



Evaluation of orally delivered ST-246 as postexposure prophylactic and antiviral therapeutic in an aerosolized rabbitpox rabbit model[☆]

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ABSTRACT

Orthopoxviruses, such as variola and monkeypox viruses, can cause severe disease in humans when delivered by the aerosol route, and thus represent significant threats to both military and civilian populations. Currently, there are no antiviral therapies approved by the U.S. Food and Drug Administration (FDA) to treat smallpox or monkeypox infection. In this study, we showed that administration of the antiviral compound ST-246 to rabbits by oral gavage, once daily for 14 days beginning 1 h postexposure (p.e.), resulted in 100% survival in a lethal aerosolized rabbitpox model used as a surrogate for smallpox. Furthermore, efficacy of delayed treatment with ST-246 was evaluated by beginning treatment on days 1, 2, 3, and 4 p.e. Although a limited number of rabbits showed less severe signs of the rabbitpox disease from the day 1 and day 2 p.e. treatment groups, their illness resolved very quickly, and the survival rates for these group of rabbits were 88% and 100%, respectively. But when the treatment was started on days 3 or 4 p.e., survival was 67% and 33%, respectively. This work suggests that ST-246 is a very potent antiviral compound against aerosolized rabbitpox in rabbits and should be investigated for further development for all orthopoxvirus diseases.

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1. Introduction

Smallpox, the disease caused by variola virus (VARV), is naturally transmissible by aerosol and is highly communicable in humans. Smallpox has been described as a devastating disease in humans, causing an estimated mortality in greater than 30% of infected individuals and significant sequelae in survivors. Although eliminated as a naturally occurring disease more than three decades ago, it has reemerged as a biological threat based on the potential for the virus to be used as an offensive military or terrorist weapon (Henderson, 2002). This has facilitated a renewed interest in developing new-generation vaccines and antiviral therapeutics that can be used to prevent infection or ameliorate effects after exposure in the event of a deliberate release.

There are few animal disease models that simulate the pathophysiology and unique clinical progress of smallpox disease in

humans. Rabbitpox virus (RPXV) was first described in the 1930s (Greene, 1933). Recent genomic sequencing studies showed that RPXV is closely related to vaccinia virus with over 95% similarity (Li et al., 2005). The disease caused by RPXV infection of rabbits is severe and has a high case-fatality rate (Greene, 1934a,b). An aerosolized dose of 15 Plaque Forming Units (PFU) of RPXV resulted in uniform lethality in rabbits (Westwood et al., 1966). The description of the clinical syndrome that follows experimental infection possesses many of the characteristics observed in active human smallpox (Bedson and Duckworth, 1963; Westwood et al., 1966). Widespread dissemination of the virus always preceded other signs of generalized infection. Rabbits began to exhibit clinical signs of disease approximately 3–5 days after exposure. Initial signs of depression and anorexia were followed by mucus membrane discharge and weight loss accompanied by a steady increase in body temperature. The clinical progression of rabbitpox in these early studies was noted to be similar to that in human smallpox, with most rabbits succumbing to disease 7–12 days postexposure (p.e.) (Westwood et al., 1966).

Currently, there is no FDA-approved drug for the prevention or treatment of smallpox infection. While vaccination is considered the front-line defense against a smallpox outbreak, adverse

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events associated with the vaccine and lag time for development of protective immunity have hindered mass vaccination campaigns of the general public and underscores the need for new antiviral drugs that are safe and effective at inhibiting VARV infection. The ideal antiviral drug would be potent, nontoxic, and highly selective for smallpox virus replication. Development of smallpox-specific compounds with superior safety profiles and improved pharmacological properties will serve as both a deterrent and a front line of defense against a possible biological attack.

ST-246 was discovered by performing high throughput cell based screen using live vaccinia virus (Yang et al., 2005). Mechanism of action studies showed that it targets the vaccinia virus F13L gene product which encodes a 37-kDa palmitylated peripheral membrane protein required for extracellular virus particle formation (Yang et al., 2005). Thus, ST-246 is a potent vaccinia virus egress inhibitor that is also effective in inhibiting and/or halting in vitro infection by other orthopoxviruses, including monkeypox virus, camelpox virus, cowpox virus, ectromelia (mousepox) virus, and VARV (Yang et al., 2005). In addition, oral administration of ST-246 can effectively inhibit infections in vivo for monkeypox (Sbrana et al., 2007), cowpox (Quenelle et al., 2007a,b), ectromelia (mousepox) (Yang et al., 2005; Quenelle et al., 2007a), and VARV (personal communication – John Huggins-USAMRIID). Phase I human safety trials have been initiated to support use of ST-246 as a therapeutic for orthopoxvirus infections.

