MONONUCLEAR CELL; Focal, Minimal	2	1		1			
KIDNEY: INFILTRATION, LYMPHOCYTIC; Cortical, Focal, Minimal	1	1					
KIDNEYS: INFILTRATION, MONONUCLEAR CELL; Multifocal, Minimal	1			1			
KIDNEYS: Regeneration; tubule; focal, minimal	1					1	
KIDNEYS: Cast; tubule; multifocal, minimal	1						1
KIDNEYS: Cast; tubule; multifocal, mild	3						3
KIDNEYS: Degeneration; tubule; multifocal, mild	3						3
KIDNEYS: Dilation; tubule; multifocal, mild	2						2
KIDNEYS: Dilation; tubule; multifocal, moderate	1						1
KIDNEYS: Necrosis; tubule; multifocal, moderate	2						2
KIDNEYS: Regeneration; tubule; multifocal, mild	1						1
KIDNEYS: Regeneration; tubule; multifocal, moderate	2						2
KIDNEYS: Necrosis; tubule; multifocal, mild	1						1
KIDNEY Examined:	**36**	**6**	**6**	**6**	**6**	**6**	**6**
LIVER: Normal	24	5	3	6	5	5	
LIVER: Hematopoiesis; multifocal, minimal	1					1	
LIVER: Infiltration; mixed, multifocal, minimal	1				1		
LIVER: Infiltration; mononuclear cell; multifocal, minimal	4		3			1	
LIVER: INFILTRATION, MONONUCLEAR CELL; Multifocal, Minimal	1	1					
LIVER: Degeneration; kupffer cell; multifocal, marked	3						3
LIVER: Degeneration; hepatocyte; multifocal, marked	3						3
LIVER: Hypertrophy; hepatocyte; diffuse, mild	3						3
LIVER: Infiltration; histiocytic, multifocal, minimal	1						1
LIVER: Infiltration; histiocytic, multifocal, mild	1						1
LIVER: Necrosis; hepatocyte; multifocal, minimal	1						1
LIVER: Necrosis; coagulative, multifocal, moderate	1						1
LIVER: Necrosis; coagulative, multifocal, marked	1						1
LIVER: Vacuolation; hepatocyte; multifocal, marked	3						3
LIVER: Vacuolation; kupffer cell; multifocal, marked	3						3
LIVER: Degeneration; hepatocyte; multifocal, minimal	1						1
LIVER: Degeneration; kupffer cell; multifocal, minimal	1						1

LIVER: Hematopoiesis; multifocal, minimal	1						1
LIVER: Hypertrophy; hepatocyte; diffuse, minimal	2						2
LIVER: Hypertrophy; hepatocyte; diffuse, mild	1						1
LIVER: Infiltration; histiocytic, multifocal, minimal	1						1
LIVER: Vacuolation; hepatocyte; multifocal, minimal	1						1
LIVER: Vacuolation; kupffer cell; multifocal, minimal	1						1
LIVER Examined:	**36**	**6**	**6**	**6**	**6**	**6**	**6**
SPLEEN: Normal	29	5	6	6	6	6	
SPLEEN: HEMATOPOIESIS; Decreased, Diffuse, Minimal	1	1					
SPLEEN: Atrophy; red pulp; diffuse, minimal	1						1
SPLEEN: Atrophy; red pulp; diffuse, mild	1						1
SPLEEN: Atrophy; red pulp; diffuse, moderate	3						3
SPLEEN: Atrophy; white pulp; diffuse, minimal	1						1
SPLEEN: Atrophy; white pulp; diffuse, mild	1						1
SPLEEN: Atrophy; white pulp; diffuse, moderate	1						1
SPLEEN: Atrophy; white pulp; diffuse, marked	3						3
SPLEEN: Infiltration; red pulp; histiocytic, diffuse, minimal	2						2
SPLEEN: Infiltration; red pulp; histiocytic, diffuse, mild	2						2
SPLEEN: Infiltration; red pulp; histiocytic, diffuse, moderate	2						2
SPLEEN: Necrosis; white pulp; lymphoid, diffuse, marked	2						2
SPLEEN: Necrosis; white pulp; lymphoid, multifocal, marked	1						1
SPLEEN Examined:	**36**	**6**	**6**	**6**	**6**	**6**	**6**
THYMUS: Normal	27	4	6	5	6	6	
THYMUS: HEMORRHAGE; Minimal	1	1					
THYMUS: HEMORRHAGE; Corticomedullary junction; Focal, Minimal	1	1					
THYMUS: Atrophy; lymphoid, diffuse, marked	1						1
THYMUS: Necrosis; lymphoid, diffuse, mild	1						1
THYMUS: Necrosis; lymphoid, diffuse, marked	3						3
THYMUS: Necrosis; lymphoid, multifocal, moderate	2						2
THYMUS Examined:	**35**	**6**	**6**	**5**	**6**	**6**	**6**

