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Genistein, a general kinase inhibitor, as a potential antiviral for arenaviral hemorrhagic fever as described in the Pirital virus-Syrian golden hamster model

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ABSTRACT

Arenaviruses are rodent-borne negative strand RNA viruses and infection of these viruses in humans may result in disease and hemorrhagic fever. To date, supportive care, ribavirin, and in some cases immune plasma remain the foremost treatment options for arenaviral hemorrhagic fever. Research with the hemorrhagic fever causing-arenaviruses usually requires a Biosafety level (BSL)-4 environment; however, surrogate animal model systems have been developed to preliminarily study and screen various vaccines and antivirals. The Syrian golden hamster-Pirital virus (PIRV) surrogate model of hemorrhagic fever provides an opportunity to test new antivirals in an ABSL-3 setting. Thus, we challenged hamsters, implanted with telemetry, with PIRV and observed viremia and tissue viral titers, and changes in core body temperature, hematology, clinical chemistry, and coagulation parameters. Physical signs of disease of the PIRV-infected hamsters included weight loss, lethargy, petechial rashes, epistaxis, ocular orbital and rectal hemorrhage, and visible signs of neurologic disorders. However, treating animals with genistein, a plant derived isoflavone and general kinase inhibitor, resulted in increased survival rates and led to an improved clinical profile. In all, the results from this study demonstrate the potential of a general kinase inhibitor genistein as an antiviral against arenaviral hemorrhagic fever.

1. Introduction

Arenaviruses are rodent-borne, RNA viruses distributed worldwide and some are capable of causing hemorrhagic fever in humans. *Lassa virus* (LASV) is an Old World arenavirus and the etiologic agent that causes Lassa fever in a quarter of a million individuals annually resulting in approximately 5000 deaths in endemic regions of West Africa (McCormick et al., 1987), while the New World arenaviruses *Junín virus*, *Machupo virus*, *Guanarito virus*, and *Sabiá virus* have the ability to cause hemorrhagic fever in humans. These viruses all require a select agent license and Biosafety level (BSL)-4 laboratories for use (Borio et al., 2002; McKee et al., 1992, 1993; Medeot et al., 1990). PIRV is a New World arenavirus that was isolated from the cotton rat (*Sigmodon alstoni*) in the Municipality of Guanarito, Venezuela in 1994 (Fulhorst et al., 1997). PIRV has not been associated with any human disease; however, infection of the Syrian golden hamster with PIRV leads to 100% mortality and pathology very similar to that observed in arenavirus-induced hemorrhagic fever in humans (Sbrana et al., 2006; Xiao et al., 2001). We hypothesize that the PIRV hamster hemorrhagic fever model can be used to test the efficacy of drugs for the treatment of human acquired arenavirus hemorrhagic fever. To test this hypothesis, we treated PIRV-infected hamsters with genistein utilizing various treatment regimens. Genistein, an isoflavone found in soybeans and soy products, acts as a general tyrosine kinase inhibitor and has been shown to inhibit viral infection *in vitro* by inhibiting viral entry (Akiyama et al., 1987; Damm et al., 2005; Pelkmans et al., 2002; Vela et al., 2008b). Efficacy may lead to a new class of antivirals against arenavirus hemorrhagic fever (Vela et al., 2008a). Additionally, the PIRV surrogate system provides for a safe and cost-effective surrogate model to study the pathology of the human pathogenic arenaviruses and to screen potential antivirals, therapeutics, and vaccine strategies.

2. Experimental procedures

2.1. Cell lines, virus, and reagents

Pirital virus (PIRV) (VAV-488) was obtained from Dr. Robert B. Tesh at the University of Texas Medical Branch (Galveston, TX). The virus was passed up to two times in Vero cells using RPMI 1640 media (Gibco) plus antibiotics (diluted 1:100) and diluted in RPMI with 1% (FBS) prior to challenge. The Pichindé virus (PICV) P18 variant, used in this study, was derived from 18 guinea pig passages of

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PICV strain CoAn 4763, as described by Jahrling et al. (1981b). The high-passage virulent PICV-P18 (Zhang et al., 1999) was utilized at an MOI of 1 for the infection experiments. The animals were challenged with 10^5 pfu/mL of PIRV. Genistein (purchased from Sigma Aldrich, St. Louis, MO) was diluted in 0.1% DMSO and 1× sterile phosphate buffered saline (PBS) to a final concentration of 10 μM, 20 μM, 50 μM, and 100 μM for the cell culture experiments. For the animal experiments, genistein was administered at a concentration of 15 mg/kg subcutaneously.

2.2. Plaque assay

Plaque assays were performed as previously described (Vela et al., 2007). Briefly, Vero E-6 cells, seeded on 6-well plates were inoculated with viral samples for 20 min. A 1.89% methyl-cellulose overlay (containing 2× EMEM, antibiotics, and non-essential amino acids) was added to the inoculum prior to incubating at 37.0 °C (5% CO_2) for 96 h. The wells were then fixed and stained in a crystal violet solution prior to visual analyses. In the case when genistein was used, the cell monolayer was pre-treated on day 0, the day of infection, with 1.0 mL of Genistein at various concentrations (described above) for 1 h with gentle rocking at 37.0 °C. The cell media containing genistein was then removed and cells were inoculated with PIRV, PICV, or media.

2.3. Animal studies

Thirty-three (33) female Syrian golden hamsters (*Mesocricetus auratus*) (13–15 weeks of age at the beginning of study) were obtained from Charles River Kingston (Kingston, NY) and randomly associated into six groups; Groups 1–5 consisted of 6 animals and Group 6 consisted of 3 animal. The animals were individually housed in isolator cages and allowed food and water ad libitum. All study procedures were approved in accordance to the guidelines by the Institutional Animal Care and Use Committee. All work involving infected animals or virus was performed in the BSL-3 laboratory.

2.4. Telemetry studies

Hamsters were surgically implanted with the radiotelemetry PhysioTel TA series transmitter (DataSciences Intl., Arden Hills, MN). Core body temperatures were recorded every 15 min. The hamsters were anesthetized with Xylazine/Ketamine prior to removal from the housing units for general observations on study days −2, 0, 2, 5, and 6 (day 6 for the control animals).

2.5. Hematology, clinical chemistry, and coagulation studies

Hematological analyses were conducted on an Advia 120 hematological analyzer. Clinical chemistry analyses were performed on the Advia 1200 serum chemistry analyzer and coagulation analyses were performed using the Trinity Biotech AMAX Destiny Plus.

2.6. Pathology studies

Necropsies were performed on all hamsters. Tissues were sampled and fixed in 10% neutral buffered formalin and processed for routine hematoxylin and eosin staining and examined microscopically.

2.7. Tissue harvesting

Various tissues (including the brain, pancreas tissue, liver, spleen, kidney, adrenals, lung, heart, lymph nodes, and intestines) were collected in sterile 1× PBS, homogenized, and centrifuged (2000 × *g*, 5 min at 4 °C). Plaque assays were conducted to measure viral titers.

2.8. Blood sampling

Samples were obtained by retro-orbital puncture and collected for viremia and/or hematology, clinical chemistry, and coagulation. Terminal samples were only obtained from animals that were euthanized due to moribundity.

2.9. Statistical analyses

Animal survival data was analyzed using the SAS® MULTTEST procedure to adjust for multiple comparisons at the 0.05 level of significance using the Bonferroni–Holm Method. A time-to-death analysis was performed based on the length of survival model and the Kaplan–Meier estimator and log-rank tests were used to determine any significant differences between groups.