In this study, we evaluated the efficacy of orally administered ST-246 against aerosolized RPXV in rabbits when the initiation of drug therapy was days 0, 1, 2, 3, or 4 p.e. These treatment days were chosen in order to evaluate the efficacy of ST-246 as postexposure prophylactic and antiviral therapeutic.

2. Materials and methods

2.1. Cells and viruses

CV-1 (ATCC CCL-70) African green monkey kidney fibroblast cells (ATCC, Manassas, VA) were maintained in Eagle's minimal essential medium (EMEM) supplemented with 1% nonessential amino acids (NEAA), 1% 200 nM L-glutamine, 7.5% fetal bovine serum (FBS), and 0.5% penicillin/streptomycin.

RPXV, strain *Utrecht*, was provided by Richard Moyer at the University of Florida. The viral seed stock was originally obtained from American Type Culture Collection (ATCC, Manassas, VA). RPXV was propagated in CV-1 cells.

2.2. Animals

Forty-eight (24 male, 24 female) New Zealand white (NZW) rabbits (*Oryctolagus cuniculus*) weighing approximately 2.5–3.0 kg were purchased from Charles River Laboratories (Wilmington, MA). The rabbits were maintained on a 12-h light/12 dark cycle and fed standard rabbit food supplemented with fresh leafy vegetables and water *ad libitum*. The rabbits were caged singly in stainless steel cages under biosafety level 3 (BSL-3) containment.

Each animal was implanted subcutaneously between its scapulae with a programmable temperature transponder chip (Bio Medic Data Systems, DE) to determine rabbit identification and subcutaneous body temperature.

2.3. Aerosol challenge

Rabbits were challenged by aerosol as previously described (Pitt et al., 2001). Briefly, the respiratory function of each of the rabbits was first measured using whole-body plethysmography (Buxco

Systems, Sharon, CT) before aerosol challenge. Thereafter, each rabbit was exposed to aerosolized RPXV using a dynamic muzzle-only (nose and mouth) inhalation chamber operated within a class III safety cabinet maintained under negative pressure. Small-particle aerosols of the virus were generated with a three-jet Collison nebulizer (BGI Inc., Waltham, MA) which provided homogenous particle size distribution (mean diameter = 1.0 μ m) so that most of the inhaled particles were delivered to lungs. The atmosphere within the inhalation chamber was continuously sampled during each exposure for aerosol concentration using an all-glass impinger (AGI; Ace Glass, Vineland, NJ). The collection fluid and starting concentrations for each exposure were assayed by plaque assay using CV-1 cells for determination of aerosol concentration. Determination of presented dose to each rabbit was calculated using respiratory minute volume (V_m) estimates derived from the respiratory function measurements performed before the exposures. The presented aerosol dose was then calculated by multiplying the total volume (V_t) of experimental atmosphere inhaled by each animal ($V_t = V_m \times$ length of exposure) by the empirically determined exposure concentration from chamber sampling (C_e) ('presented dose' = $C_e \times V_t$) (Roy and Pitt, 2006).

2.4. ST-246 treatment

ST-246 (4-trifluoromethyl-N-(3,3a,4,4a,5,5a,6,6a-octahydro-1,3-dioxo-4,6-ethenocycloprop[*f*]isoindol-2(1H)-yl)-benzamide was formulated for oral administration and provided by SIGA Technologies (Corvallis, OR). Aqueous 0.75% methylcellulose containing 1% Tween 80 (Sigma, St. Louis, MO) was used for suspension of ST-246 to produce a drug concentration of 100 mg/ml. The dose of ST-246 used to treat each rabbit was 40 mg/kg of body weight once a day.

The rabbits were divided into six groups of eight animals with equal numbers of males and females in each group. During the course of this experiment, one or two rabbits from each group, except group 2, had to be excluded from the study because of technical problems unrelated to RPXV or ST-246. Approximately 1 h after exposure to the aerosolized virus, all rabbits in group 1 were administered ST-246 by oral gavage (day 0 treatment). At the same time, rabbits in group 6, which was the control group, was treated with the drug suspension vehicle only; the volume of the vehicle given to each animal was calculated as if ST-246 were present at the stock concentration of 100 mg/ml. Twenty-four hours later (day 1 p.e.); rabbits in group 2 were given their initial treatment of oral ST-246. On day 2 p.e., rabbits in group 3 received their initial dose of ST-246 and similar treatment of rabbits in group 4 was initiated on day 3 p.e. Treatment with ST-246 for the final group of rabbits (group 5) was started on day 4 p.e. All rabbits were treated or vehicle-treated once per day for 14 consecutive days – or until an animal died.