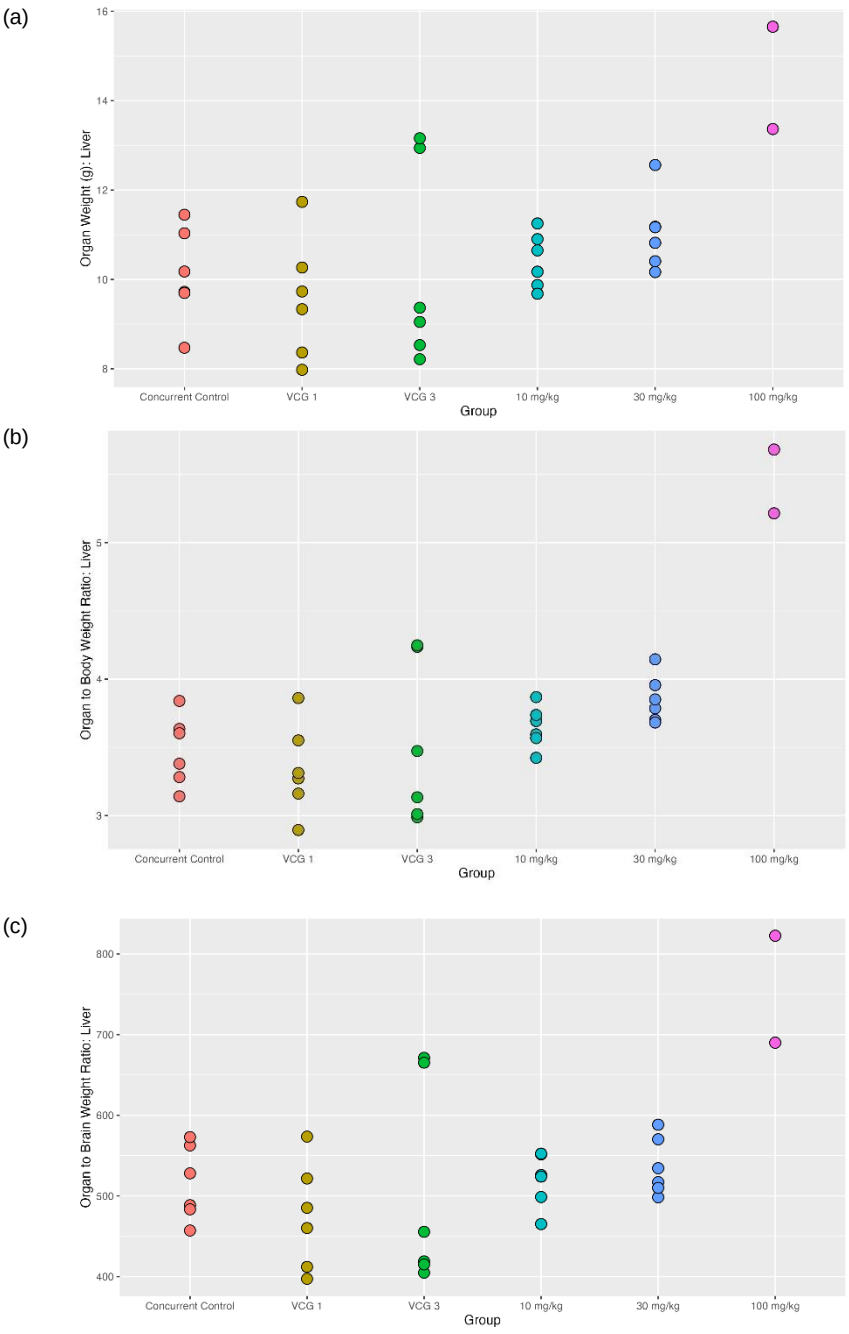
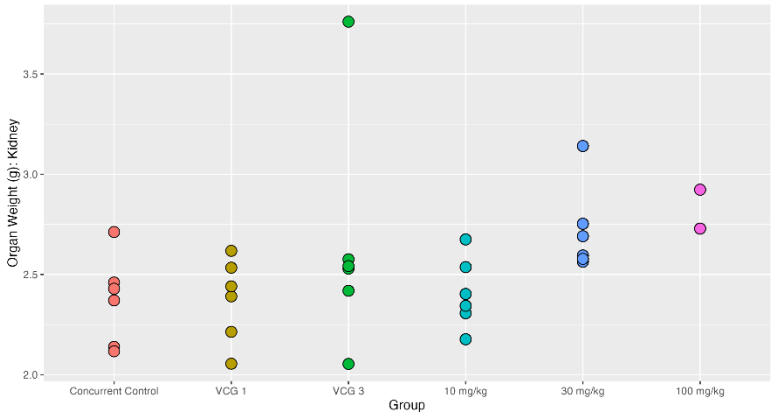