3. Results

3.1. Genistein treatment of host cells leads to an inhibition of viral infection

It has been previously shown that genistein treatment of host cells results in the inhibition of Pichindé virus (PICV) infection (Vela et al., 2008b). Therefore, cells were pre-treated with genistein to determine the antiviral properties of the drug against PIRV infection. Genistein pre-treatment of Vero cells resulted in a concentration-dependent inhibition of viral infection (Fig. 1). Treating cells with 10 μM and 20 μM of genistein resulted in a 6.5% and 28.6% decrease in PIRV infection, respectively, while treating cells with 50 μM and 100 μM of genistein resulted in a 99.3% and 99.8% decrease in PIRV infection, respectively. Genistein concentration-dependent viral infection was also observed when cells were pre-treated with genistein and infected with PICV. As a control, cells were treated with the DMSO carrier control and minimal inhibition was observed. Additionally, cell viability was not affected when cells were treated with 10–100 μM of genistein (50% effective concentration [EC50] ~30 μM) (data not shown). These data demonstrate that treating cells with genistein results in the concentration-dependent inhibition of PIRV infection, *in vitro*, without affecting cell viability.

3.2. Genistein treatment results in increased survival in hamsters infected with PIRV

After determining that genistein inhibited PIRV infection, *in vitro*, we next aimed to determine the antiviral effect of genistein in an animal model system. Syrian golden hamsters, implanted with telemetry units to measure core body temperatures, were

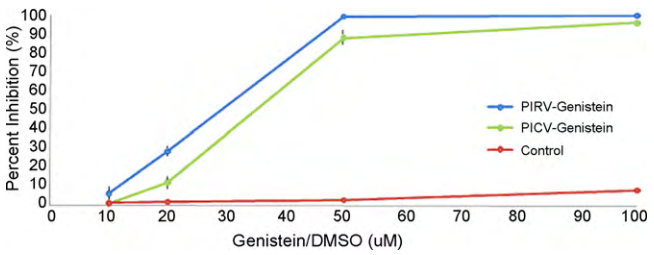

Fig. 1. Pre-treatment of host cells with genistein leads to concentration-dependent inhibition of PIRV infection. Vero cells were pre-treated with various concentrations of genistein for 1 h prior to virus infection. Plaque assays were used to determine inhibition of viral infection.

Table 1
Study design. Thirty-three (33) hamsters were used in this study; Groups 1–5 consisted of 6 hamsters and Group 6 consisted of 3 hamsters. Group 1 served as the PIRV-infected genistein carrier-control; the Group 2 animals were pre-treated with genistein once 24 h prior to challenge; the Group 3 animals were pre-treated with genistein once 24 h prior to challenge and everyday of the study; the Group 4 animals were treated with genistein 24 h post-challenge and every day after for the length of the study in a post-exposure prophylactic regimen; the Group 5 animals were treated with genistein post-challenge at the first visible signs of disease and daily thereafter in a therapeutic regimen; and the Group 6 animals served as a mock-infected control.

Group	No. of hamsters	Virus	Titer	Treatment (15 mg)	Treatment schedule
1	6	PIRV	10^5 pfu	Carrier Control	–
2	6	PIRV	10^5 pfu	Genistein	1× pre-infection (day −1)
3	6	PIRV	10^5 pfu	Genistein	Pre-infection (day −1 to end of study)
4	6	PIRV	10^5 pfu	Genistein	P.E.P. – daily (day 1 to end of study)
5	6	PIRV	10^5 pfu	Genistein	Therapeutic – daily after first signs of disease
6	3	–	–	Genistein	Daily

treated with genistein (15 mg/kg) at various time points and infected intraperitoneally (i.p.) with PIRV (10^5 pfu) (Table 1). The Group 1 animals (drug carrier control) were infected i.p. with PIRV. Animals were treated with genistein 24 h prior to challenge (Group 2-pre-exposure prophylactic [PrP] Group), 24 h prior to challenge and daily until the end of the study (Group 3-pre-exposure/post-exposure prophylactic Group [PPEP]), as a post-exposure prophylactic (PEP) (Group 4), or as a therapeutic (Group 5). The Group 6 animals served as treated non-infected controls. Animals treated with genistein in a post-exposure prophylactic regimen were treated 24 h post-infection, while animals treated with genistein in a therapeutic regimen were treated at the appearance of petechia. Infection of the hamsters with PIRV led to consistent disease progression. The Group 1 Carrier Control animals exhibited elevated temperatures 24–36 h post-infection, losses in body weight, lethargy, and exhibited huddled posture, ruffled fur. Petechial rashes were recorded in all Carrier Control animals 48 h post-infection. Ocular orbital hemorrhage and ecchymosis was observed in all of the Carrie Control hamsters. Additionally, the Group 1 Carrier Control animals that survived to day 7 or day 8 post-infection (Fig. 2), exhibited rectal hemorrhaging and neurological signs of disease including, tremors, loss of balance, seizures, and hind limb paralysis prior to death.

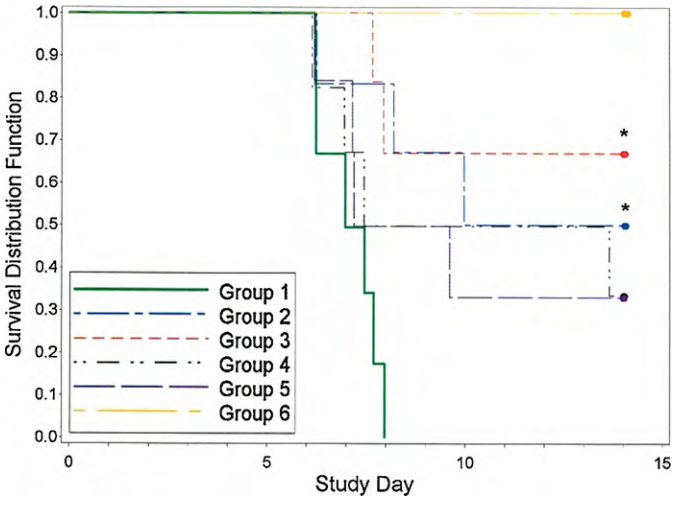

Fig. 2. Genistein treatment in hamsters infected with PIRV leads to survivability and a decrease probability of death. All Group 1–5 animals were challenged with 10^5 pfu/mL of PIRV by i.p., while the Group 6 animals were mock challenged. Group 1 represents animals treated with the carrier control and infected with PIRV. The Group 2 animals were treated with genistein once 24 h prior to challenge. The Group 3 animals were treated with genistein 1× prior to challenge and every subsequent day after challenged. The Group 4 animals were treated with genistein 24 h post-challenge, and the Group 5 animals were treated at the first signs of petechiae. The Group 6 control animals were treated with genistein 1× prior to mock-challenge and every day subsequent until the end of the study. Statistical significance ($p \leq 0.05$) is demonstrated by an * (asterisk).

Table 2
Statistical analyses of the survival among groups.

Group	Unadjusted pairwise log-rank *p*-value				
	2	3	4	5	6
1	0.0090*	0.0080*	0.1895	0.1609	0.0110*
2		0.6606	0.5436	0.4592	0.1747
3			0.2086	0.2086	0.2945
4				0.9601	0.0933
5					0.0933

* A significant *p*-value ≤0.05.

All treatment regimens with genistein resulted in some hamsters surviving challenge (Fig. 2). Administering genistein (15 mg/kg) once 24 h prior to PIRV challenge resulted in 50% survival (3/6 hamsters), yielding increases in survival in the Group 2 PrP animals when compared to the carrier-control treated animals ($p < 0.05$). Additionally, 66.6% (4/6 hamsters) of the Group 3 PPEP animals treated with genistein, 24 h prior to treatment and every day post-infection, survived challenge ($p < 0.05$). Two of six hamsters (33.3%) survived when treated with genistein in a PEP regimen. Similarly, 33.3% of hamsters survived when treated with genistein in a therapeutic regime. The time-to-death and overall survival of the Group 2 PrP and Group 3 PPEP animals was statistically significant when compared to the Group 1 carrier-control treated animals (Table 2). And though no statistical significance was observed in the Group 4 PEP and 5 therapeutic animals when compared to the Carrier Control animals, a small portion of the animals did survive. This is encouraging considering infection with PIRV (10^5 pfu/mL) in hamsters leads to 100% mortality.