Before a drug treatment or vehicle-treatment, each rabbit was lightly anesthetized with an intramuscular injection of a ketamine–xylazine mixture (10 mg/kg and 2 mg/kg respectively). While each rabbit was anesthetized, a #12 French rubber catheter (Jorgensen Laboratories, Inc., Loveland, CO) was passed orally so that the administered material could be delivered into the rabbit's stomach.

2.5. Clinical observations

Rabbits were observed two times per day during the lights-on part of the light-dark cycle by study personnel for 21 days, starting 24 h after challenge, and more frequently if warranted for clinical illness or changes in behavior. Rabbits were weighed and temperature was recorded every day, starting the day before exposure,

and continuing 21 days after exposure. The rabbits were anesthetized with intramuscular ketamine–xylazine mixture (20 mg/kg and 4 mg/kg respectively) and 1 ml of blood was collected from the ear vein of each animal for baseline values for viral load the day before exposure and then day 2 p.e. and every other day after that through day 12 p.e. When signs of severe disease were observed, such as marked lethargy, dyspnea, and/or open mouth breathing, rabbits were anesthetized with the ketamine–xylazine mixture and then euthanized with an overdose of intravenous pentobarbital solution.

2.6. Plaque assay

Viral contents of starting concentrations and AGIs were determined by plaque assay. Briefly, each sample was sonicated, serially diluted, and added to confluent monolayers of CV-1 cells. The plates were then incubated for adsorption for 1 h at 37 °C. After 1 h, 2 ml of 50/50 mixture of 2XEMEM complete medium +1.2% agarose overlay was added to each well of 6-well plates. After 48 h incubation, 5% neutral red in EMEM complete medium was added to the overlay and the plates were incubated overnight at 37 °C. Plaques were then visualized and counted.

2.7. DNA isolation and real-time PCR

DNA was isolated from blood and tissues by using BioRobot M48 (Qiagen, Valencia, CA) in accordance with the manufacturer's instructions. Real-time PCR was carried out with the LightCycler 2.0 (Roche, Indianapolis, IN) using a pan-orthopox assay as previously described (Kulesh et al., 2004). Briefly, the oligonucleotide primers and a minor groove binder (MGB) protein-containing TaqMan probe were selected from conserved regions of the orthopoxviral hemagglutinin (HA) gene, their sequences published elsewhere (Kulesh et al., 2004). Reactions were performed on a Roche Light cycler and data was analyzed by using LightCycler Software version 4.0.

2.8. Postmortem examination

The carcasses from all rabbits that died or were euthanized due to rabbitpox were submitted for a complete gross necropsy under BSL-3 containment. Samples of the following organs were aseptically collected and stored at –70 °C for real-time PCR assay: mandibular lymph node, liver, spleen, adrenal gland, gonad, lungs, and brain. A complete set of tissue samples, including sections of skull with nasal passages, was also collected from each animal and fixed in 10% buffered formalin for histology.

At the end of this study (\geq day 25 p.e.), the surviving rabbits were anesthetized and then euthanized with an overdose of intravenous pentobarbital solution as described above. Although a complete gross necropsy was performed on each of these animals, no tissue samples were collected for PCR assay and only the mandibular lymph nodes, respiratory tract, thyroid gland, esophagus, and mediastinal lymph nodes were collected and fixed in formalin for histology.

2.9. Histology

The set of formalin-fixed tissue samples from each rabbit was held for a minimum of 21 days under BSL-3 containment and then was decontaminated and transferred to the USAMRIID histology laboratory. Samples of bone were decalcified for 48–72 h in 26% formic acid solution. All tissue samples were then trimmed, routinely processed, and embedded in paraffin. Sections of the paraffin-embedded tissues 5 μ m thick were cut for histology. The

histology slides were deparaffined, stained with hematoxylin and eosin (H&E), and coverslipped.

2.10. Statistical analysis

Survival rates were compared by one-tailed Fisher exact tests with stepdown Bonferroni correction for multiple comparisons. Kaplan Meier survival analysis was used to construct survival curves. Repeated measures analysis of variance (RM-ANOVA) was used to compare temperature and weight over time between groups. Whole blood viral load data did not meet assumptions of normality and homogeneity of variance and were analyzed using Kruskal-Wallis and Wilcoxon rank-sum tests. All analyses were two-tailed except the Fisher test. Analyses were conducted using SAS Version 9.1.3.

