EFFICACY OF THE ANTIPOXVIRUS COMPOUND ST-246 FOR TREATMENT OF SEVERE ORTHOPOXVIRUS INFECTION

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Abstract. Efficacy of the new antipoxvirus compound ST-246 was evaluated as treatment of monkeypox (MPX) virus infection in a ground squirrel model of the disease. Ground squirrels were given a lethal dose of MPX virus and were then treated orally at various times post-inoculation (pi) with 100 mg/kg/day of ST-246. Morbidity and mortality, clinical laboratory results, viral load, and pathology of placebo and treatment groups were compared. All animals that started treatment with ST-246 on days 0, 1, 2, and 3 pi survived lethal challenge with MPX virus; 67% of animals treated on day 4 pi also survived. In contrast, 100% of the placebo group died. Most of the ST-246-treated animals showed no evidence of clinical disease or alteration of baseline clinical laboratory values and had minimal histopathologic changes. These results suggest that ST-246 is a promising candidate for early treatment of severe orthopoxvirus infection.

INTRODUCTION

In May 1980, the World Health Assembly certified that the world was free of smallpox.1 However, the etiologic agent, variola virus (family Poxviridae; Orthopoxvirus), remains a potential global public health threat because of the perceived danger that it might be used as a biowarfare or bioterrorist agent.2,3 Most of the world’s human population is now susceptible to variola virus because smallpox vaccination has been discontinued except for a few high-risk groups such as first responders and medical personnel, laboratory workers involved in orthopoxvirus research, and military personnel. Furthermore, there are still no proven effective drugs for the treatment of severe orthopoxvirus infection.4 The major challenge to developing new therapeutics against smallpox is the fact that the disease no longer exists and that work with the etiologic agent is essentially prohibited. This dilemma precludes most of the usual steps required to obtain formal approval for a new therapeutic agent.

Several years ago, our group developed a small animal model of severe orthopoxvirus infection, using 13-lined ground squirrels (Spermophilus tridecemlineatus) infected with monkeypox (MPX) virus.5,6 This model was selected after evaluating other rodent species because it provided the most uniform clinical presentation and outcome, and it had many of the clinical and pathologic findings seen in severe hemorrhagic smallpox in humans.5,6 The present report describes results of studies on the efficacy of a new antiviral, SIGA ST-246, in treating MPX virus infection in the ground squirrel model.

ST-246 is a small molecular weight compound (MW = 376.33g/mole), recently developed by SIGA Technologies Inc. (Corvallis, OR), which inhibits poxvirus replication.7 This compound can be administered by mouth because of its oral bioavailability. At concentrations of 0.010 μM, ST-246 inhibits monkeypox virus replication in vitro by 50%.7 It also reportedly protects mice from vaccinia virus-induced tail lesions and from lethal challenge with ectromelia and vaccinia viruses (intranasal inoculation model).7 Our results in the ground squirrel model confirm these earlier reports and indicate that ST-246 is a promising candidate for early treatment of MPX and probably other severe orthopoxvirus infections.

MATERIALS AND METHODS

Virus. A strain of monkeypox virus, MPX-ZAI-1979-005, isolated in 1979 from a fatal human case in Zaire (now Democratic Republic of the Congo),8 was used in all studies. A virus stock was prepared from infected Vero cells and had a titer of 7.68 plaque-forming units (pfu)/mL.

Animals. The animals used in the study were wild-caught 13-lined ground squirrels (Spermophilus tridecemlineatus) obtained from TLS Research (Bloomingdale, IL). Animals were cared for in accordance with guidelines of the Committee on Care and Use of Laboratory Animal Resources, National Research Council, under an animal use protocol approved by the University of Texas Medical Branch. All work with infected animals and their tissues was carried out in biosafety level-3 facilities, by persons recently immunized with the Dryvax® vaccine (Wyeth Laboratories, Marietta, PA) and wearing positive-pressure high-efficiency particulate air (HEPA)–filtered respirators.

Test compounds. Two preparations were used in the studies: the formulated compound ST-246 at a concentration of 50 mg/mL suspended in 0.75% hydroxypropyl methylcellulose (HPMC) containing 1% Tween 80; and a placebo that contained HPMC and Tween 80 without the drug.