3.3. PIRV-infection disrupts the natural diurnal temperature rhythm

Core body temperatures were collected from telemetry units surgically inserted into the animals. Baseline core body temperatures were recorded for 7 days prior to infection. Analyses of these temperatures demonstrate a natural diurnal temperature pattern in the hamsters (Fig. 3). However, PIRV infection led to a deregulation of the normal diurnal temperature pattern in the animals (Fig. 3). Furthermore, increases in core body temperatures occurred 24–36 h post-infection. Deregulation of diurnal temperature patterns and an increase in core body temperature were also observed in the animals treated with genistein. The core body temperature from the PrP and PPEP surviving animals, except for a few from Group 3 PPEP, did not return to relatively normal by the end of study at day 14 post-infection (Fig. 3B and C). Additionally, one PrP animal (549) and one PPEP animal (566) exhibited hypothermic core body temperatures that rebounded to normal temperatures by the study end. The diurnal temperature patterns of the PEP and Group 5 therapeutic surviving animals did not return to normal (Fig. 3D and E). It should be noted that temperature depressions observed on days 0, 2, and 5 for the Groups 1–5 animals and on

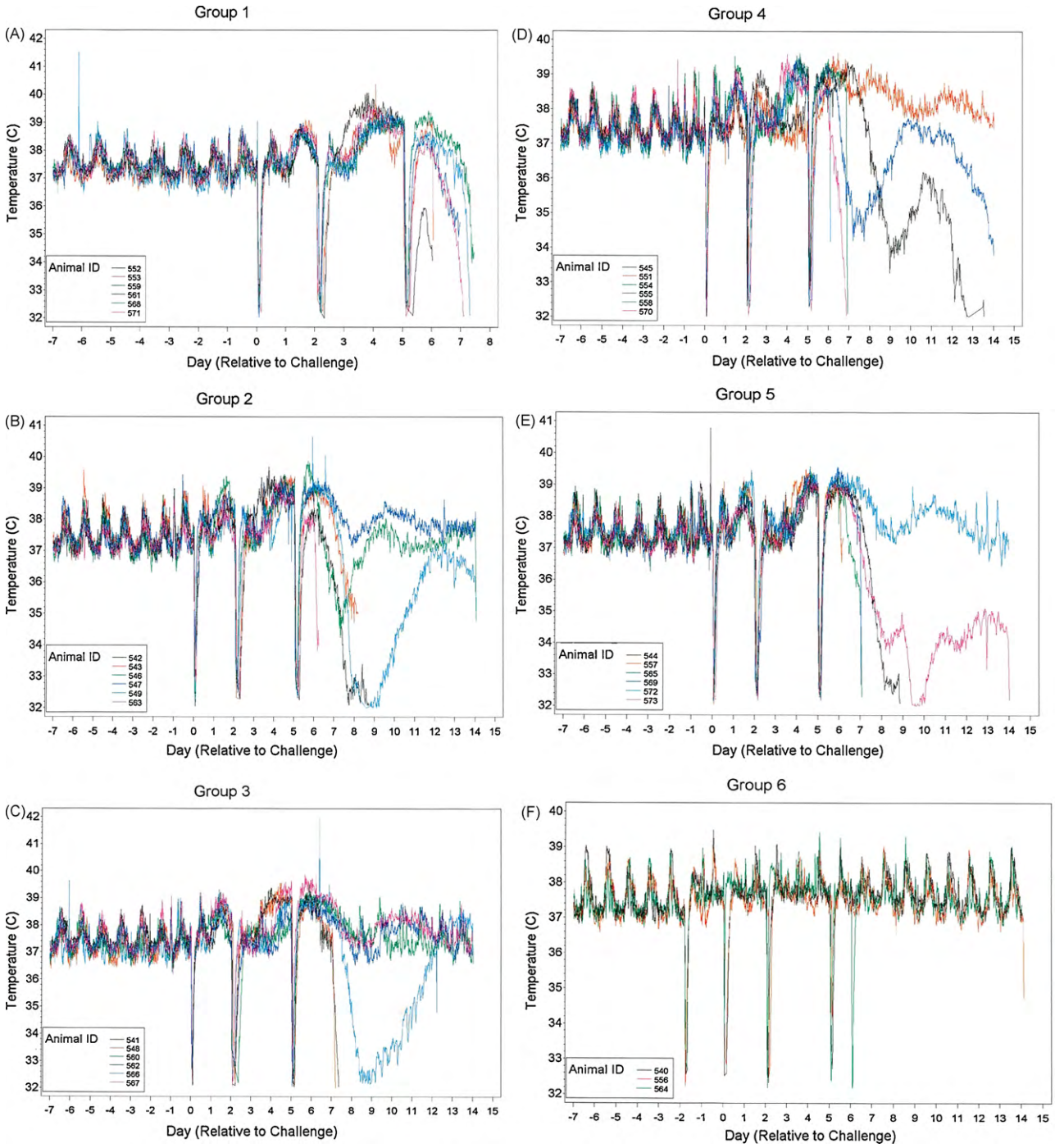

Fig. 3. PIRV-infection leads to elevated temperatures and destruction of the natural diurnal temperature rhythm; (A) Group 1 animals, (B) Group 2 animals, (C) Group 3 animals, (D) Group 4 animals, (E) Group 5 animals, and (F) Group 6 animals. Core body temperatures were measured every 15 min.

study days −2, 0, 2, 5, and 6 for the Group 6 non-infected control animals were associated with the observation period where the animals were removed from their housing, (thus causing the telemetry unit to miss temperature measurements) and also due to use of an anesthetic, which causes a decrease in core body temperature. Additionally, the biphasic temperature patterns are likely due to the administration of the anesthesia and not a product of the disease progression. The telemetry temperature data demonstrates that infection of the Syrian golden hamster leads to elevated temperatures and a disruption of the natural diurnal temperature pattern. Elevated temperatures were ameliorated and relatively normal in most of the PrP and PPEP surviving animals.

3.4. Effects of PIRV infection and genistein treatment on coagulation, hematology, and clinical chemistry

PIRV infection in Syrian golden hamsters led to abnormal increased prothrombin times (PT) and activated partial thromboplastin times (APTT) (Table 3). PT and APTT from infected Carrier Control hamsters increased when compared to baseline samples. Additionally, the PT values measured from all genistein-treated animals that died or were euthanized were increased when compared to baseline samples. The PT and APTT from surviving animals were slightly increased in samples collected from Groups 2, 4, and 5 and did not return to normal levels by the study end. Fibrinogen was

Table 3
Coagulation, hematology, and clinical chemistry parameters. PIRV infection in hamsters leads to several changes in coagulation, hematology, and clinical chemistry parameters.

