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A Kinase Inhibitor Cocktail as a Prophylaxis in the Syrian Golden Hamster Pirital Virus Model

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Abstract

Arena viruses are negative strand RNA viruses that have the potential to cause a wide spectrum of disease including hemorrhagic fever in humans and experimental animals. There are currently limited therapeutic treatments available for arena virus infection, which includes ribavirin and in some cases immune plasma. Therapeutic research testing the efficacy of products on arena virus infection can be difficult because work with the arena viruses that cause hemorrhagic fever require Biosafety level (BSL)-4 containment. However, surrogate arena virus animal models have been developed that can be utilized as a screening tool to test product efficacy of antivirals that may have broad acting anti-arena viral activity. Because there is a strong need to develop novel prophylaxis and therapeutics, we have utilized the Syrian golden hamster Pirital virus (PIRV) surrogate model to test antiviral efficacy in a BSL-3 environment. PIRV is a New World arena virus that is not known to cause human disease, but it is capable of causing hemorrhagic fever, morbidity, and complete mortality in the Syrian golden hamster. Thus, we infected hamsters with PIRV and treated with two different dosages of a kinase inhibitor cocktail consisting of genistein and tyrphostin AG1478. Drug efficacy was determined by evaluating physical signs of disease, mortality, viral titers in specific tissues, and terminal viremia. In this study, treating PIRV-infected hamsters with a kinase inhibitor cocktail led to a significant survival rate, lower viral titers, and an absence of viremia in survivors. In all, the results demonstrate the potential of the cocktail as an antiviral against arena virus infection.

Keywords: Kinase inhibitor; Viruses; Viremia

Introduction

Arena viruses are enveloped, bipartite negative single-stranded RNA viruses that can cause a myriad of disease signs in humans and host animals including hemorrhagic fever. With the exception of Tacaribe virus, the majority of these viruses are rodent-borne. The arena virus family can be divided into two groups: the Lassa-Lymphocytic choriomeningitis sero complex and the Tacaribe sero complex. Lassa virus (LASV) is an Old World arena virus and the etiologic agent that causes Lassa fever resulting in approximately 5,000 deaths in endemic regions of West Africa [1], while the New World arena viruses Junín virus, Chapare virus, Machupo virus, Guanarito virus, and Sabiá virus have the ability to cause hemorrhagic fever in humans. Pirital virus (PIRV) is a New World arenavirus that was isolated in the Municipality of Guanarito, Venezuela in 1994 from the cotton rat (*Sigmodon alstoni*) [2]. PIRV has not been associated with any human disease; yet, infection of the Syrian golden hamster with PIRV leads to hemorrhagic fever manifestations and a disease progression that is similar to the hemorrhagic fever disease observed in humans infected with the New World arena viruses [3,4]. PIRV infection of hamsters leads to elevated temperatures, loss of body weight, viremia, lethargy, petechia, epistaxis, ecchymoses, neurologic signs of disease, and complete mortality [3,5]. Post mortem examination of PIRV-infected hamsters demonstrated hemorrhage associated with the liver, lungs, heart, spleen, and brain, as well as splenomegaly, hepatomegaly, and abnormal clinical pathology including elevated ASL and AST levels, as well as an increase in times associated with coagulation. Viral titers are commonly associated with the lymph nodes, brain, liver, spleen, kidney, heart, intestines, and lungs from infected animals. Additionally, viremia can be observed 2 days post-challenge and remains constant in animals succumbing to disease, with terminal viremia measured in animals succumbing to disease. Thus, the PIRV hamster hemorrhagic fever model may be used as a tool to evaluate the efficacy of various antivirals for the treatment of arena virus hemorrhagic fever in humans.

Different kinase inhibitors have been used as tools to delineate viral entry mechanisms since treating cells with such kinase inhibitors leads to an inhibition of viral infection [6-9]. Genistein is one such tyrosine kinase inhibitor that has been shown to inhibit viral infection *in vitro* by inhibiting viral entry [10-13]. It is also known to be an immune modulator and a phytoestrogen that can bind to estrogen receptors on host cells [14]. We have previously demonstrated that treating cells with the kinase inhibitor genistein leads to the inhibition of Pichindé virus (PICV) and PIRV (New World arena viruses) infection, which likely occurs due to the inhibition of kinase activity that is necessary for viral entry and productive viral infection [15,16]. Because genistein has broad properties, we also tested the affects of tyrphostin AG1478, which is a kinase inhibitor specifically inhibits the epidermal growth factor receptor (EGFR) [17], on viral infection. We have previously demonstrated that a kinase inhibitor cocktail consisting of genistein and tyrphostin AG1478 led to an inhibition of viral infection in cells infected with LASV [18]. Altogether, these studies suggest kinase inhibitors as a potential prophylactic and/or therapeutic for viral hemorrhagic fever. To test whether kinase inhibitors can be an effective prophylactic in an animal model, we previously utilized the PIRV-Syrian golden hamster model [16]. PIRV is a rodent-borne arena virus that was isolated in Venezuela. Infection of hamsters with PIRV