Fig. S1: Distribution of (a) individual organ weights, (b) organ weight to body weight ratios, and (c) organ weight to brain weight ratios for liver
The data shows similar findings across CCG, VCGs and test groups with increased absolute and relative liver weight exhibited (especially liver to body weight ratio) by high-dose animals regardless of the control group comparator.

(a)



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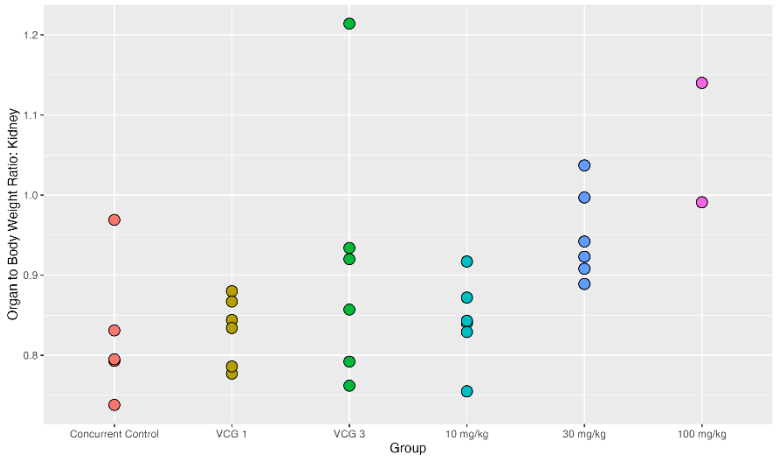

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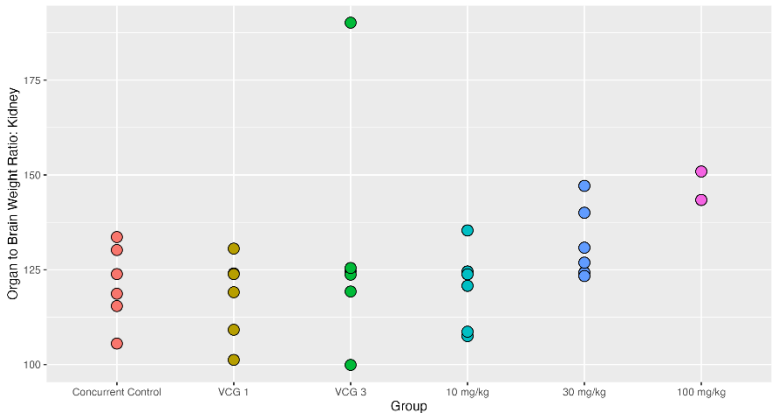


