



4-Week repeated dose rat GLP toxicity study of oncolytic ECHO-7 virus Rigvir administered intramuscularly with a 4-week recovery period

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ABSTRACT

The oncolytic ECHO-7 virus Rigvir was registered in Latvia in 2004 and later in Georgia, Armenia and Uzbekistan. No severe adverse events have been observed. During drug development good laboratory practice (GLP) pre-clinical toxicology studies are generally required by regulatory agencies. Since such studies had previously not been performed, the aim of this 4-week repeated dose GLP toxicity study was to determine the potential toxicity, and reversibility of any findings after a 4-week treatment-free period. Han-Wistar rats were randomly assigned to control, Rigvir (2×10^6 , 1×10^7 and 2×10^7 TCID₅₀) groups. Intramuscular administration was on days 1-3, 8-10, 15-17, and 22-24. Clinical signs, average food-intake, body weights, ophthalmology, clinical pathology parameters, bioanalysis, gross necropsy, organ weights, biodistribution and histopathology were evaluated. There were no unscheduled deaths, adverse clinical signs, no changes in body weight, body weight gain, food intake, ophthalmoscopy, clinical pathology, urine volume or composition, or organ weights. Slightly higher numbers of eosinophils in Rigvir treated animals returned to normal after recovery. Rigvir biodistributed to the spleen. Low incidence of inflammatory cell infiltration at administration sites and increased lymphoid cellularity at the regional (inguinal and popliteal) lymph nodes were observed; after recovery, only those in popliteal lymph nodes remained. Therefore, 4-week Rigvir at 2×10^7 TCID₅₀ administration was well tolerated in rats. The no-observed-adverse-effect level (NOAEL) was the highest dose tested, 2×10^7 TCID₅₀.

Objectives: The objectives of this study were to determine the potential toxicity of Rigvir, an ECHO-7 oncolytic virus, when administered intramuscularly for 4 weeks to rats, with a 4-week recovery period, and to evaluate the reversibility of any potential findings. In addition, the biodistribution of Rigvir in selected tissues was determined.

1. Introduction

An oncolytic ECHO-7 virus has been developed for treatment of melanoma in Riga, Latvia. The resulting medicinal product Rigvir was registered in Latvia in 2004 for treatment of melanoma, local treatment of skin and subcutaneous metastases of melanoma, and for prevention of relapse and metastasis after radical surgery [1]. Rigvir has subsequently been registered also in Georgia, Armenia and Uzbekistan. Consequently, Rigvir has been on markets for approximately 15 years. It is noteworthy that no severe adverse effects of Rigvir treatment have been observed in any of the clinical trials or during the marketing period [1].

Preclinical studies *in vitro* have shown that Rigvir reduces the viability of cancer cells of human origin, including melanoma, rhabdomyosarcoma, gastric adenocarcinoma, lung carcinoma, and pancreas

adenocarcinoma [2]. The results suggest that Rigvir has oncolytic properties.

During preregistration studies, over 700 cancer patients were involved in efficacy studies; over 540 melanoma patients, and late-stage patients with stomach, colorectal and other cancers. Patients were treated with Rigvir for 3 years after surgery and compared to immunotherapy: 3- and 5-year overall survival appeared to be increased in the Rigvir treated patients [1]. The safety of Rigvir was tested in over 180 patients with no severe adverse events observed [1].

A recent retrospective study showed that in 79 melanoma stage IB, IIA, IIB and IIC patients treated with Rigvir post-surgery the mortality was 4.39–6.57-fold lower than in patients under observation according to current guidelines [3]. These results suggest that Rigvir significantly prolongs survival in early-stage melanoma patients without any side

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effect.

Preclinical toxicology studies are commonly performed in suitable animal models. During drug development good laboratory practice (GLP) pre-clinical toxicology studies are generally required by regulatory agencies and the rat represents a standard animal model in regulatory toxicology [4–8]. Since such studies had previously not been performed, and this study had been suggested in a recent Scientific Advice procedure with the European Medicines Agency, the aim of this 4-week repeated dose GLP toxicity study was to determine the potential toxicity and reversibility of any findings after a 4-week treatment-free period. In the present study the no-observed-adverse-effect-level (NOAEL) in the rat was also determined [8–10].

2. Methods and Materials

2.1. Animals

Studies in laboratory animals are required to support regulatory submissions.

The Han Wistar rat was chosen as the animal model for this study as it is an accepted rodent species for preclinical toxicity testing by regulatory agencies. In this study, 74 male rats and 74 female rats obtained from Charles River UK Limited, Margate, Kent, UK were used. The animals were 7–8 weeks old and weighed 168–242 g (males) (target 200–300 g) and 136–186 g (females) (target 150–250 g) at the initiation of dosing. Animals were randomly assigned to the 4 groups, males and females separately.

The animals were acclimatised to the test facility for up to 14 days before the start of administration. The animals were group housed (up to 4 animals of the same sex and same dose group together). Appropriately sized polycarbonate/polypropylene cages with stainless steel grid tops and solid bottoms were used. Appropriate bedding was provided. Where possible, control group animals were housed on a separate rack from the test item-treated animals. The temperature was 19–23 °C, humidity 40–70%, light cycle of 12 hours light and 12 hours dark (except during designated procedures), and ventilation ten or more air changes per hour.

Animals were socially housed for psychological/environmental enrichment and were provided with items such as a device for hiding, an object for chewing, except when interrupted by study procedures/activities.

The animals were provided food *ad libitum* (Special Diet Services Rat and Mouse (modified) No. 1 Diet SQC), and public supply tap water *ad libitum* from water bottles, except during designated procedures.

Veterinary advice was available throughout the course of the study.

2.2. Groups and test item administration

The test item, Rigvir, batch B0319RT, with a titre of $10^{8.0}$ TCID₅₀/ml, was stored as received in a freezer at –20 °C until use. On the day of dosing, the vials were removed from the freezer, allowed to thaw at room temperature for approximately 15–20 minutes and placed in a refrigerator set to maintain 4 °C. The test item was thawed and administered as received. The control group was administered 0.9% sodium chloride w/v solution, kept in a refrigerator set to maintain 4 °C.