Pharmacokinetic analysis of ST-246. The ST-246 preparation was formulated, as noted above, for administration to the squirrels daily by oral gavage. An initial study was done to determine the bioavailability of ST-246 in the animals. Six ground squirrels were bled to get a baseline sample and were then given a single dose of 100 mg/kg of the ST-246 preparation by oral gavage. Subsequently, at time intervals of 1, 2, 4, 8, and 24 hours after treatment, the animals were bled from the retro-orbital sinus. Blood was immediately placed in EDTA-treated tubes to prevent coagulation. After centrifugation, the plasma was collected and shipped on dry ice to Absorption System (Exton, PA), where ST-246 plasma concentrations were determined by liquid chromatography (LC)–tandem mass spectrometry (MS/MS) using a Quatro Micro LC-MS/MS instrument (Waters Corp., Milford, MA). The LC was performed with a Luna C18 column (3-μm particle size,
on day 7 pi, two animals from the placebo group were used for immunohistochemical studies, as described previously.

**Efficacy of ST-246 administered post-exposure. Morbidity and mortality.** The ground squirrels inoculated with MPX virus and treated orally with ST-246 immediately after challenge (0 hours) or starting at 24, 48, or 72 hours pi all survived; none of these animals showed any apparent clinical disease. Sixty-seven percent of the animals in which treatment was started at 96 hours pi also survived. Two of the animals in this latter group showed signs of disease beginning on day 6 pi, but then recovered around day 15 pi and appeared well at the conclusion of the experiment. In contrast, the animals infected with MPX virus and treated with the placebo began to show signs of illness on day 4 pi, and all died between days 6 and 9 pi (Figure 1). The difference in mortality between the placebo group and groups ST-246-0h, 246-24h, 246-48h, 246-72h, and 246-96h was statistically significant. The probability of survival at 21 days was 0% for the placebo group, 100% for groups 246-0h, 246-24h, 246-48h, and 246-72h; and 67% for group 246-96h. Mean time to death was day 7 for the animals receiving placebo and day 13 for those squirrels in group

**Sample collection.** On day 7 pi, two animals from the placebo group and from each of the ST-246 treatment groups were humanely killed and necropsied to obtain blood and tissue samples for examination, as described below. These two animals were not included in calculation of survival rates. On day 21 pi, the experiment was terminated, and all survivors were humanely killed. The animals humanely killed were first exsanguinated by cardiac puncture under deep halothane anesthesia (Hydrocarbon Laboratories, River Edge, NJ). Blood was saved for subsequent hematologic, coagulation, and clinical chemistry studies and for virus assay, as described in the next sections. A necropsy was performed on each animal, and samples of liver, spleen, pancreas, kidney, adrenal gland, lung, heart, lymph nodes, and intestine were collected. Samples of these tissues were fixed in 10% neutral-buffered formaldehyde (100 mm × 2 mm; Phenomenex, Torrance, CA) at a flow rate of 0.2 mL/min with a mobile phase containing 80% acetonitrile, 19.7% water, and 0.3% acetic acid. WINNONLIN (Pharsight Inc., Mountain View, CA) was used to estimate pharmacokinetic values.

**Experimental infection and dose regimens.** A total of 59 randomly mixed, subadult and adult ground squirrels were divided into 7 groups. One group of nine animals received the placebo (see above) plus virus (100 pfu of MPX virus, which is equivalent to a 50% lethal dose [LD₅₀] of approximately 200 given subcutaneously) and is subsequently referred to as the placebo group. A second group of seven uninfected and untreated ground squirrels, designated as the normal group, was bled to obtain standard (normal) reference values for the clinical laboratory tests described below; their tissues were also used as normal controls for histopathologic analysis. The remaining five groups were designated ST-246-0h (n = 8), ST-246-24h (n = 9), ST-246-48h (n = 9), ST-246-72h (n = 9), and ST-246-96h (n = 8), were given 100 pfu of MPX virus subcutaneously and were then treated once a day with 100 mg/kg of ST-246 beginning at approximately 0, 24, 48, 72, and 96 hours post-inoculation (pi), respectively. All treatments were continued until day 14 pi. The animals were weighed daily and examined for signs of illness or death.

**Pharmacokinetic evaluation of ST-246.** The six animals treated with a single dose of 100 mg/kg of ST-246 by oral gavage all remained active and appeared well during the study and thereafter. Their clinical laboratory values remained unchanged when compared with the normal group, and no apparent toxicity was observed. Microscopic examination of their tissues, taken at necropsy one month after treatment, showed no changes in any of the major organ systems.