3. Results

3.1. Effects of oral ST-246 treatment on disease progress and mortality of rabbits exposed to aerosolized RPXV

Six groups of rabbits were exposed to targeted dose of 2000 PFU of aerosolized RPXV per rabbit on day 0, then treated with vehicle or 40 mg/kg ST-246 via oral gavage once a day for 14 days starting on days 0 (1 h after virus exposure), 1, 2, 3, or 4. The dose of 40 mg/kg of ST-246 was selected based upon body surface area calculations to provide a dose equivalent to those that protected mice and non-human primates from lethal poxviral infection (Yang et al., 2005; personal communication – John Huggins-USAMRIID). The average calculated dose that was presented to the rabbits was 2860 PFU/rabbit (range 1140–5000 PFU/per rabbit). In our previous studies, we found that aerosolized RPXV at this dose caused 100% mortality in exposed rabbits (A. Nalca, unpublished data).

Vehicle-only treatments (group 6) started on day 0 (1 h after virus exposure) and continued once a day until the rabbits died or were euthanized due to rabbitpox. Animals in this group started to show signs of the disease on day 3 with fever reaching a mean of 40.9 °C. Furthermore, facial and cervical area edema was observed in these rabbits at this time. Anorexia and weakness were followed by weight loss and dehydration. Purulent ocular and nasal discharges, accompanied by dyspnea, were common signs beginning on day 5, and maculopapular lesions were also observed in the skin of several of these rabbits at this time. All of the rabbits in this group succumbed to disease or were moribund and euthanized on day 6 postexposure.

When the ST-246 treatment started on day 0, day 1 p.e. or day 2 p.e., 21 of the 22 rabbits (all except one animal from the group that started the treatment on day 1) survived the aerosolized RPXV challenge. A limited number of rabbits from these same groups showed less severe signs of RPXV disease signs such as fever, anorexia, and ocular discharge, but they were resolved in 48 h. Treatment with ST-246 significantly increased the survival rate for these groups compared to vehicle-treated group. *P* value for survival was 0.0008 when treatment started on days 0 and 2 and it was 0.0019 when treatment started on day 1 p.e. Even when oral ST-246 treatment started on day 3 p.e. or 4 p.e., survival rate increased, 67% and 33%, respectively, compared to vehicle-treated group which had 100% mortality (Fig. 1).

As seen with the control group, the groups that started treatments on day 3 or 4 showed early clinical signs of the RPXV disease on day 3 p.e. Both of these groups had high mean fever reaching 40.9 °C and 40.6 °C, respectively; however, body temperatures in the surviving rabbits from these groups returned to normal levels by day 8 p.e. or day 9 p.e. Overall there was a significant difference in percent change in temperature from day 1 to day 5 between

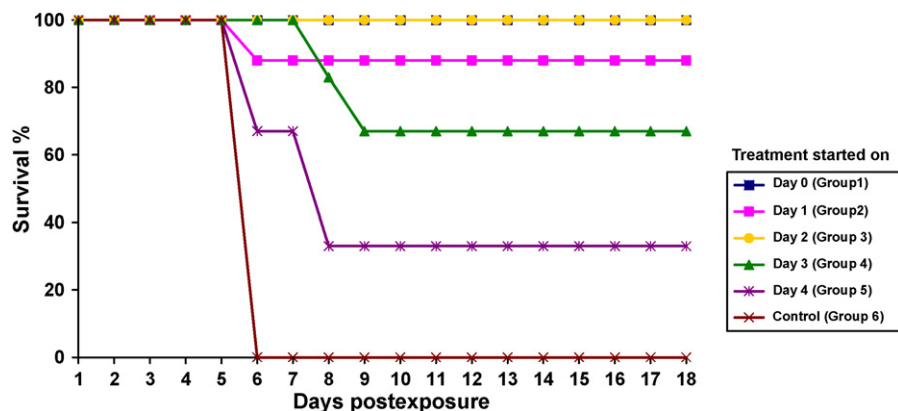


Fig. 1. The effects of ST-246 in a lethal aerosolized RPXV challenge model. Six groups of rabbits were challenged with aerosolized RPXV on day 0. Group 1 rabbits started to be treated 1 h after virus exposure with 40 mg/kg of ST-246 by oral gavage once a day for 14 days. Control group rabbits also started to be treated on same time with vehicle solution. Other groups of rabbits began treatment on day 1, day 2, day 3, and day 4 postexposure and treatment continued for 14 days.

groups ($p < 0.0001$). Post-hoc tests indicated that there were significant differences in percent change in temperature during the same time period when group 6 was compared to group 1 ($p = 0.0408$), group 2 ($p = 0.0050$) or group 3 ($p = 0.0052$) (Fig. 2a).