The test groups of 10 animals per sex were control (sterile 0.9% sodium chloride w/v solution), Rigvir (2×10^6 TCID₅₀), Rigvir (1×10^7 TCID₅₀), and Rigvir (2×10^7 TCID₅₀), and recovery animals, 5 per sex from control and 5 per sex from the highest dose group, and biodistribution animals, 6 per sex per each group. Administration was by intramuscular injection into the hind limbs using a needle and syringe.

The volumes administered on dosing days were 2 times 0.1 ml (one injection to each hind limb), 0.1 ml (injection into alternating hind limbs), and 0.02 ml (injection into alternating hind limbs) in the different groups, respectively, in compliance with [11].

The administration was on days 1–3, 8–10, 15–17, and 22–24. The

dosing cycle regimen mimics the clinical dosing regimen in a condensed manner. In patients, Rigvir (2 ml) is first administered intramuscularly (i.m.) regionally for 3 consecutive days. About 4 weeks later, this administration is repeated for another 3 consecutive days, and about 4 weeks later again for another 3 consecutive days. During the rest of the first year, a single administration of Rigvir is performed at monthly intervals. During the first half of the second year, administration is at 6-week intervals, during the second half of the second year at 2-month intervals, and during the third year at 3-month intervals [3].

The total number of animals to be used in this study is considered to be the minimum required to properly characterise the effects of the test item. This study has been designed such that it does not require an unnecessary number of animals to accomplish its objectives. There were 10 males and 10 females in each group. There were additional 6 males and 6 females in each group for biodistribution measurements. In addition, there were 5 males and 5 females in two recovery groups, control, and the highest titre group.

The study included a control group of animals that were treated in a similar manner to those receiving the test item. There was no requirement for blinding of the operators and data analysts. At study assignment, each animal was identified using a subcutaneously implanted ‘glass-sealed’ electronic cylindrical microchip.

2.3. Observations and analyses

The animals were checked twice daily for mortality, detailed clinical observation at least weekly and regular water intake. Individual body weight and food intake was recorded twice weekly from days number –5, –2, 2, 4, 8, 11, 15, 18, 22, 25, 29, 32, 36, 39, 43, 46, 50, 53, to 57.

The eyes were examined using an indirect ophthalmoscope before start of treatment (all groups), after the 4-week treatment and after the recovery period (control and highest dose group). Samples were taken for clinical pathology (haematology, coagulation, clinical chemistry, and urinalysis), and bone marrow smear evaluation after week 4 of treatment (all groups) and after the recovery period (control and highest dose group). The following haematology parameters were evaluated: red blood cell count, haemoglobin concentration, haematocrit, mean corpuscular volume, red blood cell distribution width, mean corpuscular haemoglobin concentration, mean corpuscular haemoglobin, platelet count, reticulocyte count (absolute), white blood cell count, neutrophil count (absolute), lymphocyte count (absolute), monocyte count (absolute), eosinophil count (absolute), basophil count (absolute), and large unstained cells (absolute).

The following coagulation parameters were evaluated: activated partial thromboplastin time, fibrinogen, and prothrombin time.

The following clinical chemistry parameters were evaluated: alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, gamma-glutamyltransferase, creatine kinase, total bilirubin, urea, creatinine, calcium, phosphate, total protein, albumin, calculated globulin, albumin/globulin ratio, glucose, cholesterol, triglycerides, sodium, potassium, and chloride.

The following urinalysis parameters were evaluated: colour, appearance/clarity, specific gravity, volume, pH, protein, glucose, bilirubin, ketones, and blood.

At the end of the study the weight of the organs was determined of the brain, adrenal gland, epididymis, heart, kidney, liver, ovary, pituitary gland, prostate, spleen, testis, thymus, thyroid gland, and uterus.

Blood samples were obtained from the jugular vein (no anaesthesia) or orbital sinus vein under terminal carbon dioxide anaesthesia, after week 4 and after the recovery period, 0.5 ml for haematology and coagulation, and 0.7 ml for clinical chemistry. Lithium-heparin plasma was for clinical chemistry and trisodium citrate plasma prepared for coagulation analysis was prepared using pre-coated tubes.

Tissue samples were collected at necropsy from all biodistribution animals, of injection site, heart, lung, liver, kidney, spleen, ovary, testicle, bone marrow, brain and cerebrospinal fluid (CSF). The single dose

samples were collected 24 ± 2 h post dose, and the full dose samples were collected on Day 29.

2.4. Detection of viral RNA using RT-qPCR

A one-step real-time reverse transcription quantitative polymerase chain reaction (RT-qPCR) GLP analysis method was set up and validated to measure Rigvir in rat fluid and tissue samples. ECHO-7 Wallace strain was used as reference (VR-37, ATCC). The primers and probe used for detection of Rigvir were shown to be specific during the development of this assay: forward primer 5' CAACTGAGCCCGACCAAG 3', reverse primer 5' AGTAATTGGCCGGTAACGA 3', and probe 5' (6-FAM) AGGCTGCTCGCGGCTGAAGGT 3' MGBNFQ.

2.5. Histopathology

Tissues processed for histopathology and microscopic evaluation were embedded in paraffin, sectioned, mounted on glass slides, and stained with haematoxylin and eosin.

Microscopic evaluation was conducted by a board-certified veterinary pathologist on all collected tissues from all animals in the control group and highest Rigvir dose group and all gross lesions from all animals.

The following tissues were taken for histology evaluation: artery (aorta), bone marrow (sternum), bones (femur, sternum), brain, epididymis, oesophagus, eye, gland (adrenal, Harderian, mammary, parathyroid, pituitary, prostate, salivary, submandibular, seminal vesicle, thyroid), gut-associated lymphoid tissue (Peyer's patch or solitary lymphoid follicle), heart, joint (femorotibial), kidneys, caecum, colon, rectum, liver, lung, lymph nodes draining administration sites (inguinal, popliteal), mandibular and mesenteric lymph nodes, skeletal muscle, nerves (optic, sciatic), ovaries, pancreas, administration sites (left and right hind limb), skin, duodenum, ileum, jejunum, spinal cord, spleen, stomach, testes, thymus, tongue, trachea, urinary bladder, uterus/cervix, vagina.

In addition, the following tissues were taken for macroscopic evaluation: nasal cavity, bone marrow smear, glands (clitoral, lacrimal, preputial, sublingual, parotid, Zymbals), larynx, tibial nerve, oviduct, and ureter.