The LC-MS-MS analysis showed that the blood level of ST-246 reaches a maximum of approximately 15,000 ng/mL at 8 hours after treatment, and it decreases thereafter, becoming undetectable before the 24-hour post-treatment. The levels of ST-246 in blood of the ground squirrels were lower than those observed in mice, but were almost three-fold higher than those observed in non-human primates (Jordan R, unpublished data).

**Virologic and immunologic assays.** Viral load in blood was determined by plaque assay in 24-well microplate cultures of Vero cells using a double agar overlay and adding the second overlay on the fourth day. Plaques were counted on the sixth day after inoculation. The MPX virus titers were calculated as the number of pfu per milliliter of blood or per milliliter of a 10% tissue homogenate. Antibody titers were determined by complement fixation test as previously described.

**Statistical analyses.** The Kaplan-Meier method and log rank test were used to analyze survival data. The two animals per group killed on day 7 pi were not included in the survival analysis. The biochemical and hematologic results were analyzed statistically with one-way analysis of variance (ANOVA), and the level of significance was established for \( P \leq 0.05 \). The Tukey–honestly significantly different (HSD) test was used for posthoc analysis. Weight change was analyzed with repeated measures ANOVA followed by the Tukey-HSD test. Data are expressed as the mean ± SD.

**RESULTS**

**Hematologic, biochemical, and coagulation studies.** For coagulation studies, citrated blood was centrifuged at 2,500 rpm for 10 minutes at 4°C. After centrifugation, the plasma was transferred to clean tubes and analyzed on a Star-t 4 coagulation analyzer (Drew Scientific, Oxford, CT), which determined the total white blood cells (WBCs) and differential counts, neutrophils, monocytes, lymphocytes, basophils, eosinophils, red blood cells, hemoglobin level, hematocrit, and platelets. For clinical chemistry, whole blood collected in plain glass tubes was allowed to clot at room temperature for several hours and then centrifuged for 5 minutes at 2,500 rpm. Serum was transferred to clean tubes and analyzed promptly on a Prochem-V clinical chemistry analyzer (Drew Scientific), according to the manufacturer’s instructions. The following biochemical parameters were determined: serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin, serum amylase, blood urea nitrogen, and serum potassium.
ST-246-96 that died. All of the other animals survived. Likewise, there was no significant weight change among surviving animals in the five ST-246 treatment groups or in the normal group.

**Virologic and antibody data.** None of the animals in the normal group developed detectable viremia. All of the animals inoculated with MPX virus and receiving placebo had high MPX virus titers, a result that is consistent with what was previously observed. None of the squirrels challenged with MPX virus and treated with ST-246 at 0, 24, 48 and 72 hours pi developed detectable viremia at any time. The two squirrels in the ST-246-96h group (challenged with MPX virus and treated at 96 hours pi) that were humanely killed on day 7 pi had low-titer viremia (0.2 and 2.1 pfu/mL, respectively) (Table 1).

None of the animals in the placebo group or in treatment arms ST-246-0h, ST-246-24h, ST-246-48h, and ST-246-72h developed detectable antibodies to MPX virus. In contrast, the two squirrels in the ST-246-96h group that got sick and then recovered had detectable MPX virus antibody titers at the end of the experiment (day 21 pi).

Table 1 shows the mean virus titers in blood and organ (liver, lung, and spleen) samples taken from ground squirrels in the placebo and five ST-246 treatment groups on day 7 pi. The latter six groups were bled on the seventh day after virus inoculation.

The mean WBC count among surviving animals in the placebo group on day 7 was significantly elevated compared with the five ST-246 treatment groups and the normal group (Table 2). Analysis of the differential WBC counts indicated that the levels of neutrophils, lymphocytes, and monocytes in the placebo group were elevated (17,200, 10,940 and 3,130/µL, respectively) compared with the normal and five treatment groups. The number of eosinophils was in the normal range for the groups beginning treatment at 0, 24, and 48 hours pi. However, the levels in the animals receiving placebo and those treated at later time points (72 and 96 hours pi) were significantly increased. No statistically significant difference was observed among the level of basophils in the various study arms.