Fig. S2: Distribution of (a) individual organ weights, (b) organ weight to body weight ratios, and (c) organ weight to brain weight ratios for kidney

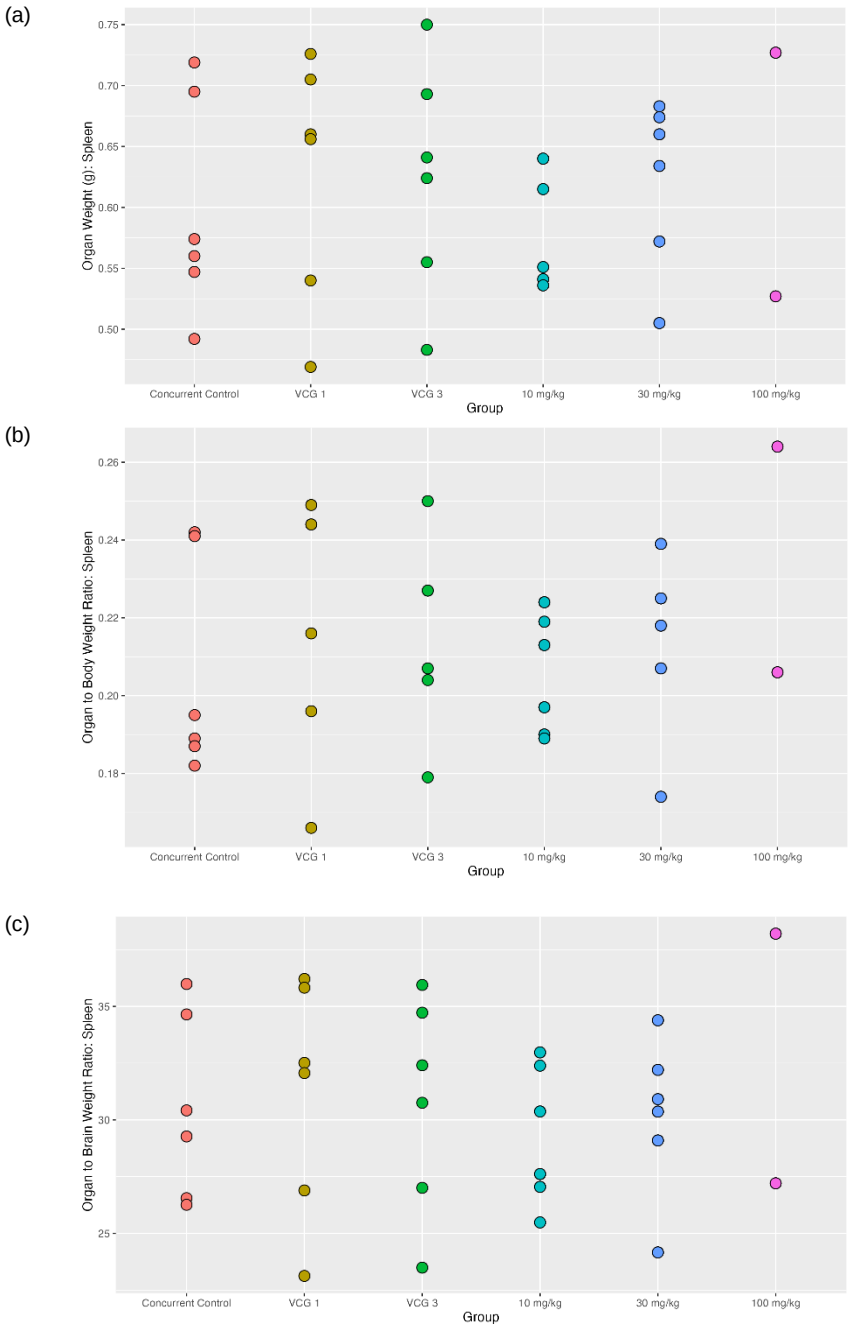
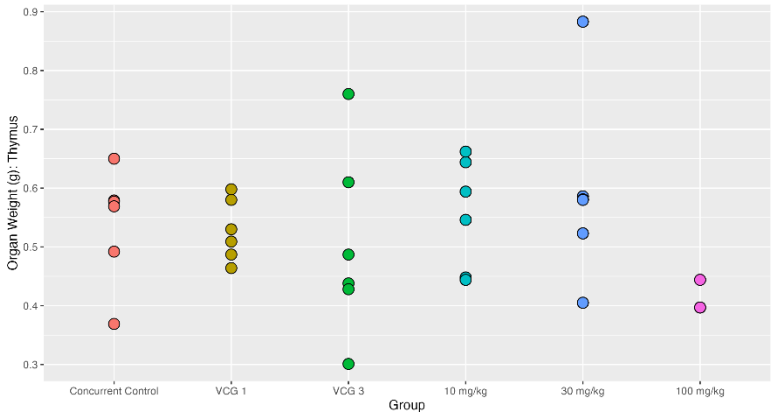