A total of 1438 slides were evaluated for histology.

2.6. Biodistribution

At necropsy, $24 \text{ h} \pm 2 \text{ h}$ after the first administration and on Day 29, samples of injection site, heart, lung, liver, kidney, spleen, ovary, testicle, bone marrow (femur), brain and CSF were collected from all biodistribution animals.

A total of 1051 samples were collected for biodistribution analysis.

2.7. Statistical analysis

Levene's test was used to assess the homogeneity of group variances.

The groups were compared using an overall one-way Anova F-test if Levene's test was not significant or the Kruskal-Wallis test if it was significant. If the overall F-test or Kruskal Wallis test was found to be significant, then pairwise comparisons were conducted using Dunnett's or Dunn's test, respectively. Datasets with two groups were compared using a Dunnett's test or Dunn's test. Statistical difference was if $P \leq 0.05$.

2.8. Ethical approval

The study complied with national legislation covering the use of animals in scientific research. UK Home Office controls scientific procedures on animals in the UK and does so by the issue of licences under the Animals (Scientific Procedures) Act 1986. The regulations conform

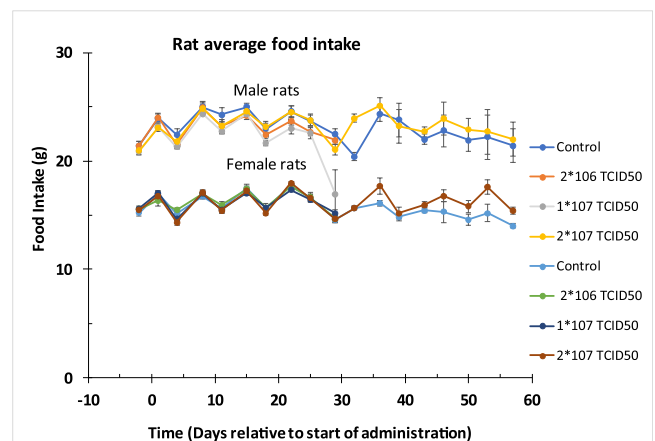


Fig. 1. Average food intake per group for male and female rats shown separately. Control, $N = 15$, Rigvir (2×10^6 TCID₅₀), $N = 10$, Rigvir (1×10^7 TCID₅₀), $N = 10$, and Rigvir (2×10^7 TCID₅₀), $N = 15$; recovery animals $N = 2-5$. The average food intake was measured as averages over 3-4 days. The results are expressed as mean \pm SEM.

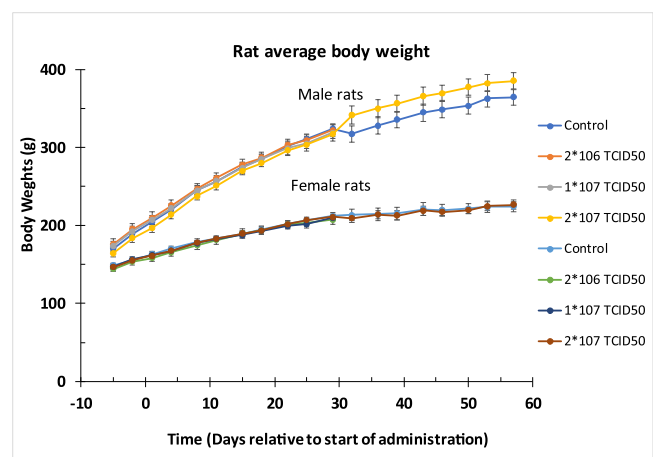


Fig. 2. Average body weight per group for male and female rats shown separately. Control, $N = 15$, Rigvir (2×10^6 TCID₅₀), $N = 10$, Rigvir (1×10^7 TCID₅₀), $N = 10$, and Rigvir (2×10^7 TCID₅₀), $N = 15$; recovery animals $N = 2-5$. The results are expressed as mean \pm SEM.

to the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (Strasbourg, Council of Europe) and achieve the standard of care required by the US Department of Health and Human Services' Guide for the Care and Use of Laboratory Animals. The Home Office licence governing this study strictly specifies the limits of severity of effects on the animals; the procedures described here did not cause any effects which exceed the severity limit of the procedure. This study was performed under the Home Office Project Licence No. PBAD559F8, Toxicology of Pharmaceuticals, Protocol No. 1.

The present study was performed for regulatory purposes and was performed in accordance with International Council for Harmonisation (ICH) guidelines. The study was performed in accordance with the OECD Principles of Good Laboratory Practice [12] as incorporated into the United Kingdom Statutory Instrument for GLP and as accepted by Regulatory Authorities throughout the European Union, United States of America (FDA and EPA), Japan (MHLW, MAFF and METI), and other countries that are signatories to the OECD Mutual Acceptance of Data Agreement.

Table 1
Eosinophil count after the 4-week Rigvir treatment and after the 4-week recovery period.

Dose (TCID ₅₀)	Eosinophil count (10 ⁹ /L)			
	Male		Female	
	Day 29	Day 53	Day 29	Day 53
0 (Control)	0.12 ± 0.04 (10)	0.10 ± 0.03 (5)	0.12 ± 0.05 (10)	0.08 ± 0.01 (5)
2 × 10 ⁶	0.18 ± 0.05 (10)	n.d.	0.19 ± 0.06 (10)	n.d.
1 × 10 ⁷	0.16 ± 0.06 (9)	n.d.	0.25 ± 0.10 ** (10)	n.d.
2 × 10 ⁷	0.22 ± 0.09 ** (10)	0.12 ± 0.04 (5)	0.26 ± 0.10 ** (10)	0.09 ± 0.02 (5)

Mean ± SD, number of animals (N), not determined (n.d.). Statistical significance vs. control (Anova & Dunnet): P ≤ 0.01 (**).

3. Results

The observed average food intake and body weights were not significantly different between the groups (Figs. 1 and 2). In the male middle dose group at day 29 an unexpectedly low value of food consumption was observed; it did not influence the body weight and was considered incidental.

There were no unscheduled deaths, adverse clinical signs, ophthalmoscopic observations, nor test-item related organ weight differences (Tables A1 and A2).

Clinical pathology parameters, bioanalysis, gross necropsy, organ weights, and histopathology were evaluated.