	Study day		
	0[a]	Terminal	14
Coagulation parameter			
Prothrombin time (PT)	10.8 ± 3.4	Group 1: 87.7 ± 119.42 Group 2: 26.7 ± 5.86 Group 3: 47.6 ± 0 Group 4: 29.8 ± 0 Group 5: 168.1 ± 152.3	Group 1: no survivors Group 2: 23.3 ± 5.6 Group 3: ND Group 4: 17.9 ± 8.2 Group 5: 17.9 ± 6.9 Group 6: 8.9 ± 3.2
Activated partial thromboplastin time (APTT)	24.9 ± 2.1	Group 1: >300 ± 0[b] Group 2: 172 ± 111.07 Group 3: 84.6 ± 0 Group 4: >300 ± 0[b] Group 5: >300 ± 0[b]	Group 1: no survivors Group 2: 77.2 ± 25.3 Group 3: ND Group 4: 44.3 ± 15.3 Group 5: 0 Group 6: 16 ± 7.0
Fibrinogen	154.5 ± 3.5	Group 1: 284.2 ± 88.71 Group 2: 361.7 ± 11.93 Group 3: 300 ± 0 Group 4: 300 ± 0 Group 5: 282.8 ± 70.76	Group 1: no survivors Group 2: 612 ± 205.2 Group 3: ND Group 4: >900 Group 5: >900 Group 6: 157 ± 52.7
D-dimer	86.5 ± 20.5	Group 1: 222.9 ± 345.33 Group 2: 97.5 ± 31.85 Group 3: 93.4 ± 0 Group 4: 87.2 ± 0 Group 5: 64.9 ± 33.59	Group 1: no survivors Group 2: 72.2 ± 29.2 Group 3: ND Group 4: 92.6 ± 24.6 Group 5: 72.9 ± 21.1 Group 6: 116 ± 52.4
Hematology parameter			
White blood cell count	7.44 ± 3.0	Group 1: 35.18 ± 15.39 Group 2: 33.37 ± 16.18 Group 3: 38.81 ± 30.09 Group 4: 41.76 ± 30.04 Group 5: 35.08 ± 6.0	Group 1: no survivors Group 2: 17.70 ± 23.09 Group 3: 7.01 ± 1.67 Group 4: 7.14 ± 7.18 Group 5: 12.88 ± 13.85 Group 6: 4.46 ± 1.90
Thombocyte count	620.5 ± 67.4	Group 1: 705.2 ± 207.9 Group 2: 322.5 ± 272.9 Group 3: 395.5 ± 335.9 Group 4: 417.3 ± 210.3 Group 5: 732.0 ± 98.9	Group 1: no survivors Group 2: 592.0 ± 818.8 Group 3: 489.0 ± 330.3 Group 4: 400.0 ± 276.5 Group 5: 460.0 ± 316.8 Group 6: 690.0 ± 240.0
Clinical chemistry parameter			
Blood urea nitrogen (BUN)	20.2 ± 2.6	Group 1: 112.8 ± 31.8 Group 2: 155.9 ± 106.3 Group 3: 144.2 Group 4: 105.6 ± 43.1 Group 5: 57.5	Group 1: no survivors Group 2: 81.3 ± 90.9 Group 3: 15.9 ± 1.2 Group 4: 179.8 ± 233.5 Group 5: 178.0 ± 231.6 Group 6: 25.0 ± 3.0
Creatinine	0.21 ± 0.03	Group 1: 1.4 ± 1.2 Group 2: 1.39 ± 1.4 Group 3: 0.73 Group 4: 1.0 ± 0.5 Group 5: 0.3	Group 1: no survivors Group 2: 0.5 ± 0.6 Group 3: 0.2 ± 0.0 Group 4: 1.5 ± 1.9 Group 5: 1.4 ± 1.8 Group 6: 0.3 ± 0.1
Alanine aminotransferase	53.1 ± 18.1	Group 1: 4708.0 ± 1678.4 Group 2: 3307.6 ± 2399.8 Group 3: 2657.3 Group 4: 3429.4 ± 167.4 Group 5: 11012.9	Group 1: no survivors Group 2: 127.7 ± 61.2 Group 3: 112.4 ± 23.7 Group 4: 321.5 ± 230.9 Group 5: 250.2 ± 188.8 Group 6: 38.0 ± 10.1
Aspartate aminotransferase	123.6 ± 80.5	Group 1: 6529.2 ± 4013.3 Group 2: 5145.8 ± 4611.7 Group 3: 4484.6 Group 4: 6590.4 ± 1889.0 Group 5: ND[b]	Group 1: No Survivors Group 2: 325.4 ± 131.1 Group 3: 134.8 ± 38.5 Group 4: 644.2 ± 704.4 Group 5: 440.4 ± 425.4 Group 6: 84.4 ± 6.3

NA: not available.
ND: no data due to blood clots in sample.
[a] Pooled samples.
[b] Sample did not clot in the alloted 300 s (system restriction).

also higher in terminal samples and samples collected from surviving animals when compared to baseline. Furthermore, the D-dimer values were higher in the samples collected from the Carrier Control terminal animals when compared to baseline. All of the other D-dimer values from the treated groups (Groups 2–5) were within the baseline range.

Complete blood counts were determined in animals infected with PIRV. Animals that died post-challenge showed elevations in WBCs, neutrophils, and neutrophils/lymphocyte ratio and decreases in lymphocytes and eosinophils (in comparison to the overall baseline) typical of a viral infection (Table 3 and Supplementary Table 1). Surviving animals also showed alterations; however, the degree of alteration for each parameter was less than that of animals that died. Animals that received genistein prior to challenge [PrP (1 treatment) and PPEP (15 treatments)] demonstrated less hematology parameter changes when comparing against all of the treatment groups. This infers that the presence of genistein prior and throughout the post-challenge period provides some level of protection against infection. Groups receiving genistein post-challenge (PEP and therapeutic groups) showed alterations in hematology parameters that mirrored those that died (elevated WBC (except the PPEP Group), neutrophils, and neutrophil/lymphocyte ratio and decreased lymphocytes); however, these alterations were not as significant indicating a capability of genistein to provide some protection against viral infection even post-onset of clinical signs. Thrombocytopenia was detected in most PIRV-infected animals. Interestingly, thrombocytosis was observed among terminal samples collected from Carrier Control Group 1 and Therapeutic Group 5 animals, which may suggest a deregulation in thrombocyte function. Surviving animals of Groups 2–5 showed increased mean platelet volume over baseline levels suggesting an increase in release of new platelets in response to the disease process.

Increases in alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase, gama glutamyl transferase and sorbitol dehydrogenase were seen in all terminal samples regardless of treatment (Table 3 and Supplementary Table). In surviving animals, the levels of these enzymes were elevated; however, they were lower than levels seen in terminally ill animals. Enzyme levels in the groups receiving genistein pre-challenge (PrP and PPEP) were consistently nearer baseline levels than those receiving genistein in a PEP (Group 4) or therapeutic (Group 5) regimen. Levels in Group 3 (PPEP), which received both pre- and post-exposure treatments, were consistently the closest to baseline levels (a trend also seen in the hematologic parameters and overall mortality). The high elevations in liver enzymes in these animals are expected due to the extensive tissue damage and necrosis seen in these animals. Elevations were also observed in blood urea nitrogen (BUN), creatinine, and BUN/creatinine ratios in the terminal samples demonstrating some level of kidney damage or decreased kidney function in PIRV-infected animals (Table 3 and Supplementary Table). These elevations were lessened by pre-treatment with genistein while PEP or therapeutic treatments did not provide the same trend (Table 3).

3.5. Effects of genistein treatment on viremia and tissue viral loads

Infection of animals with PIRV results in viremia two days post-infection in most animals which persisted until the death of the animal (Fig. 4A). Eight of eleven surviving animals had no detectable viremia. Viral loads were detected in the lymph nodes, liver, spleen, kidney, heart, lungs, brain, and intestines in the PIRV-infected animals (Fig. 4B). Comparison of animals that survived challenge and animals that succumbed to infection yielded significant viremia differences (day 5 and day 14/terminal bleeds) (Table 4). Furthermore, significant differences in viremia were detected when comparing the Group 1 Carrier Control Group and PPEP Group 3 day 14/terminal samples ($p = 0.01$). Additionally, viral loads detected in the liver and spleen were significantly different in the animals that survived when compared to the animals that succumbed to infection (Table 4).