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results in hemorrhagic fever and mortality [3-5]. However, treating the animals with the kinase inhibitor genistein led to a significant increase in survival and the amelioration of hemorrhagic fever disease signs [16]. Therefore, this study served as a proof-of-concept to using a kinase inhibitor as a therapeutic or prophylactic in an animal model. Thus, this current study aimed to determine whether a prophylactic treatment of PIRV-infected hamsters with a kinase inhibitor cocktail results in further protection and increased survival.

Materials and Methods

Cell lines, virus, and reagents

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 five times in Vero E6 cells (ATCC) using RPMI 1640 media (Gibco) plus antibiotics (diluted 1:100) and diluted in Minimal Essential Media prior to challenge. The animals were challenged with 1×10^4 pfu of PIRV administered by intraperitoneal injection. Genistein and tyrphostin AG1478 (purchased from Sigma Aldrich, St. Louis, MO) were diluted in 0.1% DMSO and 1x sterile phosphate buffered saline (PBS). The drugs were administered to the animals subcutaneously at a final dosage of 15 or 30 mg/kg.

Plaque assay

Plaque assays were performed as previously described [19]. Briefly, Vero E6 cells (ATCC) were seeded 24 hours prior to inoculation. After 10 fold dilutions of the samples, 100 µL was added to each well (in triplicate). The plates were incubated at 37.0°C (5% CO_2) for 60 minutes with gentle rocking every 15 minutes. A 0.7% methyl-cellulose overlay that contained EMEM, antibiotics, non-essential amino acids, and 10% FBS was added to the inoculums. The plates were then incubated for 96 hours at 37.0°C (5% CO_2). Following this incubation, the overlay was aspirated and replaced with an identical overlay supplemented with 4% Neutral Red (Sigma) and incubated an additional 24 hours at 37.0°C (5% CO_2). The wells were then fixed with a 2% paraformaldehyde solution prior to visual analyses.

Animal studies

Thirteen (13) female Syrian golden hamsters (*Mesocricetus auratus*) (13-15 weeks of age) were received from Charles River Kingston (Kingston, NY) and randomly distributed into three groups. Group 1 consisted of three animals, while Groups 2 and 3 each contained five animals. 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.

Blood sampling

Terminal blood samples were obtained from animals via cardiac puncture. Sera were used for viremia analysis.

Tissue harvesting

Various tissues including the spleen, liver, intestines, brain, pancreas, kidney, lymph nodes, heart, adrenal glands, and lung were harvested from the animals and collected in sterile 1x PBS. The tissues were homogenized and plaque assays were conducted to measure the viral titers associated with the tissues.

Statistical analyses

The SAS® MULTTEST procedure (adjusting for multiple comparisons at the 0.05 level of significance using the Bonferroni-Holm Method) was used for animal survival data analyses. The time-to-death analysis was performed based on the length of survival model and the Kaplan-Meier estimator and the log-rank tests were used to determine any significant differences among the groups.

Results and Discussion

Syrian golden hamsters (*Mesocricetus auratus*) (13-15 weeks of age at the beginning of the study) were randomly distributed into three groups. The Group 1 control animals were treated intraperitoneally (i.p.) with the DMSO carrier control, while the Group 2 (15 mg/mL) and Group 3 (30 mg/mL) animals were treated with the kinase inhibitor cocktail consisting of both genistein and tyrphostin AG1478. These prophylactic treatments occurred once daily; beginning one day prior to infection and occurring until the end of the study or until the animals succumbed to disease. All animals were challenged (by i.p.) with a lethal dose of PIRV (1×10^4 pfu) on Study Day 0. Infection of the carrier-control animals with PIRV resulted in complete mortality. However, 60% of the hamsters treated with 15 mg/kg of the kinase inhibitor cocktail survived, while 80% of the hamsters treated with 30 mg/kg of the cocktail survived (Figure 1). The Kaplan-Meier estimator and log-rank tests were used to determine the significance of survival among groups. Significant survival was observed when the Group 3 animals were compared to the of the Group 1 animals (Table 1).