There were no signs of clinical pathology: haematology (Tables B1 and B2), coagulation (Table C1), clinical chemistry (Tables D1 and D2), and urinalysis, urine volume or composition (Table E1).

On Day 29, there were slightly higher numbers of circulating eosinophils (increased from 0.12 × 10⁹/L in both males and females up by 1.79-fold in males and by 2.11-fold in females) in animals receiving Rigvir, when compared with controls, achieving statistical significance at 2 × 10⁷ TCID₅₀ in males, and ≥ 1 × 10⁷ TCID₅₀ in females. There was evidence of a relationship with dosage. After the recovery period, the numbers of eosinophils in animals that had received Rigvir were similar to controls (Table 1).

No measurable amount of Rigvir was detected in any control group samples. Rigvir was present at quantifiable levels in plasma samples on Day 1 at 24 h in all three Rigvir treatment groups with up to 1.83 × 10⁵ copies/mL plasma and in Rigvir (2 × 10⁷ TCID₅₀), plasma samples at 48 h with up to 8.81 × 10³ copies/mL. The levels showed a dose-dependency, the highest detected levels of Rigvir were found in the high-dose group, 2 × 10⁷ TCID₅₀. On Day 1, Rigvir was detected in spleen samples from all treatment groups with up to 2.57 × 10³ copies/μg RNA, and in one kidney and one testis sample from 2 different animals receiving 1 × 10⁷ TCID₅₀. On Day 29, Rigvir was only detected in the spleen samples of 2 animals (one male and one female) receiving 2 × 10⁷ TCID₅₀. Thus, Rigvir biodistributed to the spleen.

There were no treatment-related necropsy findings. At histopathological examination, there were very minor treatment-related findings at the administration sites and regional lymph nodes. There was a low incidence of treatment-related inflammatory cell infiltration at both administration sites at all dose levels. This was composed of mixed cells, mononuclear cells, or lymphocytic cells, and the severity was generally minimal, except for a few mild infiltrates in high dose animals. Treatment-related increased lymphoid cellularity was present at the regional (inguinal and popliteal) lymph nodes. This was mainly minimal in severity, with increased lymphoid follicles. Mild increased cellularity was present in a small proportion of animals at the high dose, and in occasional other animals, including one control male animal. These findings were non-adverse, and there was complete recovery at the administration sites and inguinal lymph node; after recovery, only those

in popliteal lymph nodes remained. Other microscopic findings observed were of the nature commonly observed in this strain and age of rat, or occurred at a similar incidence in control and treated animals, and, therefore, were considered not to be test item-related.

Due to the limited quantifiable plasma concentrations of test item in male and female rats across doses and days, the generation of toxicokinetic parameters was not possible.

4. Discussion

The safety and efficacy of Rigvir has been studied in approximately 800 patients [1,3]. While Rigvir increased survival and reduced mortality, few if any severe adverse events were observed [1,3].

The results of the present biodistribution study show that target tissues were the spleen, inguinal lymph node, popliteal lymph node, and administration sites. This is consistent with similar findings that have been observed with other oncolytic viruses. For example, while there were no changes to the lymph nodes, the highest levels of parvovirus H1 after intravenous or intracerebral administration in Wistar rats were found in the liver and spleen [13,14]. After a single administration of a measles virus to mice it was found mostly in lymphoid organs, lymph nodes and spleen, with no adverse events reported [15]. Daily intravenous administration of an adenovirus to rats for four weeks was associated with slight toxicities, which included increased red blood cell count, platelet count, prothrombin time, and decreased food intake, albumin, total cholesterol, total bilirubin, total protein, and creatinine [16]. Daily intramuscular administration of an adenovirus to rats for two weeks was associated with slight toxicities that included increased white blood cell count, reticulocyte count, platelet count, aspartate aminotransferase, and decreased food intake, red blood cell count, haemoglobin, haematocrit, albumin, and total cholesterol [17]. Currently, only one other oncolytic virus, a genetically modified, weakened form of Herpes Simplex Virus Type 1, Human alphaherpesvirus 1, with the generic name talimogene laherparepvec, has been approved by regulatory agencies in Europe and the USA. Only few adverse events were found in the mice and rat toxicology studies with talimogene laherparepvec, including enlarged spleens and local irritation at injection sites that were reversed at terminal necropsy [18,19]. Tumour-bearing mouse biodistribution studies following intravenous administration of talimogene laherparepvec showed the presence of the viral DNA in the tumour, blood, lymph nodes, spleen, and liver, indicating that these tissues are “likely associated with immune-mediated viral clearance” [19–21]. Similarly, some findings in immune cells and spleen have been observed in a mouse toxicology study with an adenovirus [22].

The results show that there were no significant test item-related adverse events. Therefore, it is concluded that the results suggest that oncolytic ECHO-7 virus Rigvir is safe to administer to rats at the doses used in this study. The dosing regimen used was chosen to mimic the clinical dosing regimen in a condensed manner.

Based on the results, the no-observed-adverse-effect level (NOAEL) was considered to be the highest dose tested of 2 × 10⁷ TCID₅₀.

Author statement

The study was planned, the manuscript was written and approved by KP, GJ, AR and PA, and the study was performed by KP and GJ.

Declaration of Competing Interest

The authors declare no competing interest.

Acknowledgements

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Appendix A

A statistically significant lower spleen weight was present in all male treated groups when compared with the control group, however, there was no dose-relationship, there were no correlating microscopic findings and not observed in females, therefore this was considered an incidental finding.

There were individual organ weight values that were different from

their respective controls. There were, however, no patterns or correlating data to suggest these values were test item-related.

There were individual organ weight values that were different from their respective controls. There were, however, no patterns or correlating data to suggest these values were test item-related.

Table A1

Organ weights after the 4-week Rigvir treatment on day 29 and after the 4-week recovery on day 57. Males.