Table 4
Comparisons of viremia and viral loads in dead and surviving animals.

Comparison of viremia and viral loads	
Sample	Dead vs survivors (p value)
Day 5 viremia	0.01*
Day 14/terminal viremia	0.002*
Lymph nodes	0.09
Brain	0.17
Liver	0.0001*
Spleen	0.00002*
Kidney	0.33
Adrenal	0.06
Heart	0.23
Intestines	0.2
Lung	0.23

* A significant p-value ≤ 0.05.

3.6. PIRV infection in the Syrian golden hamster leads to lesions and mineralization within several tissues

Gross red or dark discoloration was noted in the brain, small and large intestines, kidneys, liver, lungs, lymph nodes, spleen and/or skin at necropsy in all hamsters that died following PIRV infection. Histological sections are shown in Fig. 5A–O. Enlarged bronchial and mediastinal lymph nodes and fluid within the thoracic and abdominal cavities were also noted in several infected animals (data not shown). Gross lesions were more commonly seen in hamsters that died during the study, although occasional qualitatively similar lesions were noted in infected animals surviving to study termination on day 14. In addition, uninfected genistein-treated control hamsters were euthanized at the study end to determine whether the drug caused any gross lesions. In all, tissues from uninfected genistein-treated animals lacked gross lesions associated with drug treatment. The tissue discolorations attributed to some of the tissue samples were associated with euthanasia artifacts or agonal passive congestion (Fig. 6A–M) and not the drug.

Microscopically, the most severely affected organs were the liver, lungs, spleen, brain, heart and adrenal glands. Lesions were similar in incidence and severity among hamsters that died with or without genistein treatment, although more genistein-treated hamsters survived virus infection than did untreated controls. No remarkable microscopic findings were evident in uninfected, genistein-treated hamsters. Individual necrotic and mineralized hepatocytes were present throughout the liver in hamsters that died following PIRV infection, often in association with a few neutrophils or histiocytes (Fig. 5A). Cellular debris and fibrin accumulation within hepatic sinusoids and sinusoidal extramedullary hematopoiesis were noted in animals that died but were reduced in incidence and severity in survivors, which had mild neutrophilic, histiocytic and lymphoplasmacytic inflammation (data not shown). Interstitial pneumonia was also apparent in many hamsters that died (data not shown). Neutrophils, necrotic cellular debris and fibrin were visible within capillaries and the supporting tissue of the interstitium (Fig. 5B). Few survivors had residual lung inflammation. Splenic lesions in hamsters that died included striking acute coagulation necrosis of the red pulp and more subtle individual lymphocyte necrosis in the white pulp. The red pulp was largely replaced by fibrin and a few neutrophils, with rare mineralization of necrotic cells (Fig. 5C), and these lesions were absent in

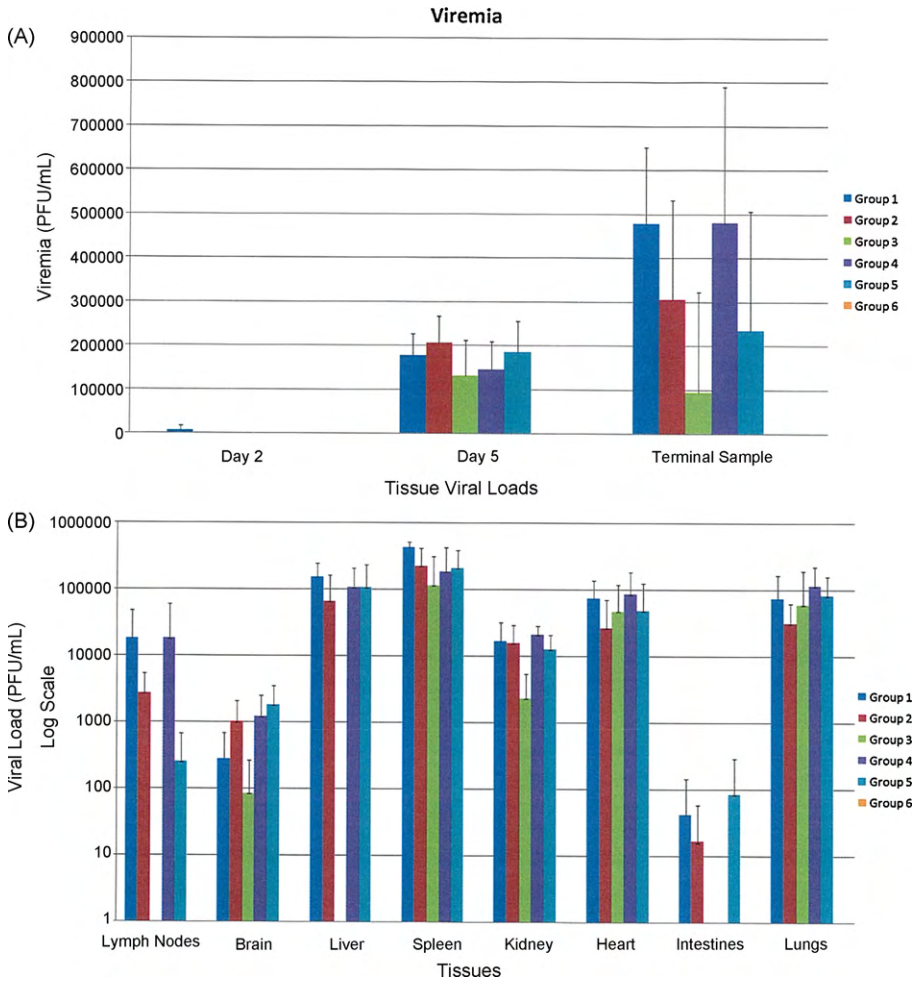

Fig. 4. Pre-treating the animals with genistein 24 h prior to treatment and everyday thereafter for 14 days leads to decreased viremia PIRV-infection leads to (A) decreased viremia and (B) a decrease in tissue viral loads as measured by plaque assays (limit of detection: 50 pfu/mL). Tissues were harvested from all animals that were found dead, euthanized after moribundity, or euthanized 14 days post-infection (at the end of the study).

the survivors. Microscopic lesions were prevalent but mild in the brains of hamsters that died after PIRV infection. Necrotic individual cells and cellular debris, representing endothelial cells, glia and a few neurons, were distributed throughout all brain sections (Fig. 5D–G). Hemorrhage was common in the meninges and perivascular regions. Residual necrosis of glial and/or endothelial cells and gliosis, both of minimal severity, remained evident in the brains of four survivors on day 14. Chronic lymphohistiocytic inflammation of minimal to mild severity was also noted in the perivascular, meningeal and periventricular regions in the majority of survivors.

Cardiac myofiber necrosis and mineralization was a fairly common finding among hamsters that died. Small vessels within the myocardium contained fibrin and necrotic cellular debris, potentially representing both damaged endothelial cells and leukocytes in circulation (Fig. 5H). Several survivors had developed chronic histiocytic and lymphocytic valvular endocarditis (Fig. 5I). Mild individual cell necrosis and mineralization were noted in all three layers of the adrenal cortex in dead hamsters, most obvious in the zona fasciculata (Fig. 5J). Many survivors had necrosis and mineralization, in addition to mixed inflammatory cell infiltrate or extramedullary hematopoiesis (Fig. 5K). Granulation tissue formation and mild chronic inflammation were noted on the peritoneal wall and the serosal surface of the intestines. Because the lesions occurred in PIRV-infected hamsters regardless of genistein treatment and were absent in the uninfected, genistein-treated control group, they are interpreted as local consequences of virus inoculation.