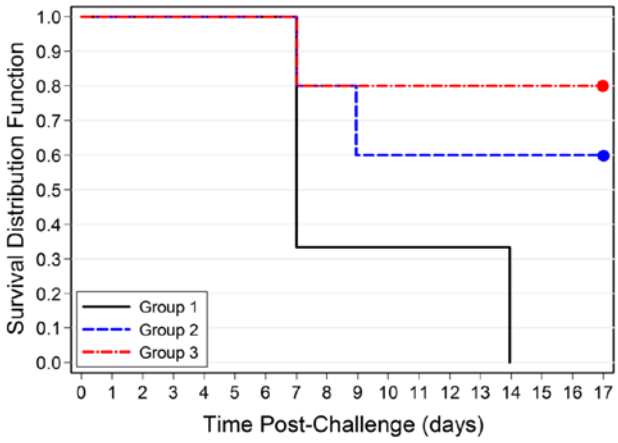

Figure 1: Kinase inhibitor cocktail treatment in hamsters infected with PIRV leads to increased survival. The Group 1 animals were treated with the DMSO carrier control; the Group 2 animals were treated with 15 µg/kg of the kinase inhibitor cocktail; and the Group 3 animals were treated with 30 µg/kg of the kinase inhibitor cocktail. The animals were treated 1x 24 hrs prior to infection and 1x daily every day after challenge. Statistical significance ($p\leq0.05$) was observed when comparing the survival of the Group 3 animals to the Group 1 animals.

Group	2	3
1	0.0831	0.0245*
2		0.5485

*Comparison between the two groups was significantly different at the 0.05 level of significance.

Table 1: P-values from the Pairwise Log-Rank Tests Comparing Time to Death and Overall Survival between Groups.

Study Day:		-3	-1	4	5	8	11	14
Animal ID	**Group**							
Hamster 1	1	136.8	136.9	125.3	119.86	106.12		
Hamster 2		151.6	147.8	139.52	136.52	117.72		
Hamster 3		157.7	161.9	159.49	153.38	136.92	128.87	122.87
Hamster 1	2	140.7	140.4	139.94	137.04	138.94	138.4	138.9
Hamster 2		146.5	146.3	145.42	145.41	147.66	148.94	150.48
Hamster 3		132.7	132.1	132.66	131.22	114.4		
Hamster 4		135.2	140.5	141.22	130.93	115.93		
Hamster 5		152.3	153.8	159.78	160.63	163.48	156.87	165.05
Hamster 1	3	157.5	156.3	155.3	154.71	150.3	151.2	154.87
Hamster 2		147.3	150.7	149.99	152.18	149.49	152.98	161.49
Hamster 3		152.9	152.8	152.18	146.83	146.56	143.68	142.89
Hamster 4		137.6	132.8	133.7	133.13	121.95		
Hamster 5		147.1	148.6	151.56	150.36	147.77	150.37	149.05

Represents animals that succumbed to disease

Hamster 3 from Group 1 died on Study Day 14 after the animal was weighed
Weights are reported in grams

Table 2: Comparison of Weight Changes.

Though no statistical significance was observed when comparing the Group 2 animals to the Group 1 animals, 60% survival of the Group 2 treated animals is encouraging since infection with PIRV leads to complete mortality.

Additionally, the PIRV-infected animals that succumbed to disease experienced a large amount of weight loss (11.4%-22.4%) when compared to their baseline body weight (Table 2). Any animal that lost at least 11.4% of its body weight did not survive PIRV challenge. The average weight loss for the Group 1 carrier-control treated animals was 22.3%. The Group 2 animals that succumbed to infection lost an average of 14.1% of their body weight, while the Group 3 animal that died lost 11.4% of its body weight when compared to baseline. The surviving treated-animals gained weight or only lost a minimal amount of weight by the study end. One Group 2 surviving animal lost 1.3% of its body weight; while the other Group 2 animals all gained weight by the study end. Of the four Group 3 animal survivors, 2 animals experienced weight gain by the study end, while 2 animals experienced an average of 4.1% weight loss. Thus, it appears that treatment of the animals with the kinase inhibitor protected the animals from severe weight loss, which is characteristic of PIRV infection. Additionally, all animals that succumbed to disease demonstrated overt signs of hemorrhagic fever including loss of body weight, lethargy, petechial rash, hind limb paralysis or weakness, and hemorrhage (data not shown). Surviving animals only transiently demonstrated signs of hemorrhagic fever and all survivors completely recovered by the end of the study. At no time did any of the treated-survivors exhibit any neurological signs of disease. For instance, hind limb paralysis is a common sign of late stages of PIRV disease in hamsters and none of the surviving-treated hamsters ever exhibited paralysis. In all, these data demonstrate that treatment of the animals with a kinase inhibitor cocktail protected them from sequelae associated with PIRV-induced hemorrhagic fever.