The weight loss was very dramatic for the groups 4, 5 and 6 but survivors from groups 4 and 5 gained weight back through the end

of treatment period (Fig. 2b). Post-hoc tests indicated that there were significant differences in % change in weight during the same time period when group 6 was compared to group 2 ($p = 0.0136$) or group 3 ($p = 0.0005$) (Fig. 2b).

3.2. Evaluation of whole blood and tissue viral load in rabbits exposed to aerosolized RPXV and then treated with ST-246

Starting the day before exposure and continuing every second day after exposure, all rabbits were bled for viral load analysis in whole blood. The rabbits that succumbed to disease were necropsied and selected tissue samples were collected for viral load determination by real-time PCR. A pan-orthopox assay was used to measure viral load in whole blood and tissues (Kulesh et al., 2004). The limit of detection (LOD) for this assay was 5000 genomes/ml.

Fig. 3a shows the geometric mean of whole blood viral load from each group of rabbits. With the exception of a single rabbit from group 2 and one from group 3, there was no detectable viral load in the whole blood samples from the first three groups (Fig. 3a). The viremic rabbit from group 2 succumbed to disease on day 6. On the other hand, the blood viral load for the rabbit from group 3 started to increase on day 4, peaked on day 6 and then dropped to below detection level by day 12. This rabbit survived to the end of the study.

The rabbits that succumbed to RPXV disease from groups 4 and 5 showed increased viral loads in whole blood compared to surviving rabbits. Overall there was significant difference in viral load between all groups at day 4 ($p = 0.0003$) and day 6 ($p < 0.0001$). Post-hoc tests showed significant differences on day 4 when group 6 was compared to group 1 ($p = 0.0165$), group 2 ($p = 0.0168$) and group 3 ($p = 0.0286$). Similarly for day 6, there were significant differences when group 6 was compared group 1 ($p = 0.0165$), group 2 ($p = 0.0168$), group 3 ($p = 0.0182$) and group 4 ($p = 0.0374$).

Fig. 3b shows the average tissue viral loads from each group of rabbits that succumbed to the disease. Although it did not completely inhibit the viral replication, ST-246 appeared to reduce the viral load in tissues other than lungs and mandibular lymph nodes for the treated groups compared to vehicle-treated control group. Sample sizes were too low to test the hypothesis.

3.3. Pathology

Animals in the vehicle-treated control group had lesions typical of those present in rabbits after exposure to a fatal dose of