Other common sequelae of PIRV infection in hamsters that died after infection included renal tubular necrosis (nephrosis) (Fig. 5L), pancreatic acinar (Fig. 5M) and islet cell necrosis and mineralization and lymphocyte necrosis in multiple lymph nodes (Fig. 5N) and the gastrointestinal lymphoid tissue (GALT) (Fig. 5O).

4. Discussion

We have previously performed a kinomic profiling study demonstrating inhibition of PICV infection in cells treated with genistein (Vela et al., 2008b). Results from this published report suggest genistein-mediated inhibition of both (1) arenavirus entry events and (2) post-entry events in the viral life cycle. Inhibition of PICV infection was hypothesized to be due to inhibiting kinase activity necessary for viral infection. In this current report, we have (1) expanded on the previous research to demonstrate inhibition of PIRV infection in genistein-treated cells and (2) determine the efficacy of the drug in different antiviral regimens in the PIRV-Syrian golden hamster animal system.

We first wanted to test whether genistein treatment of host cells results in the inhibition of PIRV infection similar to what was observed previously with PICV. Therefore, Vero cells were treated with different concentrations of genistein prior to PIRV infection and inhibition of PIRV infection in Vero cells was observed in a

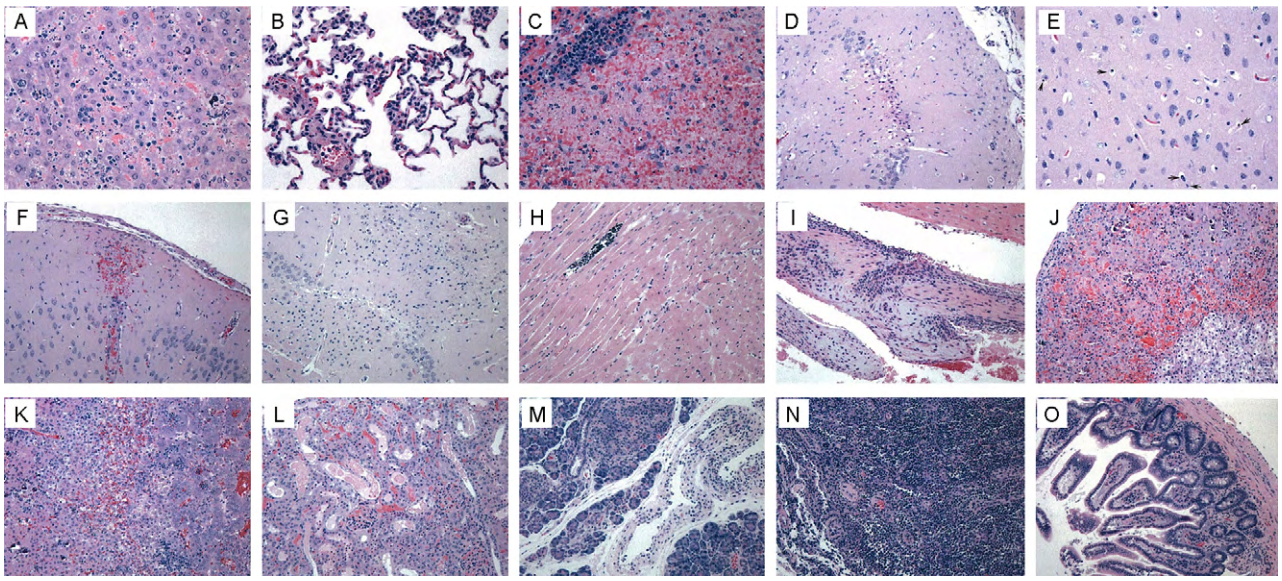

Fig. 5. PIRV infection leads to lesions consisting of a dark or red discoloration in the brain, small and large intestines, kidneys, liver, lungs, lymph nodes, spleen, skin and skeletal muscle correlating microscopically with hemorrhage, inflammation and necrosis. All samples were collected from infected animals. (A) Liver (collected 7 days p.i.): hepatocellular necrosis and mineralization were observed. Individual hepatocytes are hypereosinophilic with pyknotic and karyorrhectic nuclei. Sinusoids contain fibrin, cellular debris and a few neutrophils. One cluster of hematopoietic cells is present (H&E 40× magnification). (B) Lung (collected 10 days p.i.): interstitial pneumonia demonstrating alveolar septae and blood vessels containing fibrin, cellular debris, and a few neutrophils (H&E 40× magnification). (C) Spleen (collected 7 days p.i.): acute coagulation necrosis and fibrin accumulation in the splenic red pulp. Lymphoid tissue at the upper left is modestly depleted (H&E 40× magnification). (D) Brain-hippocampus (collected 8 days p.i.): a cluster of necrotic neurons in the hippocampus, as evident by hypereosinophilia, pyknosis and vacuolization of the neuropil (H&E 40× magnification). (E) Brain-cerebral cortex (collected 8 days p.i.): scattered necrotic individual cells (H&E 40× magnification). (F) Brain-cerebral cortex (collected 8 days p.i.): focal neuropil necrosis, perivascular hemorrhage, and scattered necrotic individual cells (pyknotic or karyorrhectic nuclei) in the cerebrum (H&E 20× magnification). (G) Brain-hippocampus (collected 14 days p.i.): a focus of gliosis, cellular debris and neuropil spongiosis in the hippocampus of a hamster surviving to scheduled termination 14 days after PIRV infection. The focal absence of neurons in the hippocampus suggests neuronal necrosis with a secondary glial response (H&E 20× magnification). (H) Heart (collected 7 days p.i.): coagulated, fragmented and mineralized myocardial fibers were observed. Necrotic cellular debris is visible in the interstitium, possibly representing capillary endothelial cells (H&E 40× magnification). (I) Heart-mitral valve (collected 14 days p.i.): chronic lymphoplasmacytic and histiocytic valvular endocarditis in a surviving hamster (H&E 20× magnification). (J) Adrenal gland (collected 7 days p.i.): necrosis and mineralization of cortical cells with accumulation of fibrin, neutrophils, and cellular debris and hemorrhage. Note the hypereosinophilic acutely necrotic cortical cells in addition to the deeply basophilic granular mineralized cells (H&E 20× magnification). (K) Adrenal gland (collected 14 days p.i.): mineralization of cortical cells, congestion and extramedullary hematopoiesis in the adrenal cortex of a surviving hamster (H&E 20× magnification). (L) Kidney (collected 6 days p.i.): acute tubular necrosis was observed. Note the hypereosinophilic tubular epithelial cells with pyknotic nuclei, sliding and flattening of remaining adjacent tubular epithelial cells and the amorphous protein accumulation in tubular lumens (H&E 20× magnification). (M) Pancreas (collected 7 days p.i.): necrosis of individual pancreatic islet and acinar cells. Chronic lymphoplasmacytic serositis is visible along the right edge of the organ, likely resulting from peritoneal virus inoculation (H&E 20× magnification). (N) Mesenteric lymph node (collected 8 days p.i.): lymphocyte necrosis and depletion (H&E 20× magnification). (O) Jejunum (collected 7 days p.i.): lymphocyte necrosis in the lamina propria (gut-associated lymphoid tissue) (H&E 20× magnification). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

concentration-dependent manner. These results suggest genistein as an antiviral for various arenaviruses. Next, we wanted to determine the efficacy of genistein in an animal model system. Therefore, the PIRV-Syrian golden hamster animal model was used to test for drug efficacy. Additionally, 15 mg/kg of genistein was used, which was based on a published study that tested the affects of the drug utilizing the guinea pig asthma model (Duan et al., 2003). Infection of female Syrian golden hamsters (13–15 weeks of age) with PIRV resulted in a disease progression of elevated temperatures, weight loss, lethargy, the formation of petechial rashes, huddled posture and ruffled fur, epistaxis, and ocular orbital hemorrhage. Petechial rashes were the trigger used to treat the Group 5 animals (the therapeutic group) in this study. Animals surviving to the later stages of disease exhibited neurological signs of disease such as whole body tremors, loss of balance, shaking, and hind limb paralysis, in addition to rectal hemorrhage.