Viral titers were also measured in specific tissues. Animals that succumbed to disease demonstrated higher viral titers in most of the collected tissues (Figure 2). In most cases, detectable virus (by plaque assay analysis) was isolated in the spleen, liver, intestines, brain, pancreas, kidney, lymph nodes, heart, adrenal gland, and lung from animals that died. The highest viral titers were consistently observed in the kidneys from animals that succumbed to infection. Additionally, high viral titers were also associated with the spleen and liver from animals that died as a result of PIRV infection. The two Group 2 animals and one Group 3 animal that died also exhibited high viral titers in the tissues collected. Surviving animals exhibited low viral

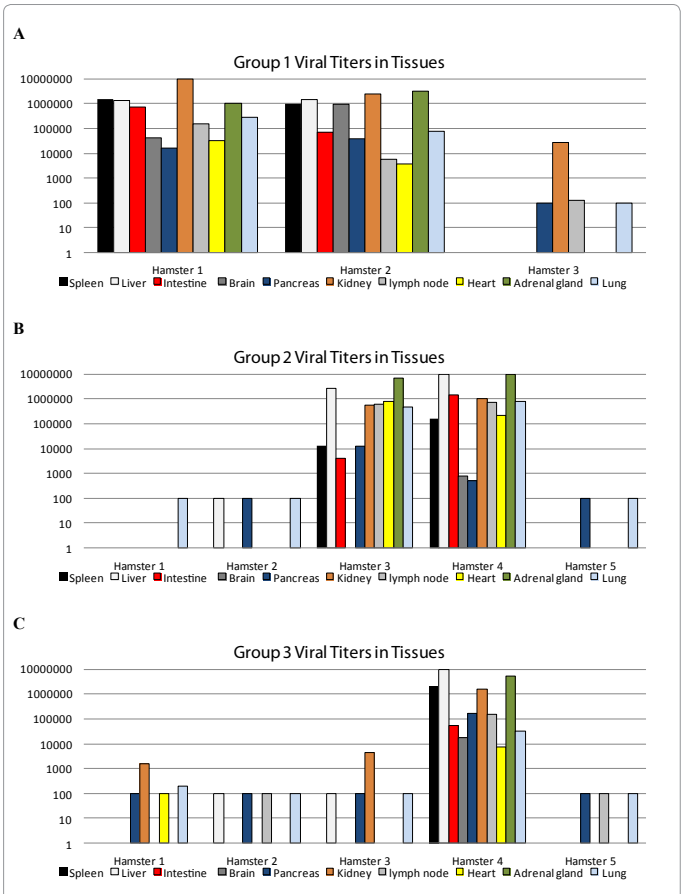

Figure 2: Treating animals with the kinase inhibitor cocktail leads to less viral tissue titers in the surviving animals. Viral tissue titers were measured in the (A) Group 1 animals, (B) Group 2 animals, and (C) Group 3 animals. Tissues were harvested from all animals that were found dead, euthanized due to disease, or euthanized at the study end. The viral loads were measured by plaque assay analysis (limit of detection: 50 pfu/mL).

titers in the pancreas, lungs, liver, and in some cases, the kidneys. It should be mentioned that the measured viral titers were at the limit of quantitation of the plaque assay. In all, these data suggest less viral replication or viral infection in specific tissues collected from the kinase inhibitor cocktail treated-animals.

Terminal viremia was also analyzed. Blood was collected into SST tubes by cardiac puncture and viremia was analyzed by a standard plaque assay as previously described [16]. All Groups 1, 2, and 3 animals that succumbed to disease exhibited terminal viremia, while none of the survivors exhibited any terminal viremia (Figure 3). These results were not surprising since a previous study demonstrated the presence of virus in the blood at the time of the death [16]. Additionally, limited viremia (8 of 11 surviving animals had no detectable viremia at the end of the study) has been associated with surviving animals in previous studies [16]. In this current study, no viremia was observed among surviving animals. This may be due to the treatment of the animals with two kinase inhibitors; however, it is possible that the limited number of animals may have led to the lack of opportunity to measure viremia in survivors.