Organ	Organ weight (g)					
	Dose (TCID ₅₀)					
	Day 29				Day 57	
	0 (Control)	2 × 10 ⁶	1 × 10 ⁷	2 × 10 ⁷	0 (Control)	2 × 10 ⁷
Brain	1.95 ± 0.09	1.97 ± 0.10	1.95 ± 0.08	1.96 ± 0.11	2.02 ± 0.05	2.07 ± 0.06
Epididymis	1.05 ± 0.17	1.03 ± 0.11	0.96 ± 0.20	1.01 ± 0.14	1.20 ± 0.12	1.20 ± 0.12
Adrenal	0.060 ± 0.009	0.057 ± 0.009	0.0059 ± 0.011	0.063 ± 0.012	0.054 ± 0.006	0.055 ± 0.007
Pituitary	0.0084 ± 0.0019	0.0082 ± 0.0019	0.0071 ± 0.0016	0.0080 ± 0.0023	0.008 ± 0.001	0.008 ± 0.002
Prostate	0.38 ± 0.12	0.30 ± 0.11	0.31 ± 0.12	0.26 ± 0.06	0.45 ± 0.08	0.41 ± 0.09
Thyroid	0.015 ± 0.004	0.015 ± 0.003	0.015 ± 0.004	0.015 ± 0.004	0.020 ± 0.003	0.022 ± 0.005
Heart	1.09 ± 0.08	1.00 ± 0.11	1.01 ± 0.13	1.01 ± 0.10	1.17 ± 0.13	1.16 ± 0.11
Kidney	2.18 ± 0.20	2.24 ± 0.19	2.09 ± 0.24	2.01 ± 0.17	2.12 ± 0.14	2.19 ± 0.17
Liver	12.77 ± 1.13	12.44 ± 1.16	12.17 ± 1.33	11.78 ± 0.54	13.49 ± 0.81	13.43 ± 0.70
Spleen	0.62 ± 0.08	0.52 ± 0.06 **	0.51 ± 0.08 **	0.51 ± 0.05 **	0.59 ± 0.09	0.73 ± 0.06 *
Testis	3.39 ± 0.19	3.40 ± 0.21	3.54 ± 0.21	3.40 ± 0.22	3.48 ± 0.50	3.28 ± 0.28
Thymus	0.67 ± 0.07	0.53 ± 0.11 *	0.60 ± 0.12	0.55 ± 0.12 *	0.43 ± 0.15	0.50 ± 0.10

Mean ± SD, number of animals, day 29 (N = 10), day 57 (N = 5). Statistical significance vs. control (Anova & Dunnet): P ≤ 0.05 (*), P ≤ 0.01 (**).

Table A2

: Organ weights after the 4-week Rigvir treatment on day 29 and after the 4-week recovery on day 57. Females.

Organ	Organ weight (g)					
	Dose (TCID ₅₀)					
	Day 29				Day 57	
	0 (Control)	2 × 10 ⁶	1 × 10 ⁷	2 × 10 ⁷	0 (Control)	2 × 10 ⁷
Brain	1.86 ± 0.06	1.80 ± 0.05	1.82 ± 0.05	1.85 ± 0.08	1.93 ± 0.06	1.86 ± 0.07
Adrenal	0.078 ± 0.008	0.077 ± 0.013	0.070 ± 0.014	0.070 ± 0.010	0.062 ± 0.011	0.066 ± 0.016
Pituitary	0.0098 ± 0.0021	0.0093 ± 0.0021	0.0094 ± 0.0038	0.0104 ± 0.0018	0.0088 ± 0.0046	0.0115 ± 0.0012
Thyroid	0.011 ± 0.003	0.011 ± 0.003	0.013 ± 0.003	0.014 ± 0.003	0.019 ± 0.004	0.023 ± 0.004
Heart	0.75 ± 0.08	0.71 ± 0.06	0.72 ± 0.08	0.72 ± 0.10	0.77 ± 0.04	0.82 ± 0.05
Kidney	1.50 ± 0.09	1.48 ± 0.11	1.47 ± 0.14	1.47 ± 0.13	1.39 ± 0.14	1.45 ± 0.15
Liver	7.84 ± 0.69	7.69 ± 0.98	7.54 ± 0.94	7.81 ± 1.30	8.08 ± 0.69	8.14 ± 0.50
Ovary	0.091 ± 0.019	0.113 ± 0.013 **	0.088 ± 0.010	0.101 ± 0.015	0.083 ± 0.020	0.101 ± 0.009
Spleen	0.41 ± 0.05	0.39 ± 0.06	0.41 ± 0.05	0.41 ± 0.07	0.50 ± 0.08	0.53 ± 0.05
Thymus	0.47 ± 0.12	0.46 ± 0.08	0.48 ± 0.07	0.52 ± 0.10	0.36 ± 0.06	0.35 ± 0.02
Uterus	0.61 ± 0.32	0.58 ± 0.14	0.62 ± 0.26	0.50 ± 0.13	0.74 ± 0.39	0.63 ± 0.13

Mean ± SD, number of animals, day 29 (N = 10), day 57 (N = 5). Statistical significance vs. control (Anova & Dunnet): P ≤ 0.01 (**).

There were individual organ weight values that were different from their respective controls. There were, however, no patterns or correlating data to suggest these values were test item-related.

Appendix B

Table B1

Clinical Pathology: Haematology. Males.