Infection of the carrier-treated control Group 1 animals with PIRV resulted in 100% mortality, while genistein treatment of the Groups 2, 3, 4, and 5 animals resulted in 50%, 66.6%, 33.3%, and 33.3% survival, respectively. Moreover, statistically significant survival rates were calculated for the PrP (Group 2) and PPEP (Group 3) animals when compared to the Carrier Control animals (Group 1). No statistical significance was calculated when comparing the PEP and therapeutic animals to the Carrier Control animals. This is due to a 33% survival rate for the Group 4 (PEP) and 5 (therapeutic) animals, which at first glance does not look optimal. However, it should be noted that PIRV infection (10^5 pfu/mL) in Syrian golden hamsters results in 100% mortality, which corresponds to some level of protection resulting from the drug. There is very little information about the toxicity of the drug, *in vivo*, and it is possible that the optimal amount of the drug was not used. Furthermore, the half-life and activity of the drug in the hamster system has not been described. Future studies should be focused on assessing the toxicity of the drug in hamsters, in addition to absorption, distribution, metabolism, and excretion (ADME) studies to determine whether higher doses of the drug or more frequent administration of the drug can be performed in hamsters. This may allow for a greater survival rate in the hamsters treated with genistein in a post-exposure prophylactic or therapeutic regimen. It should also be noted that the appearance of petechial rashes was the "trigger" used to treat the hamsters in Group 5 (therapeutic group). Petechial rashes did not occur until 4–5 days post-infection, and the use of petechial rashes as a trigger may not be optimal for therapeutic studies. Future studies should be aimed at delineating the biomarkers associated with hemorrhagic fever in the hamster model to determine a proper biomarker signature to be used as a therapeutic trigger. It is likely that the therapeutic trigger may occur prior to the appearance of petechial rashes, and thus treating earlier may results in a higher survival rate. A rise in core body temperature in combination with weight loss may be a more useful trigger. Also,

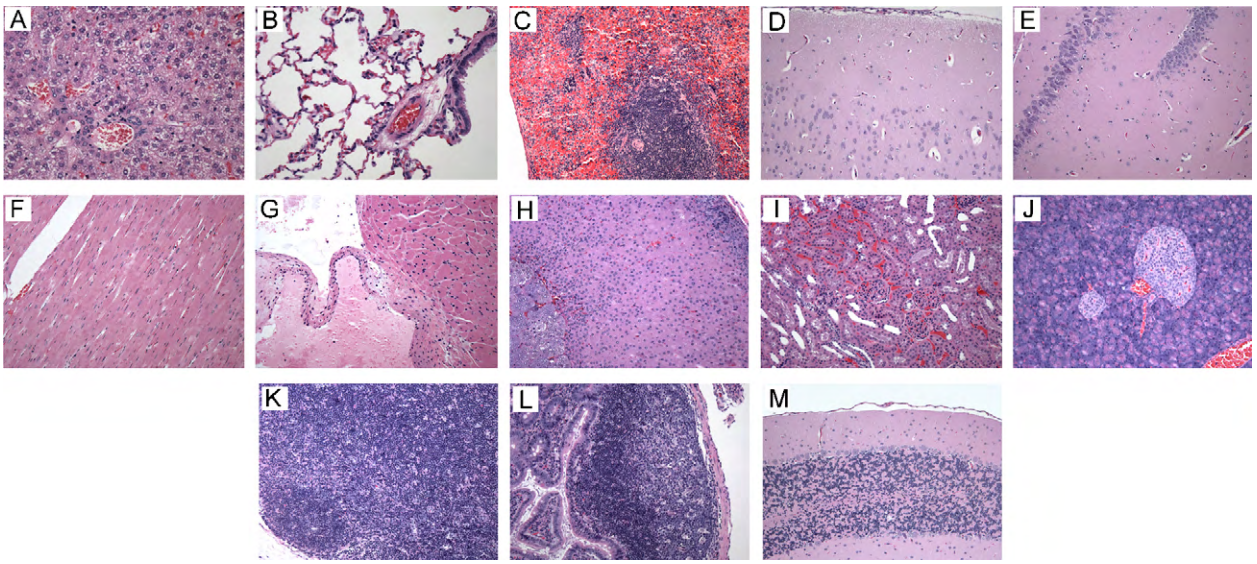

Fig. 6. Genistein treatment in uninfected control animals does not lead to lesions. Lesions, consisting of dark or red discoloration, associated with genistein treatment were not detected in the (A) liver (H&E 40× magnification, (B) lung (H&E 40× magnification), (C) spleen (H&E 20× magnification), (D) cerebral cortex of the brain (parietal cortex) (H&E 20× magnification), (E) hippocampus of the brain (H&E 20× magnification), (F) myocardium of the heart (H&E 20× magnification), (G) mitral valve of the heart-lysed RBC are an artifact of euthanasia (H&E 20× magnification), (H) adrenals (H&E 20× magnification), (I) kidney (H&E 20× magnification), (J) pancreas (H&E 20× magnification), (K) mesenteric lymph node (H&E 20× magnification), (L) gut-associated lymphoid tissue (jejunum) (H&E 20× magnification), and (M) cerebellum of the brain (H&E 20× magnification). These are representative samples from the 3 genistein-treated uninfected hamsters. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

the upregulation of the immune response in respect to cytokines and chemokines may be a useful trigger for treatment. Determining the optimal therapeutic trigger and optimal drug delivery concentration and frequency may result in higher survival rates of animals treated with genistein in a post-exposure prophylactic and therapeutic regimen.

A disruption of the natural diurnal core body temperature pattern was observed in all PIRV-infected animals. All PIRV-infected animals, regardless of treatment, developed increased core body temperatures 1 day after infection. This core body temperature increase remained constant in the Group 1 (Carrier Control animals) and began to decline prior to the death of the animal. However, the Group 2 (PrP) and Group 3 (PPEP) surviving animals all returned to relatively normal temperature levels. Some animals (546, 549, and 566) recovered after exhibiting low core body temperatures. Additionally, a diurnal temperature pattern was observed by some of the PPEP animals 8–9 days post-infection. These animals demonstrated increased core body temperatures and a disruption of the diurnal rhythm following PIRV infection, but the core body temperature of these animals appeared to recover as evident by the diurnal temperature patterns observed at the end of the study. In all, these demonstrate the ability of the PPEP animals to recover from fever after being treated with genistein. However, the diurnal temperature rhythm of most of the Groups 2, 4, and 5 animals that survived until the end of study did not recover, which suggest that pre-treatment and a constant administration of genistein may ameliorate the disruption of the diurnal temperature pattern and elevated core body temperatures. As previously stated, a better understanding of the drug kinetics may shed some insight into a more optimal concentration that needs to be administered to provide better efficacy and aid in ameliorating elevated core body temperatures.

Observed thrombocytopenia was associated with a large mathematical variation when comparing the terminal samples from the different groups, which may suggest that dysfunctioning thrombocytes may be playing a role in vascular collapse. A similar phenomena has been noted in patients suffering from Argentine hemorrhagic fever (caused by JUNV infection) and LASV-infected primates (Cummins et al., 1990; Lange et al., 1985). Changes in clinical chemistry suggest liver and kidney damage, while coagulation parameters demonstrate a disruption of coagulation and clotting. It should be noted that unhealthy anesthetized animals may not have been able to properly metabolize the anesthetic due to the liver and kidney damage, which may have had an effect on some of the animals anesthetized on study day 5. Additionally, the data suggest a certain level of protection to the livers and kidneys of the animals that received multiple genistein treatments as a prophylactic since ALT, AST, BUN, and creatinine levels returned to normal in the surviving animals. Moreover, a disruption in coagulation was also less apparent in the PPEP survivors that received several doses of genistein as a prophylactic.

