Antiviral Research 97 (2013) 301–311

Contents lists available at SciVerse ScienceDirect

Antiviral Research

journal homepage: www.elsevier.com/locate/antiviral

Increased susceptibility of Cantagalo virus to the antiviral effect of ST-246®

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ARTICLE INFO

Article history:
Received 25 August 2012
Revised 26 November 2012
Accepted 28 November 2012
Available online 17 December 2012

Keywords:
Cantagalo virus
ST-246
Antiviral agent
Vaccinia virus
Poxvirus

ABSTRACT

Cantagalo virus (CTGV) is the etiologic agent of a pustular disease in dairy cows and dairy workers in Brazil with important economical and occupational impacts. Nevertheless, no antiviral therapy is currently available. ST-246 is a potent inhibitor of orthopoxvirus egress from cells and has proved its efficacy in cell culture and in animal models. In this work, we evaluated the effect of ST-246 on CTGV replication. Plaque reduction assays indicated that CTGV is 6–38 times more susceptible to the drug than VACV-WR and cowpox virus, respectively, with an EC_{50} of 0.0086 μM and a selective index of >11,600. The analysis of β-gal activity expressed by recombinant viruses in the presence of ST-246 confirmed these results. In addition, ST-246 had a greater effect on the reduction of CTGV spread in comet tail assays and on the production of extracellular virus relative to VACV-WR. Infection of mice with CTGV by tail scarification generated primary lesions at the site of scarification that appeared less severe than those induced by VACV-WR. Animals infected with CTGV and treated with ST-246 at 100 mg/kg for 5 days did not develop primary lesions and virus yields were inhibited by nearly 98%. In contrast, primary lesions induced by VACV-WR were not affected by ST-246. The analysis of F13 (p37) protein from CTGV revealed a unique substitution in residue 217 (D217N) not found in other orthopoxviruses. Construction of recombinant VACV-WR containing the D217N polymorphism did not lead to an increase in the susceptibility to ST-246. Therefore, it is still unknown why CTGV is more susceptible to the antiviral effects of ST-246 compared to VACV-WR. Nonetheless, our data demonstrates that ST-246 is a potent inhibitor of CTGV replication that should be further evaluated as a promising anti-CTGV therapy.

1. Introduction

Cantagalo virus (CTGV) was isolated during an outbreak of a pustular skin disease affecting dairy cows and milkers in the Rio de Janeiro state of Brazil. The virus was characterized as a strain of vaccinia virus (VACV; *Orthopoxvirus*; *Poxviridae*) and it shares important genotypic and phenotypic features with the smallpox vaccine used in Brazil until the late 1970s (Damaso et al., 2000). Most outbreaks of CTGV-like infections have been reported in Southeastern Brazil (Damaso et al., 2007; de Souza Trindade et al., 2003; Megid et al., 2008; Nagasse-Sugahara et al., 2004), but the spread of the disease in cattle and humans and its establishment in northern states in the Amazon biome has been described recently (Medaglia et al., 2009; Quixabeira-Santos et al., 2011).

Infected animals develop pustular lesions on the teats and udder, accompanied by fever and sometimes secondary mastitis. Dairy workers usually acquire the disease during milking activities, developing lesions on hands and arms, with associated lymphadenopathy, fever, and prostration. Generalized infections are rarely observed. Nevertheless, infected workers are incapable of working for 3–4 weeks (Moussatche et al., 2008). The economical and occupational aspects of this emerging zoonosis require attention because of the increasing number of affected animals and individuals (Damaso et al., 2007; Medaglia et al., 2009; Moussatche et al., 2008). Attack rates vary from 11% to 80% of the herd depending on the farm size and herd density. Farms with high animal density combined with poor sanitation conditions usually have the highest rates (Donatele et al., 2007; Quixabeira-Santos et al., 2011).

There is no antiviral therapy commercially available to treat CTGV-infected animals or humans. The emergence of other orthopoxvirus infections worldwide poses similar concerns, such as outbreaks of monkeypox virus in Africa and cowpox virus infections in

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http://dx.doi.org/10.1016/j.antiviral.2012.11.010

Europe (Reynolds and Damon, 2012; Vorou et al., 2008). In addition, complications following smallpox vaccination are still a problem (Golden and Hooper, 2011). Therefore, considerable efforts have been recently invested towards the search for effective anti-orthopoxvirus drugs.