Parameter	Dose (TCID ₅₀)					
	Day 29				Day 53	
	0 (Control)	2 × 10 ⁶	1 × 10 ⁷	2 × 10 ⁷	0 (Control)	2 × 10 ⁷
WBC (10 ⁹ /L)	8.52 ± 1.92	8.40 ± 1.78	9.11 ± 2.45	8.19 ± 2.05	7.11 ± 1.55	8.16 ± 1.23
Neut (10 ⁹ /L)	1.00 ± 0.27	1.31 ± 0.28 *	1.22 ± 0.24	1.10 ± 0.18	1.03 ± 0.20	1.85 ± 0.77
Lym (10 ⁹ /L)	7.20 ± 1.74	6.70 ± 1.60	7.50 ± 2.33	6.64 ± 1.79	5.90 ± 1.37	5.89 ± 0.54
Mon (10 ⁹ /L)	0.14 ± 0.08	0.14 ± 0.06	0.15 ± 0.06	0.17 ± 0.14	0.05 ± 0.02	0.21 ± 0.16 *
Eos (10 ⁹ /L)	0.12 ± 0.04	0.18 ± 0.05	0.16 ± 0.06	0.22 ± 0.09 **	0.10 ± 0.03	0.12 ± 0.04
Baso (10 ⁹ /L)	0.02 ± 0.01	0.02 ± 0.01	0.02 ± 0.01	0.02 ± 0.01	0.01 ± 0.01	0.01 ± 0.01
Luc (10 ⁹ /L)	0.05 ± 0.02	0.05 ± 0.02	0.05 ± 0.03	0.05 ± 0.03	0.02 ± 0.01	0.07 ± 0.05
RBC (10 ¹² /L)	7.94 ± 0.37	7.76 ± 0.49	7.96 ± 0.35	7.90 ± 0.33	8.87 ± 0.59	8.26 ± 0.66
Hbg (g/L)	152.9 ± 8.2	149.6 ± 6.9	153.7 ± 5.5	151.2 ± 5.1	164.0 ± 5.6	148.6 ± 12.4
Hct (L/L)	0.44 ± 0.02	0.43 ± 0.02	0.44 ± 0.01	0.43 ± 0.02	0.47 ± 0.02	0.43 ± 0.03
Mcv (fl)	54.8 ± 1.0	55.0 ± 1.5	55.1 ± 1.4	55.0 ± 1.3	52.8 ± 1.9	52.2 ± 1.3
Mch (pg)	19.3 ± 0.5	19.3 ± 0.7	19.3 ± 0.6	19.2 ± 0.6	18.6 ± 0.9	18.0 ± 0.7
Mchc (g/L)	351.2 ± 5.9	351.4 ± 8.4	350.8 ± 5.3	349.1 ± 5.7	351.4 ± 10.3	344.8 ± 7.9
Rdwg (%)	11.2 ± 0.6	11.3 ± 0.5	11.2 ± 0.6	11.0 ± 0.4	12.1 ± 0.8	12.1 ± 0.8
Plt (10 ⁹ /L)	807.7 ± 85.7	836.5 ± 68.1	790.0 ± 72.0	845.4 ± 129.5	655.4 ± 98.7	706.2 ± 141.4
Retic (10 ⁹ /L)	209.5 ± 37.8	212.9 ± 51.3	218.7 ± 52.1	217.1 ± 40.9	232.3 ± 32.0	213.7 ± 21.2

Mean ± SD, number of animals, day 29 (N = 10), day 53 (N = 5). Statistical significance vs. control (Anova & Dunnet): P ≤ 0.05 (*), P ≤ 0.01 (**).

Table B2

Clinical Pathology: Haematology. Females

Parameter	Dose (TCID ₅₀)					
	Day 29				Day 53	
	0 (Control)	2 × 10 ⁶	1 × 10 ⁷	2 × 10 ⁷	0 (Control)	2 × 10 ⁷
WBC (10 ⁹ /L)	7.30 ± 1.66	6.63 ± 1.23	7.72 ± 2.05	6.86 ± 2.22	4.48 ± 1.72	4.26 ± 1.02
Neut (10 ⁹ /L)	0.91 ± 0.25	0.89 ± 0.21	0.90 ± 0.23	1.00 ± 0.44	0.97 ± 0.52	0.74 ± 0.34
Lym (10 ⁹ /L)	6.07 ± 1.52	5.37 ± 1.13	6.32 ± 1.85	5.41 ± 2.29	3.68 ± 1.20	3.37 ± 0.71
Mon (10 ⁹ /L)	0.14 ± 0.07	0.12 ± 0.03	0.18 ± 0.09	0.13 ± 0.06	0.09 ± 0.08	0.05 ± 0.02
Eos (10 ⁹ /L)	0.12 ± 0.05	0.19 ± 0.06	0.25 ± 0.10 **	0.26 ± 0.10 **	0.08 ± 0.01	0.09 ± 0.02
Baso (10 ⁹ /L)	0.02 ± 0.01	0.01 ± 0.01	0.02 ± 0.01	0.02 ± 0.01	0.01 ± 0.01	0.01 ± 0.01
Luc (10 ⁹ /L)	0.05 ± 0.02	0.04 ± 0.01	0.06 ± 0.04	0.04 ± 0.02	0.03 ± 0.01	0.01 ± 0.01
RBC (10 ¹² /L)	7.62 ± 0.33	7.46 ± 0.35	7.47 ± 0.49	7.42 ± 0.48	8.15 ± 0.78	8.11 ± 0.38
Hbg (g/L)	146.5 ± 5.0	142.5 ± 7.5	142.8 ± 8.0	143.0 ± 4.9	152.6 ± 12.2	148.2 ± 5.8
Hct (L/L)	0.42 ± 0.01	0.41 ± 0.02	0.41 ± 0.02	0.41 ± 0.02	0.43 ± 0.03	0.43 ± 0.02
Mcv (fl)	54.8 ± 1.5	54.4 ± 1.5	54.4 ± 1.5	54.7 ± 1.7	53.2 ± 1.6	53.0 ± 1.6
Mch (pg)	19.2 ± 0.7	19.1 ± 0.6	19.2 ± 0.7	19.3 ± 0.8	18.8 ± 0.7	18.3 ± 0.6
Mchc (g/L)	351.4 ± 6.2	351.6 ± 5.6	351.9 ± 7.4	352.9 ± 8.2	352.8 ± 4.7	345.8 ± 4.7 *
Rdwg (%)	10.4 ± 0.3	10.9 ± 0.3	10.7 ± 0.6	11.5 ± 0.5 **	11.3 ± 0.4	11.4 ± 0.3
Plt (10 ⁹ /L)	867.0 ± 94.3	777.6 ± 80.9	779.1 ± 72.0	848.1 ± 100.4	729.2 ± 146.8	781.8 ± 136.3
Retic (10 ⁹ /L)	204.9 ± 42.2	239.2 ± 42.2	236.0 ± 71.6	264.6 ± 92.2	204.7 ± 35.2	222.6 ± 39.6

Mean ± SD, number of animals, day 29 (N = 10), day 53 (N = 5). Statistical significance vs. control (Anova & Dunnet): P ≤ 0.05 (*), P ≤ 0.01 (**).

White blood cell (WBC) count, neutrophil count (Neut), lymphocyte count (Lym), monocyte count (Mon), eosinophil count (Eos), basophil count (Baso), and large unstained cells (Luc), red blood cell (RBC) count, haemoglobin (Hbg) concentration, haematocrit (Hct), mean corpuscular volume (MCV), mean corpuscular haemoglobin (Mch), mean corpuscular haemoglobin concentration (Mchc), red blood cell distribution width gated (Rdwg), platelet count (Plt), reticulocyte count (Retic).