In all, the findings from this study demonstrate that treatment of PIRV-infected hamsters with kinase inhibitor cocktail results in some level of protection and increased survival. The increased survival appeared to be concentration-dependent on the amount of the kinase inhibitor cocktail administered. Additionally, the surviving animals demonstrated less weight loss when compared to the animals that succumbed to disease. Mortality also appeared to correlate with viral titers in tissues and viremia. All animals that died as a result of infection exhibited higher viral titers in most of the tissues harvested, when compared to the survivors. Previously, genistein treatment of PIRV-infected hamsters resulted in lower viral titers in the lymph nodes, brain, liver, spleen, kidney, heart, intestines, and lungs [16]. In this previous study, viral titers were observed in the tissues harvested from the genistein-treated animals, albeit at lower levels when compared to the carrier-control group. In the current study, low levels of virus were only isolated from a few of the tissues in the animals treated with a cocktail. When compared to the previous kinase inhibitor studies, the PIRV-infected animals treated with the kinase inhibitor cocktail demonstrated lower titers of virus. This result may demonstrate a greater amount of protection when animals are treated with the kinase inhibitor cocktail, as opposed to a single kinase inhibitor. Similarly, no viremia was detected in the terminal blood draws from the infected animals that survived challenge. These data further demonstrate a level of protection due to the kinase inhibitor cocktail treatment. Because lower virus titers was observed in the tissues and blood of animals that survived challenge, the kinase inhibitor cocktail may be playing a role in inhibiting viral replication and infection in target tissues and cells. Previous studies, utilizing the arena virus PICV, demonstrated that entry and productive infection appears to be mediated by a cholesterol-dependent, non-caveolar, clathrin endocytic pathway that is dependent on endosomal trafficking through dynamin 2, Rab5 and RAb7-mediated endosomes [13,15,19]. Collectively, these studies identified an infection process that is dependent on an endosomal pathway that requires a number of phosphorylation-dependent events for successful and productive infection in host cells. It is possible that treatment of the animals with the kinase inhibitor cocktail interferes with the viral entry process required for productive infection of host cells. Thus, the virus could not establish a natural reservoir within the tissues of infected animals that are treated with the kinase inhibitor cocktail.

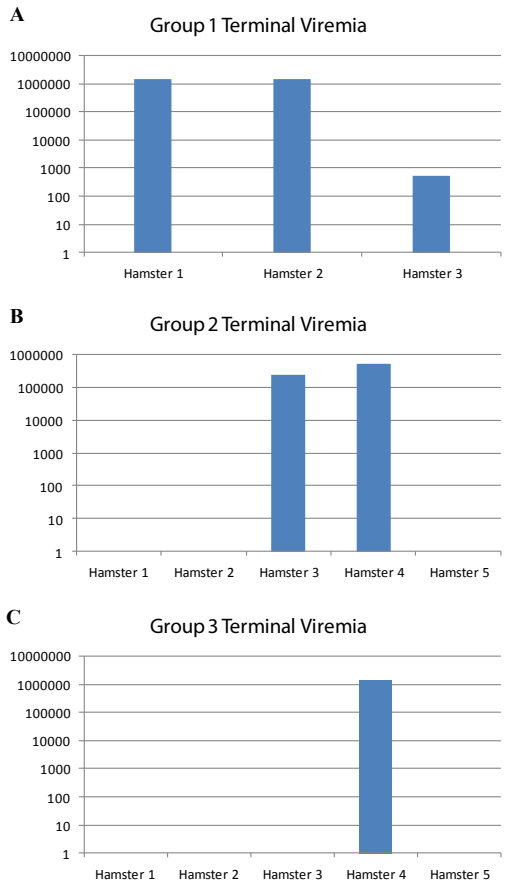

Figure 3: No viremia was associated with surviving-treated animals. Viremia was measured in the (A) Group 1 animals, (B) Group 2 animals, and (C) Group 3 animals. Blood collected from all animals that were found dead, euthanized due to disease, or euthanized at the study end. Viremia was measured by plaque assay analysis (limit of detection: 50 pfu/mL).

Currently, there are few treatment options for humans or experimental animals with suspected arena virus infection. However, results from the current study, taken together with the previous kinase inhibitor studies, add credence to the use of specific kinase inhibitors as a prophylactic option. In all, the data from this study suggest the use of a kinase inhibitor cocktail as a potential prophylactic for arena virus infection.

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