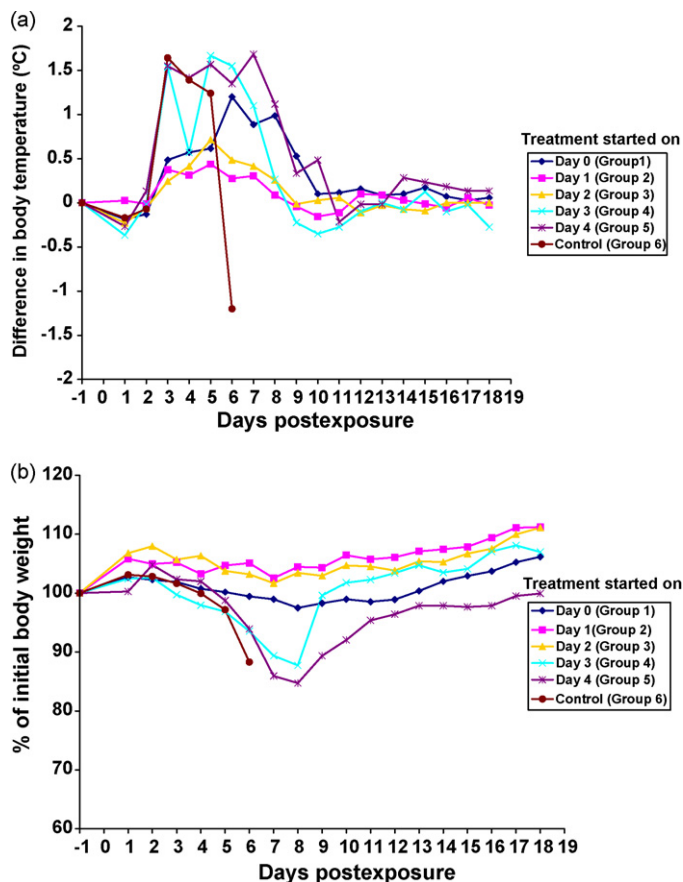


Fig. 2. The effects of 40 mg/kg/day ST-246 on weight and temperature of rabbits challenged with lethal dose of aerosolized RPXV. (A) Each rabbit was monitored daily for temperature pre- and postexposure with aerosolized RPXV. Each data point represents the mean temperature of rabbits in each group for indicated day. Groups 2 and 3 were significantly different from the controls group ($p < 0.05$). (B) Rabbits were weighed daily from day 1 to day 18. Percentage weight of surviving rabbits was calculated as the percentage of initial weight for each rabbit. Data show daily mean percentage weight for each group. Groups 1, 2 and 3 were significantly different from the controls group ($p < 0.05$).

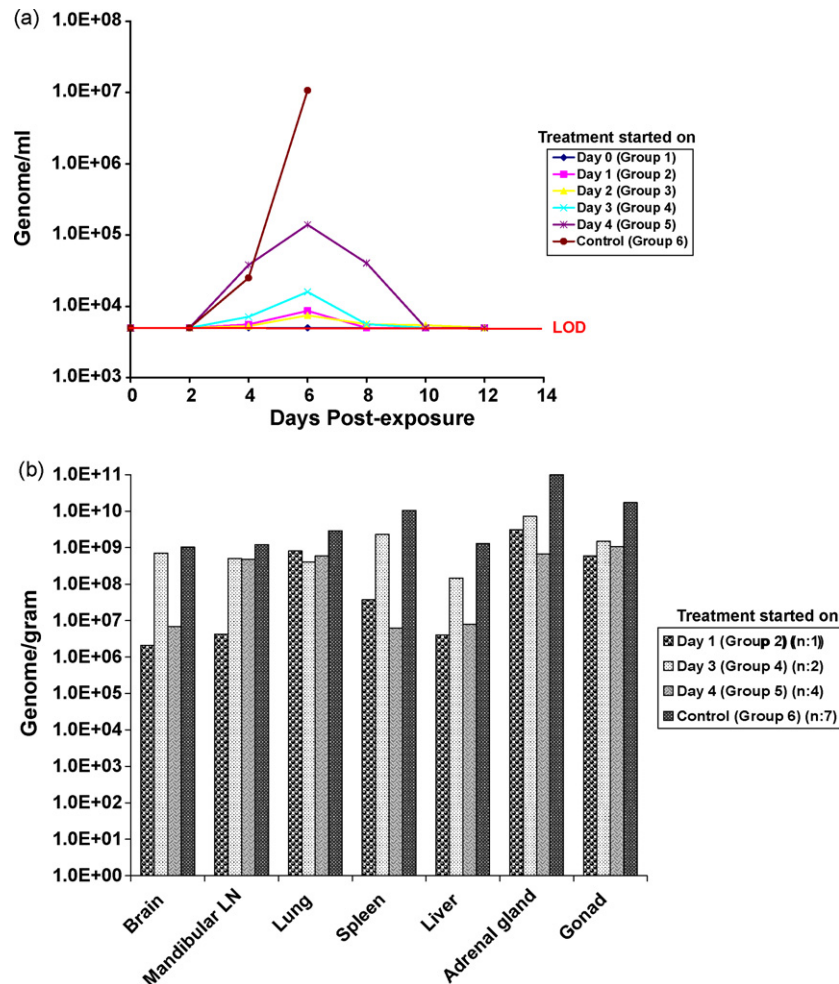


Fig. 3. The effects of 40 mg/kg/day ST-246 on whole blood viral load and tissue viral load. (A) Rabbits were bled on day 1, day 2 p.e. and then every other day through day 12 p.e. DNA was isolated from whole blood and used for real-time PCR assay as explained in Section 2. Group 1 started treatments on day 0 (1 h after virus exposure), group 2 started on day 1, group 3 started on day 2, group 4 started on day 3, group 5 started on day 4, and the control-group 6 started the vehicle treatment on day 0 (1 h after virus exposure). The graph shows the geometric mean of whole blood viral load from each group of rabbits. LOD: limit of detection. (B) Necropsy was performed on all the rabbits that succumbed to rabbitpox disease during the study. Selected tissues were collected and viral load was determined in these tissues. The graph shows the average rabbit tissue viral load from each group.

aerosolized RPXV (Nichols et al., 2006). Briefly, these lesions consisted of multifocal proliferation and/or necrosis of respiratory epithelium lining bronchi, bronchioles, and other airways, and marked acute to subacute inflammation of pulmonary blood vessels with edema and bronchopneumonia (Fig. 4a). Viral dissemination beyond the respiratory tract resulted in necrosis and inflammation in the liver, gonads, and other organs.