Appendix C

Table C1
Clinical Pathology: Coagulation.

Parameter, Sex	Dose (TCID ₅₀)					
	Day 29				Day 53	
	0 (Control)	2 × 10 ⁶	1 × 10 ⁷	2 × 10 ⁷	0 (Control)	2 × 10 ⁷
Males						
Pt (sec)	10.6 ± 0.2	10.6 ± 0.3	10.7 ± 0.3	10.7 ± 0.4	10.9 ± 0.4	10.3 ± 0.2 *
Aptt (sec)	10.5 ± 2.3	9.8 ± 2.3	9.6 ± 1.3	10.4 ± 3.3	12.6 ± 1.7	10.8 ± 0.8
Fib (g/L)	2.4 ± 0.25	2.4 ± 0.20	2.2 ± 0.18	2.3 ± 0.23	2.3 ± 0.31	2.6 ± 0.25
Females						
Pt (sec)	10.2 ± 0.1	10.1 ± 0.2	10.2 ± 0.1	10.2 ± 0.2	9.9 ± 0.2	10.1 ± 0.3
Aptt (sec)	10.8 ± 2.2	10.0 ± 1.3	9.4 ± 2.2	9.3 ± 1.7	9.8 ± 1.4	10.6 ± 1.0
Fib (g/L)	1.9 ± 0.12	1.8 ± 0.20	1.8 ± 0.20	1.8 ± 0.17	1.9 ± 0.34	1.5 ± 0.20

Mean ± SD, number of animals, day 29 (males, N = 9-10, females, N = 79), day 53 (males, N = 5, females, N = 4-5). Statistical significance vs. control (Anova & Dunnett): P ≤ 0.05 (*).

Activated partial thromboplastin time (Aptt), fibrinogen (Fib), prothrombin time (Pt).

Appendix D

Table D1
Clinical Pathology: Clinical chemistry. Males.

Parameter	Dose (TCID ₅₀)					
	Day 29				Day 53	
	0 (Control)	2 × 10 ⁶	1 × 10 ⁷	2 × 10 ⁷	0 (Control)	2 × 10 ⁷
AST (U/L)	68.3 ± 5.4	71.0 ± 5.4	65.8 ± 4.0	68.8 ± 6.7	68.3 ± 8.7	78.5 ± 10.4
ALT (U/L)	46.7 ± 6.1	51.1 ± 7.1	44.6 ± 4.9	44.9 ± 9.2	44.7 ± 1.5	48.8 ± 8.7
ALP (U/L)	147.7 ± 37.2	144.0 ± 30.3	133.5 ± 21.5	156.9 ± 49.9	142.3 ± 37.2	142.5 ± 28.8
GGT (U/L)	1.5 ± 0.0	1.5 ± 0.0	1.5 ± 0.0	1.5 ± 0.0	1.5 ± 0.0	1.5 ± 0.0
CK (U/L)	226.4 ± 53.0	230.3 ± 43.2	227.5 ± 67.8	270.3 ± 117.8	314.0 ± 54.1	570.8 ± 131.1 *
Bil (μM)	1.25 ± 0.00	1.25 ± 0.00	1.25 ± 0.00	1.25 ± 0.00	1.25 ± 0.00	1.25 ± 0.00
Urea (mM)	5.0 ± 0.7	5.1 ± 0.7	5.3 ± 0.4	5.4 ± 0.6	5.9 ± 0.6	5.5 ± 0.7
Crea (μM)	25.0 ± 3.9	24.6 ± 2.4	24.7 ± 2.4	27.1 ± 4.3	31.0 ± 1.0	29.8 ± 3.2
PGlu (mM)	9.9 ± 1.2	9.6 ± 0.9	10.1 ± 1.4	9.5 ± 1.3	7.1 ± 0.6	7.3 ± 0.7
Chol (mM)	1.5 ± 0.3	1.4 ± 0.2	1.5 ± 0.2	1.5 ± 0.2	2.0 ± 0.3	1.5 ± 0.4
TG (mM)	1.5 ± 0.4	1.6 ± 0.4	1.8 ± 0.7	1.8 ± 0.6	1.6 ± 0.6	1.7 ± 0.2
Prot (g/L)	61.3 ± 1.9	61.0 ± 2.3	61.4 ± 1.6	62.0 ± 1.3	70.1 ± 2.3	66.0 ± 2.5
Alb (g/L)	40.0 ± 1.8	40.6 ± 2.3	40.3 ± 2.4	40.4 ± 2.2	45.0 ± 1.1	41.7 ± 1.7 *
Glob (g/L)	21.3 ± 1.3	20.4 ± 1.7	21.0 ± 1.9	21.6 ± 2.2	25.1 ± 1.6	24.3 ± 1.2
Alb/Glob	1.9 ± 0.2	2.0 ± 0.2	1.9 ± 0.3	1.9 ± 0.3	1.8 ± 0.1	1.7 ± 0.1
Ca ²⁺ (mM)	2.6 ± 0.1	2.6 ± 0.1	2.6 ± 0.1	2.6 ± 0.1	2.8 ± 0.1	2.6 ± 0.02 *
Na ⁺ (mM)	141.6 ± 1.3	141.7 ± 0.9	141.7 ± 1.3	142.2 ± 1.0	141.0 ± 1.0	140.0 ± 1.2
K ⁺ (mM)	4.4 ± 0.3	4.3 ± 0.2	4.3 ± 0.3	4.3 ± 0.3	4.8 ± 0.2	5.2 ± 0.2 *
Cl ⁻ (mM)	100.4 ± 1.1	100.6 ± 1.8	100.3 ± 2.5	100.8 ± 1.7	99.3 ± 1.5	99.3 ± 0.5
PO ₄ ³⁻ (mM)	1.9 ± 0.4	1.9 ± 0.3	2.0 ± 0.5	1.9 ± 0.5	1.6 ± 0.2	1.7 ± 0.4

Mean ± SD, number of animals, day 29 (N = 10), day 53 (N = 3-4). Statistical significance vs. control (Anova & Dunnett): P ≤ 0.05 (*).

Table D2
Clinical Pathology: Clinical chemistry. Females.