ST-246® (Tecovirimat) is a synthetic, tricyclononene antiviral compound being developed to treat pathogenic orthopoxvirus infections of humans (Yang et al., 2005). ST-246 targets VACV p37, a viral palmitoylated protein encoded by VACV-Cop F13L gene and required for production of extracellular forms of virus. ST-246 prevents formation of a wrapping complex required for production of egress competent virus particles by inhibiting interaction of p37 with components of late endosomal transport vesicle biogenesis (Chen et al., 2009). The compound is orally bioavailable and protects multiple animal species from lethal orthopoxvirus challenge (Duraffour et al., 2007, 2010; Quenelle et al., 2007; Smith et al., 2009, 2011). Human clinical trials have shown that ST-246 is safe and well tolerated in healthy human volunteers with pharmacokinetic parameters consistent with once per day dosing (Jordan et al., 2008, 2010).

In the present study we have evaluated the antiviral effect of ST-246 on Cantagalo virus replication in cell culture and in mice. We show that ST-246 is more efficient at inhibiting CTGV replication *in vitro* when compared with other VACV strains and cowpox virus. In addition, ST-246 prevented the formation of lesions in mice inoculated with CTGV using the tail scarification model.

2. Materials and methods

2.1. Cells and viruses

BSC-40 cells (African green monkey kidney), RK-13 (rabbit kidney) and BHK-21 (baby hamster kidney) were propagated in monolayer cultures at 37 °C in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 5% heat-inactivated fetal bovine serum, as described (Damaso and Moussatche, 1992). Cantagalo virus reference isolate CM-01 (Damaso et al., 2000), clinical samples of CTGV isolates (Damaso et al., 2007), VACV strains IOC, Wyeth (Damaso et al., 2000), WR and cowpox virus strain Brighton Red (CPXV) were available in the laboratory's collection. Recombinant viruses, expressing the *E. coli* β-galactosidase gene under control of a VACV early/late promoter (p7.5) inserted into the thymidine kinase locus, were constructed in our laboratory (CTGV-βGal) or kindly provided by Dr. Peter Turner of the University of Florida (VACV-WR-βGal). The recombinant virus vvWR-GFP-F13L in which the GFP gene replaces the WT F13L sequence was described previously (Chen et al., 2009). Viruses were routinely propagated and titered by plaque assay in BSC-40 cells, as described (Damaso and Moussatche, 1992).

2.2. ST-246

ST-246 was synthesized and supplied by SIGA Technologies (Corvallis, OR). The drug was dissolved in DMSO and was stored at −20 °C as a 10 mM stock solution.

2.3. Plaque reduction assay and cytopathic effect (CPE) reduction assay

BSC-40, RK13 or BHK-21 monolayers (1×10^6 cells per plate) were infected with the indicated multiplicity of infection (MOI) of CTGV or other orthopoxviruses. After a 90-min adsorption period, viral inocula were removed (Time zero; 0 h), the cells were washed with phosphate buffered saline and were incubated with medium with 0.01, 0.02, 0.05, 0.1, or 0.5 μM ST-246 or 0.1% DMSO (vehicle). After 48 h post-infection, the cells were stained with 0.1% crystal violet, and the viral plaques were counted. For the 96-h assays and for experiments using recombinant VACV-WR expressing mutated F13L, 1% 2-methylcellulose was added to the medium at 0 h. The percentage of inhibition of plaque formation was calculated as follows: 100 − [(mean number of plaques in test × 100)/(mean number of plaques in control)]. The EC_{50} values (effective concentration of drug required to inhibit 50% of virus replication) were derived from the plots.

In some experiments, cytopathic effect reduction assays were conducted to measure the effective concentration of compound that inhibited 50% of the virus induced CPE. BSC-40 monolayers were seeded in 96-well plates at 1×10^4 cells per well in 180 μl of growth media. ST-246 was added directly to the assay plates at 24 concentrations (0.001–5 μM) using the HP D300 digital titration instrument (Hewlett Packard, Corvallis, OR). Cell monolayers were infected with wild-type vaccinia virus or the vaccinia virus recombinants containing the D217N amino acid substitution using an amount of virus that would cause 95% CPE at 3 days post-infection. The assay was terminated at 3 days post-infection by fixing the cells in 5% glutaraldehyde solution and the amount of CPE was visualized by staining the monolayers with 0.1% crystal violet. Virus-induced CPE were quantified by measuring absorbance at 570 nm. The EC_{50} values were calculated by fitting the data to a four-parameter logistic model to generate dose–response curve using XLfit 4.1 (IBDS, Emeryville, CA).