The four rabbits in group 5 and two rabbits in group 4 that died or were euthanized due to RPX also had lesions characteristic of the disease (as described above in the control animals). In addition, both of the rabbits in group 5 that survived to the end of the study had marked inflammation in the respiratory tract as a result of necrosis and other damage caused by the viral infection; the lesions present in one of these rabbits were so severe that the long-term survival of this animal was questionable.

Two of the four rabbits in group 4 that survived to the end of the study also had clinically significant pulmonary lesions caused by the viral infection. These consisted of scattered foci of granulomatous inflammation centered around areas of bronchial or bronchiolar necrosis (Fig. 4b). However, these lesions were not life-threatening and there was no indication of active viral infection at the time the rabbits were euthanized.

Only one of the 22 rabbits in groups 1, 2, and 3 died during this study. This rabbit was in group 2 and was viremic on day 4 and day 6 after viral challenge; it died on day 6 and had lesions typical of fatal aerosolized RPXV infection.

One of the animals in group 3 that survived to the end of the study had numerous foci of granulomatous inflammation surrounding necrotic airways in the lungs (Fig. 4c). This rabbit had clinical signs compatible with rabbitpox during days 4–9 after viral challenge and it was viremic from day 4 through day 10 postchallenge.

One of the surviving rabbits in group 2 and one animal in group 1 also had residual virus-induced lesions in their respiratory tracts (Fig. 4d). However, these rabbits were never viremic and their lesions were less severe than those present in the surviving rabbits from the other groups.

4. Discussion

The orthopoxviruses, such as VARV and monkeypox viruses, represent a significant biological threat because they can cause severe disease in humans exposed to aerosols of these agents. The discontinuation of routine smallpox vaccination of military and civilian