Parameter	Dose (TCID ₅₀)					
	Day 29				Day 53	
	0 (Control)	2 × 10 ⁶	1 × 10 ⁷	2 × 10 ⁷	0 (Control)	2 × 10 ⁷
AST (U/L)	66.2 ± 5.5	69.7 ± 7.1	68.7 ± 8.1	64.9 ± 6.9	79.8 ± 9.5	61.6 ± 6.4 **
ALT (U/L)	40.5 ± 8.7	41.6 ± 12.6	42.5 ± 7.7	43.9 ± 15.1	43.8 ± 6.5	37.4 ± 8.1
ALP (U/L)	71.4 ± 14.7	73.8 ± 16.2	82.8 ± 15.7	78.9 ± 17.1	52.8 ± 14.0	66.8 ± 14.4
GGT (U/L)	1.5 ± 0.0	1.5 ± 0.0	1.5 ± 0.0	1.5 ± 0.0	1.5 ± 0.0	1.5 ± 0.0
CK (U/L)	243.3 ± 70.0	274.1 ± 128.5	207.4 ± 59.1	249.9 ± 61.8	418.0 ± 366.1	210.0 ± 102.1
Bil (μM)	1.25 ± 0.00	1.25 ± 0.00	1.25 ± 0.00	1.25 ± 0.00	1.25 ± 0.00	1.25 ± 0.00
Urea (mM)	5.7 ± 0.7	5.4 ± 0.6	4.9 ± 0.7	5.5 ± 0.7	5.4 ± 0.5	5.5 ± 0.6

(continued on next page)

Table D2 (continued)

Parameter	Dose (TCID ₅₀)					
	Day 29				Day 53	
	0 (Control)	2 × 10 ⁶	1 × 10 ⁷	2 × 10 ⁷	0 (Control)	2 × 10 ⁷
Crea (μM)	26.9 ± 1.5	28.0 ± 3.1	26.3 ± 2.4	27.2 ± 4.8	35.6 ± 4.0	35.6 ± 5.9
PGlu (mM)	9.1 ± 1.5	9.2 ± 1.2	8.8 ± 1.0	8.9 ± 0.7	6.7 ± 0.8	6.9 ± 0.6
Chol (mM)	1.4 ± 0.3	1.4 ± 0.3	1.3 ± 0.1	1.4 ± 0.2	1.5 ± 0.3	1.8 ± 0.2
TG (mM)	1.1 ± 0.5	1.1 ± 0.3	1.2 ± 0.5	1.4 ± 0.6	1.7 ± 0.6	1.7 ± 1.2
Prot (g/L)	62.7 ± 2.5	63.6 ± 1.5	62.8 ± 3.7	64.5 ± 2.9	69.9 ± 2.3	75.8 ± 8.1
Alb (g/L)	44.9 ± 2.9	45.8 ± 2.0	44.4 ± 3.0	46.5 ± 4.0	50.3 ± 3.7	52.4 ± 5.0
Glob (g/L)	17.8 ± 2.8	17.8 ± 2.3	18.5 ± 3.0	18.0 ± 1.9	19.6 ± 2.5	23.4 ± 3.2
Alb/Glob	2.6 ± 0.6	2.6 ± 0.5	2.5 ± 0.6	2.6 ± 0.4	2.6 ± 0.6	2.3 ± 0.1
Ca ²⁺ (mM)	2.6 ± 0.1	2.6 ± 0.1	2.6 ± 0.03	2.7 ± 0.1	2.7 ± 0.1	2.8 ± 0.1
Na ⁺ (mM)	141.6 ± 0.8	141.6 ± 0.8	141.5 ± 0.5	141.9 ± 1.1	141.4 ± 0.5	141.8 ± 1.9
K ⁺ (mM)	3.8 ± 0.3	4.0 ± 0.3	4.0 ± 0.3	3.9 ± 0.2	4.5 ± 0.2	4.8 ± 0.5
Cl ⁻ (mM)	101.0 ± 1.3	100.9 ± 1.3	101.4 ± 1.7	101.4 ± 2.4	100.6 ± 1.9	100.2 ± 1.9
PO ₄ ³⁻ (mM)	2.0 ± 0.3	1.9 ± 0.1	1.9 ± 0.3	2.0 ± 0.3	1.3 ± 0.3	1.3 ± 0.3

Mean ± SD, number of animals, day 29 (N = 10), day 53 (N = 5). Statistical significance vs. control (Anova & Dunnet): P ≤ 0.01 (**).

Alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), gamma-glutamyltransferase (GGT), creatine kinase (CK), total bilirubin (Bil), urea, creatinine (Crea), calcium (Ca²⁺), phosphate (PO₄³⁻), total protein (Prot), albumin (Alb), calculated globulin (Glob), albumin/globulin ratio, plasma glucose (PGlu), cholesterol (Chol), triglycerides (TG), sodium (Na⁺), potassium (K⁺), and chloride (Cl⁻).

Appendix E

Table E1

Clinical Pathology: Urinalysis.

Parameter, Sex	Dose (TCID ₅₀)					
	Day 29				Day 54	
	0 (Control)	2 × 10 ⁶	1 × 10 ⁷	2 × 10 ⁷	0 (Control)	2 × 10 ⁷
Males						
Vol (ml)	2.4 ± 1.2	2.8 ± 1.4	2.5 ± 1.7	2.0 ± 1.8	5.7 ± 1.4	3.3 ± 2.3
sg	1.02 ± 0.01	1.02 ± 0.01	1.03 ± 0.02	1.02 ± 0.01	1.01 ± 0.002	1.02 ± 0.02
pH	8.4 ± 0.4	8.3 ± 0.5	8.2 ± 0.5	8.5 ± 0.3	8.1 ± 0.9	8.5 ± 0.6
Females						
Vol (ml)	2.8 ± 1.6	2.3 ± 1.4	2.2 ± 1.9	1.9 ± 1.4	2.3 ± 1.5	2.2 ± 1.1
sg	1.02 ± 0.004	1.02 ± 0.01	1.03 ± 0.02	1.02 ± 0.01	1.01 ± 0.003	1.01 ± 0.004
pH	7.8 ± 1.1	7.8 ± 1.3	7.5 ± 1.4	7.6 ± 1.0	7.5 ± 1.2	7.2 ± 1.2

Mean ± SD, number of animals, day 29 (males, N = 8–10, females, N = 9–10), day 54 (males, N = 5, females, N = 4–5).

Specific gravity (sg), volume (Vol).

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