The hemoglobin and hematocrit levels were also significantly elevated in the placebo group. However, these animals were very ill when bled, and the higher levels may have been due to dehydration and hemoconcentration. The serum ALT and AST levels were also significantly elevated in the placebo group compared with the drug control and the 0, 24, 48, and 72 hour treatment groups (Table 2). Transaminase levels in the ST246-96h group also appeared elevated, but the increase was not statistically significant. The levels of total bilirubin were also significantly increased in the placebo group. There was no significant difference in the amylase levels between the various groups. The alkaline phosphatase levels were increased in the animals treated with ST-246 at 48 and 72 hours pi and in the placebo group.

Prothrombin time, activated partial thromboplastin time, and thrombin time were significantly elevated for the placebo group compared with times in the normal and the various treatment groups (Table 2). The levels of factor VIIa were also significantly higher in the animals receiving placebo compared with animals receiving ST-246. The time to coagulation for factor X-deficient plasma was higher in the placebo group compared with the normal and the treatment groups; therefore, the levels of factor X were significantly lower in the placebo animals compared with the others. Protein C levels were likewise undetectable in the animals receiving placebo. The percentages of protein C were different among the other groups. Protein C levels were likewise undetectable in the animals receiving placebo. There was not statistically significant. The levels of total bilirubin were very ill when bled, and the higher levels may have been due to dehydration and hemoconcentration. The serum ALT and AST levels were also significantly elevated in the placebo group compared with the drug control and the 0, 24, 48, and 72 hour treatment groups (Table 2). Transaminase levels in the ST246-96h group also appeared elevated, but the increase was not statistically significant. The levels of total bilirubin were also significantly increased in the placebo group. There was no significant difference in the amylase levels between the various groups. The alkaline phosphatase levels were increased in the animals treated with ST-246 at 48 and 72 hours pi and in the placebo group.

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**Histopathologic and immunohistochemical analysis.** Tissue specimens collected from animals in the normal group appeared grossly and microscopically normal and were MPX

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**Table 1**

Mean virus titers in blood and organ samples taken from ground squirrels on the seventh day after inoculation with 100 plaque-forming units of monkeypox virus*

<table>
<thead>
<tr>
<th>Tissue samples</th>
<th>Placebo</th>
<th>ST246-0h</th>
<th>ST246-24h</th>
<th>ST246-48h</th>
<th>ST246-72h</th>
<th>ST246-96h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood</td>
<td>4.9 ± 1.63</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1.2 ± 1.34*</td>
</tr>
<tr>
<td>Liver</td>
<td>7.3 ± 0.42</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2.1 ± 0.28</td>
</tr>
<tr>
<td>Lung</td>
<td>8.2 ± 0.14</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2.6 ± 0.70</td>
</tr>
<tr>
<td>Spleen</td>
<td>7.0 ± 0.56</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1.9 ± 0.42</td>
</tr>
</tbody>
</table>

* Virus titers are expressed as log<sub>10</sub> plaque-forming units/mL of blood or/mL of 10% organ homogenates. 0 = < 0.7.
antigen negative by immunohistochemical analysis. Animals in the placebo group had markedly enlarged spleens and livers and visible lung consolidation at necropsy. On microscopic examination, fibrinoid necrosis was observed in the mantle and marginal zone of the splenic lymphoid follicles (Figure 2A), liver necrosis was prominent (Figure 2B), and the lungs showed edema and hemorrhage (Figure 2C). A higher proliferation of macrophages was also seen in the spleen and the lungs of these squirrels compared with the other groups.

In contrast, organs of the animals in the normal group appeared grossly and microscopically normal (Figures 2D–F). Similarly, no remarkable changes were observed in the animals beginning treatment 0 or 24 hours after infection (Figure 2G–I), except that a proliferation of macrophages was seen in the follicular area of the spleen (Figure 2G) of some animals beginning at 48 hours pi, a few inflammatory foci were visible in the 24 hour treatment group. The group (ST246-96h) of animals that was treated beginning at 96 hours pi showed severe lymphoid depletion with fibrinoid necrosis in the spleen sections (Figure 2M), periporal hepatocellular necrosis (Figure 2N), and marked hemorrhage in the lungs, with an elevated number of macrophages per high power field (Figure 2O).

The liver sections of two animals, one killed on day 7 pi from group ST246-24h and one killed on day 21 pi from group ST246-72h, showed ballooning degeneration in the first case (day 7 pi) and centrilobular necrosis and steatosis in the second case (day 21 pi). No MPX viral antigen was detected in the tissue sections of animals from the normal group or in animals that were started on ST246 treatment at 0, 24, 48 or 72 hours pi. In contrast, animals receiving placebo showed diffuse and intense viral antigen staining in the liver, spleen, and lungs. The MPX viral antigen was also detected in the spleen, lung, and liver of the animals treated 96 hours pi (group ST246-96h). However, no viral antigen was detected in the liver sections of the two animals noted above in groups 1 (ST246-24h) and 3 (ST246-72h) that showed hepatic lesions.