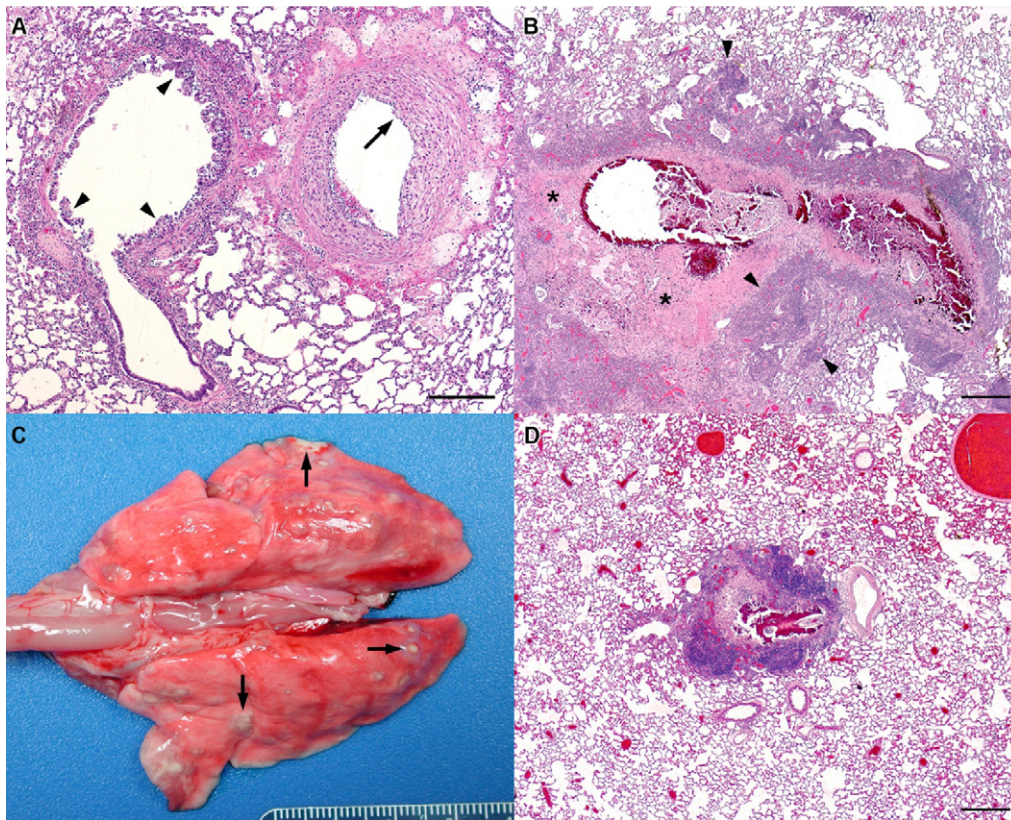


Fig. 4. Lung lesions in rabbits caused by aerosolized RPXV infection. (A) Histologic section from a control animal shows a large bronchus with an extensive area of epithelial proliferation and ballooning degeneration (arrowheads) accompanied by inflammation. An adjacent pulmonary artery (arrow) is inflamed and has perivascular inflammation and edema. Bar = 200 μ m. (B) Histologic section from one of the rabbits in group 4 that survived to the end of the study. A large necrotic bronchus is partially-mineralized (dark purple material) and surrounded by necrosis (asterisks) and a wide band of granulomatous inflammation (arrowheads). Bar = 300 μ m. (C) Rabbit 3D has multiple firm nodules scattered throughout the lung lobes; arrows point to three of the larger nodules. Histologically, these nodules closely resemble the lesion illustrated in Fig. 4B. Scale units are mm. (D) A small focus of granulomatous inflammation surrounding a necrotic and mineralized bronchiole is present in one rabbit that survived to the end of the study from group 2. Bar = 300 μ m.

populations has rendered the majority of the existing human population susceptible to disease by these viruses. Currently, there are no antiviral medications approved by the U.S. Food and Drug Administration to treat orthopoxviral infections.

Aerosolized rabbitpox in rabbits represents an important and useful model for studying orthopoxviruses, primarily because it produced a definable dose-response between route-specific exposure and disease (A. Nalca, unpublished data). This model shows disease progression similar to human smallpox (Bedson and Duckworth, 1963; Westwood et al., 1966). When rabbits are given a lethal amount of aerosolized RPXV, the virus replicates in the respiratory system primarily and then quickly spreads to other organs; infected animals typically succumb to the disease on days 6–8 p.e. (Nichols et al., 2006).

In this study, we showed that ST-246 can be used in rabbits as a p.e. prophylactic and/or antiviral therapeutic agent against an aerosolized RPXV infection. ST-246 was 100% effective in protecting rabbits from death if it was used immediately after viral challenge and it was almost as effective when treatments were initiated on day 1 or day 2 p.e.; only one of the 15 animals from these latter groups died.

While there was no detectable viral load in the blood of rabbits from group 1, one rabbit from group 2 showed a high viral load and this rabbit had succumbed to the disease by day 6 p.e. One rabbit from group 3 also was viremic; blood viral load in this animal peaked on day 6 p.e. and then dropped to below detection levels by day 12 p.e. Although this rabbit survived to the end of the study, necropsy revealed that it did have lung lesions

caused by the viral infection. The reasons that the virus was able to cause disease in these two rabbits were not determined. It is possible that viral mutation in these particular rabbits led to resistance to ST-246. Another possibility is that for some reason the drug levels in the blood of these rabbits did not reach therapeutic levels.

When ST-246 treatments were started on day 3 p.e. or 4 p.e., the rabbits were already showing signs of rabbitpox such as high fever, anorexia, and ocular discharge. Although all of the control rabbits died or were euthanized due to rabbitpox by day 6 p.e., the drug treatment of group 4 and 5 animals protected 67% and 33% of the rabbits, respectively, from death. In addition, almost all of the rabbits from groups 4 and 5 showed some level of increase in their blood viral load, which peaked on day 6, and then returned to below detection levels in surviving rabbits. These results suggest that when treatment was started on these groups, the virus was already replicating in the blood and ST-246 inhibited further replication in surviving rabbits.

ST-246 was previously reported to be efficacious *in vivo* against cowpox, vaccinia, and ectromelia viral infections in mice (Yang et al., 2005; Quenelle et al., 2007a,b), monkeypox viral infections in ground squirrels (Sbrana et al., 2007), and VARV infection in cynomolgus macaques (personal communication – John Huggins-USAMRIID). The results of this study indicated that ST-246 was also an effective antiviral drug for treating aerosolized RPXV infection in rabbits and thus presented another animal model system for *in vivo* studies with this drug. Human clinical evaluation and further animal efficacy studies are ongoing to support licensure of ST-246 for

preventing and treating diseases caused by orthopoxviruses, such as VARV and monkeypox virus.

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