Viremia and high viral titers were observed in the livers and spleens in PIRV-infected animals, while lower titers were detected in the lymph nodes, heart, kidney, and lungs. Viral tissue loads were reduced in the Group 3 (PPEP) animals when compared to the Group 1 (carrier control) animals, further demonstrating a level of protection from the drug. The animals that had measureable viral loads in the brain demonstrated neurologic signs of disease such as tremors, loss of balance, and hind limb paralysis. Viral titers observed in these specific tissues also correlate to the observed pathology. For example, the animals with viral loads in the brain demonstrated microscopic hemorrhage and lesions and correlated with the animals that presented with neurologic signs of disease. Additionally, gross lesions, microscopic hemorrhage, inflammation, necrosis, and mineralization was associated with lungs, spleen, kidneys, small and large intestines, liver, lymph nodes, and skin, and skeletal muscle, correlated with the viral titers observed in the specific tissues. In all, fewer animals died after genistein treatment and the incidence of severity of the microscopic lesions were similar among the animals that died. Furthermore, no microscopic lesions, necrosis, inflammation, and mineralization were associated with the uninfected genistein-treated animals, which suggest a level of safety associated with the drug at the concentration used in this study (15 mg/kg).

Overall, the disease progression is similar to previous reports (Sbrana et al., 2006; Xiao et al., 2001). However, there were

some notable differences amongst the studies. The current study describes more neurologic complications as a result of PIRV infection when compared to the previous studies, which may be due to the presence of infectious virus in the brain as observed in the current study. It should be noted that both studies report hind limb paralysis, which may be attributed to neurological complications associated with viral infection. Neurologic signs are more commonly observed in humans infected with a South American hemorrhagic fever arenavirus, as opposed to humans infected with LASV (an Old World arenavirus). However, it should be noted that neurologic complications are frequent in critically ill people suffering from Lassa fever (Cummins et al., 1992; Gunther et al., 2001).

Additionally, necrosis was observed in the kidneys, heart, and intestines of the PIRV-infected hamsters in the current study. Previous studies utilizing PIRV and female Syrian golden hamsters did not report such findings (Sbrana et al., 2006). This discrepancy may be a result of the age of the hamsters since the current study used hamsters that were 13–15 weeks of age when compared to previous studies that utilized 5–6-week-old hamsters. Further studies aimed at comparing the disease progression and resulting pathology in PIRV-infected younger and older hamsters may be valuable as a surrogate model to study hemorrhagic fever in younger and older humans. It has been shown that infection of guinea pigs with GTOV led to lesions in the intestines, and in some cases minimal lesions in the kidneys and heart (Hall et al., 1996). GTOV antigen was associated with capillary endothelial cells of the brain in infected guinea pigs; however, no lesions were observed in the brain in association with GTOV antigen. Thus, the isolated virus may be a result of blood-borne virus and not viral replication in the brain. Additionally, liver necrosis, associated with PIRV infection in hamsters, was not observed in GTOV-infected guinea pigs (Hall et al., 1996). However, liver necrosis has been observed in guinea pigs infected with PICV, in addition to viral antigen in blood vessels in the brain (Jahrling et al., 1981a). Virus was concluded to be mainly excluded from the brain of PICV-infected guinea pigs, other than small vessel involvement. As with the GTOV model, virus in the brain is likely a result of blood-borne virus and not viral replication. LASV infection in guinea pigs also led to viral titers in the kidneys and heart (Jahrling et al., 1982). Virus recovered from the brain of LASV-infected animals was also attributed to blood-borne virus. Therefore, it is possible for the virus observed in the brains of PIRV-infected hamsters to be attributed to blood-borne virus as well, since very low titers were observed when compared to other tissues. A correlation of viral antigen and necrosis data may aid in concluding whether the presence of virus is due to low levels of replication or blood-borne virus. Interestingly, infectious virus has been recovered from the brain of white-throated woodrats (*Neotoma albigula*) infected with *Whitewater arroyo virus* (WWAV) (Fulhorst et al., 2001). However, it is unclear whether the isolated virus was associated with viral replication or blood-borne virus.

The mechanistic inhibitory mode of action of genistein in the PIRV-hamster model has not been established. The inhibitory antiviral properties associated with genistein may occur because the drug is (1) an immunomodulator, (2) a phytoestrogen that can bind an estrogen receptor, or (3) a kinase inhibitor. Previous reports have shown that genistein inhibits arenavirus infection *in vitro*, and treatment leads to inhibition of phosphorylation induced by arenavirus infection (Vela et al., 2008b). Plus, genistein may act to inhibit phosphorylation events that are triggered through viral entry and replication. Thus, genistein may lead to an inhibition of signaling through kinases necessary for arenavirus infection. However, genistein also acts as an immunomodulator and treatment of hamsters with genistein may lead to a change in the numbers of splenic Tcells and NK cells, which may lead to a change in the robustness of the immune response in the PIRV-infected hamsters treated with genistein. But, it is important to note that immunomodulation of genistein may be affected by gender because genistein may act as an estrogen agonist or antagonist, as observed in the mouse model (Guo et al., 2006). Genistein may engage the estrogen receptor, thus allowing for antiviral action through estrogen receptor signaling. Lastly, it is possible for these three properties to contribute to an additive antiviral effect. The utilization of the hamster as an animal model leads to assay difficulties because of the limited availability of reagents. Thus, the analysis of mRNA levels may prove useful. Analyzing cytokine and chemokine mRNA level changes in infected animals versus infected genistein-treated animals may demonstrate the upregulation and/or downregulation of these various proteins. Studies should also be conducted in both male and female hamsters to determine whether genistein leads acts as an estrogen agonist or antagonist and whether the drug affects male and female hamsters infected with PIRV differently.

In conclusion, the survival of the genistein-treated animals suggests the use of a kinase inhibitor as a prophylactic against arenaviral hemorrhagic fever in the Syrian golden hamster. Additionally, the data may infer that petechial rash appears too late in the disease progression to be used as an effective therapeutic trigger for the study parameters that were tested at a dose of 15 mg/kg of genistein. Treating animals at the first signs of elevated temperatures or at a combination of triggers that occur prior to the appearance of the petechial rash may have resulted in more significant therapeutic results. However, there was not sufficient statistical power in the experimental design to detect a significant difference in survival below the 50% level. Additionally, more viral and genistein data are required to determine the optimal viral titer and the drug dose and frequency of administration prior to determining whether genistein may be used as a potential therapeutic. The amount of virus used in this study and previously published studies (Sbrana et al., 2006; Xiao et al., 2001) resulted in severe disease and 100% mortality. A natural history study aiming to deduce the 50% lethal dose (LD_{50}) would be valuable in future efficacy studies. The viral titer used in this study may not be optimal due to the severity of disease; infecting with a lower viral titer may be advantageous. Nevertheless, the survival of some of the animals demonstrates some level of drug-induced protection, which demonstrates the potential for genistein to be used as an antiviral against hemorrhagic fever, perhaps in combination with ribavirin. In addition, future drug efficacy studies will involve studying the effects of the drug on long-term survival in PIRV-infected hamsters. These studies will examine the long-term neurological complications associated with PIRV infection in surviving genistein-treated hamsters. Lastly, uninfected control animals treated with genistein demonstrated no visible clinical signs of drug-related illness or any pathology associated with the drug in the control uninfected animals. In all, this study demonstrates survival of PIRV-infected hamsters and the potential for using a general kinase inhibitor as a prophylactic antiviral against arenavirus hemorrhagic fever.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.antiviral.2010.06.007.

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