Fig. S3: Distribution of (a) individual organ weights, (b) organ weight to body weight ratios, and (c) organ weight to brain weight ratios for spleen

(a)



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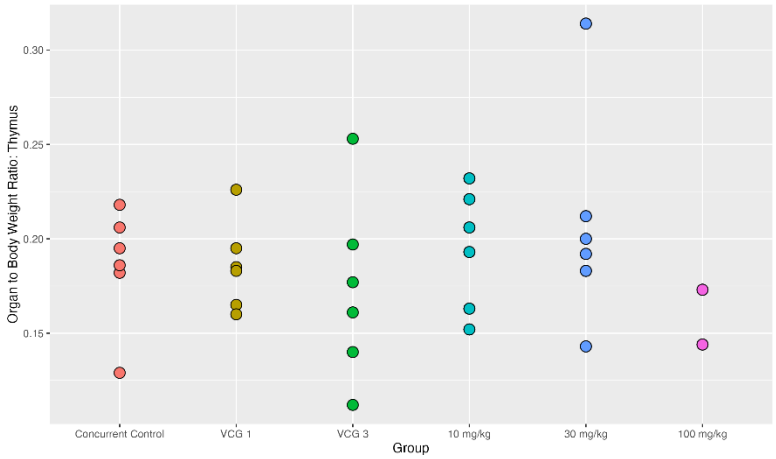

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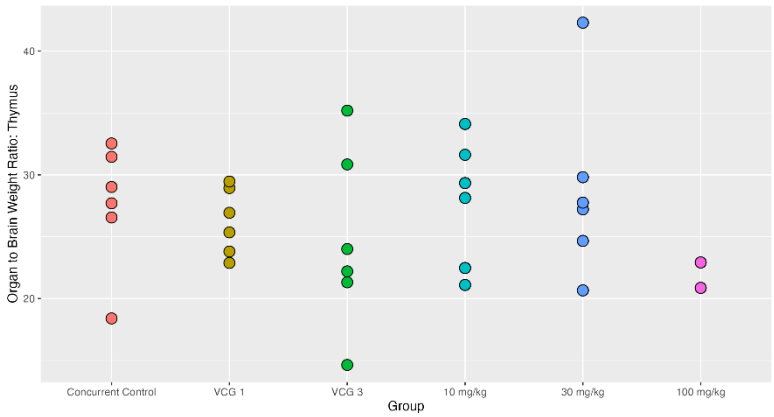

Fig. S4: Distribution of (a) individual organ weights, (b) organ weight to body weight ratios, and (c) organ weight to brain weight ratios for thymus