**DISCUSSION**

To evaluate the novel anti-poxvirus compound SIGA ST-246, we tested its efficacy as a post-exposure therapy in ground squirrels challenged with a lethal dose (100 pfu, corresponding to approximately 200 LD50 in this model) of MPX virus. Animals in the treatment groups ST246-0h, ST246-24h, ST246-48h, and ST246-72h survived a lethal challenge with MPX virus without any evidence of clinical disease, and animals in the placebo group were all dead by the end of day 8 pi (Figure 1). In the group started on treatment 96 hours pi (ST246-96h), 67% survived. Overall, ST-246 protected 100% of the ground squirrels from clinical disease and death when treatment was started before day 4 pi.

Animals in the MPX virus-infected placebo group showed lethargy, anorexia, frequent nosebleeds, and terminal respiratory distress (mostly observed 24 hours before death), as previously described. To contrast, the clinical appearance of the treated animals in groups ST246-0h, ST246-24h, ST246-48h, and ST246-72h was normal, and most of their laboratory values remained in the normal range (Table 2). Furthermore, none of the animals in these four treatment groups had detectable virus in their blood or organs, and none of them
FIGURE 2. Photomicrographs of tissue sections taken on day 7 post-inoculation (p.i.) from ground squirrels inoculated with monkeypox virus and treated with either ST-246 or placebo. Original magnification × 20 in A–D, H, K–M, and O, and × 10 in E–G, I, J, and N.
developed antibodies to MPX virus. These results suggest that early treatment with ST-246 protected the animals from death, clinical illness, and infection.

Animals in the placebo group all died and had markedly altered laboratory values. This result is consistent with our previous observations that MPX virus-infected ground squirrels develop markedly increased WBC counts, impaired coagulation functions, and elevated levels of liver transaminases. These changes in laboratory values were not observed in the ST-246-treated animals, except in the group that began the antiviral therapy at 96 hours pi. Animals in the latter group also had detectable virus in their blood and organs, and 33% of them died. However, the virus titers observed in the ST246-96h animals were significantly lower than those seen in the placebo group, which suggested that treatment started as late as 4 days pi still had some inhibitory effect on viral replication and subsequent virus spread. The surviving animals in the ST246-96h group also had detectable MPX antibody in their convalescent sera on day 21 pi.

The pathologic changes observed in the infected animals that received the placebo were consistent with our previous findings: hepatocellular necrosis in the liver sections, lymphoid depletion and fibrinoid necrosis in the spleen, and hemorrhage and edema in the lungs. Similar histopathologic changes were also observed in some of the animals in the group (ST-246-96h) beginning treatment on day 4 pi, although they were less severe. However, they were absent or very mild in the animals treated earlier after MPX virus infection. Mild histopathologic changes were observed in a few of the animals in the ST246-48h and ST246-72h groups, but because no virus was detected in their organs or blood and they did not seroconvert, it is uncertain whether these changes were MPX virus-related.

The development of therapeutic agents for the post-exposure treatment of smallpox and other severe orthopoxvirus infections in humans is of high priority for the reasons discussed earlier. However, because of the global eradication of smallpox and prohibition on experimental work with variola virus, other closely related orthopoxvirus models must be used as substitutes. Therapeutic agents effective against the substitute could be approved by the U.S. Food and Drug Administration after testing in appropriate animal models.

We have reported the results of a series of experiments conducted to evaluate the novel compound ST-246 against lethal MPX virus infection in ground squirrels. Under our experimental conditions, all animals treated orally with 100 mg/kg of ST-246 beginning on days 0, 1, 2, or 3 pi survived a lethal challenge with MPX virus. Sixty-seven percent of the animals treated on day 4 pi also survived, and 100% of the animals receiving placebo succumbed to the infection. In conclusion, we believe that these results are promising and that ST-246 should be further investigated as an anti-orthopoxvirus candidate drug.

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Disclosure: Robert Jordan and Dennis E. Hruby are employees of SIGA Technologies, Inc., the company that produced ST-246. This statement is made in the interest of full disclosure and not because the authors consider this to be a conflict of interest.


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