2.4. Visualization of viral plaques and detection of β-galactosidase activity in infected cells

Monolayers of BSC-40 cells (1×10^6 cells/well) were infected with 200 PFUs of the recombinant viruses CTGV-βGal or VACV-WR-βGal and cells were either treated with 0.01, 0.02 or 0.05 μM ST-246 or with 0.05% DMSO (control). At 48 h post-infection, the monolayers were fixed with 4% paraformaldehyde, washed twice with PBS 1× and incubated 18 h at room temperature with a solution containing 0.4 mg/ml X-Gal, 4 mM potassium ferrocyanide, 4 mM potassium ferricyanide, and 2 mM $MgCl_2$ (Sanes et al., 1986). The sites of enzyme activity were detected through the visualization of blue viral plaques.

For measurement of β-galactosidase activity, the monolayers were infected and treated with ST-246 as described above, and after 48 h the cells were processed as described (Chakrabarti et al., 1985). Cellular extracts were mixed vigorously with chloroform/SDS, and incubated with 4 mg/ml ONPG [O-nitrophenyl-B-D-galactopyranoside] until a light yellow color was developed. The samples were quantified at A_{420nm}.

2.5. Comet tail inhibition assay

BSC-40 cells grown in 6-well plates (1×10^6 cells/well) were infected with 50 PFU of CTGV or VACV-WR and either treated with 0.05% DMSO (control) or with different concentrations of ST-246. The plates were incubated tilted at a 5° angle for 3–4 days at 34.6 °C and then stained with 0.1% crystal violet. Comet tail formation in vehicle-treated group and ST-246 treated cells was compared by visual inspection.

2.6. Determination of the production of intracellular and extracellular virus

For evaluating the production of intracellular virus, BSC40 and RK-13 cells were infected at a MOI of 0.001 for 2 h. After the adsorption period, the virus inocula were removed, the cells were washed and fresh medium was added to the monolayers. After 0, 24 and 48 h post-infection, the cells were harvested in sterile water, and were submitted to three cycles of freezing and thawing.

Virus yield was determined by plaque assay in BSC-40 cells. Alternatively, in experiments to determine virus yield of recombinant VACV-WR expressing mutated F13L, an MOI of 0.1 was used and virus titers were determined after 24 h post-infection, as described above.

For analysis of extracellular virus, BSC-40 monolayers were infected with an MOI of 0.001 of CTGV or VACV-WR, and at the time of infection (0 h) the cells were incubated in the absence or presence of ST-246 at different concentrations. After 48 h, the medium was removed and centrifuged at 1000*g* for 10 min. Samples of fresh supernatant were incubated with IMV-neutralizing monoclonal antibodies directed against A28 protein kindly provided by Dr. Chwan Foo of the University of Pennsylvania (Foo et al., 2009). Antibody dilution was previously tested for neutralizing VACV-WR and CTGV. After 1 h at 37 °C, the yield of extracellular virus particles was determined in the supernatant depleted of IMV by plaque assay in BSC-40 cells. The values represent the mean of 3 independent experiments.

2.7. Infection assays in mice

Groups of female BALB/c mice ($n \geqslant 5$; 5–7 weeks of age) were anesthetized with a ketamine–xylazine mixture (100 and 6 mg/kg, respectively). Samples of purified CTGV or VACV-WR (1×10^6 PFU) diluted in 10 μl of PBS were deposited on the base of the tail, followed by scarification with a 24-gauge needle (Melamed et al., 2007). The animals were housed in filter-top microisolator cages. Treatment with different doses of ST-246 was initiated 4 h post-infection by oral gavage and continued every 24 h for 7 days. Control animals were treated with the vehicle (0.5% v/v Tween 80; 1% w/v hydroxypropylmethylcellulose) (Grosenbach et al., 2008; Yang et al., 2005). Mice were evaluated daily for clinical signs of disease. For determination of virus yield, infected mice were euthanized, and the primary lesions were removed with a blade and kept in PBS at −80 °C. The tissue was frozen and thawed twice, ground in a tissue homogenizer, and after low-speed centrifugation, the supernatant was used for determination of virus yield by plaque assay in BSC-40 cells. Protein concentration was determined in a duplicate sample. All animal experiments were performed according to the NIH Guidelines for the Care and Use of Laboratory Animals, and the protocols were approved by the Animal Ethics Committee of the Centro de Ciências da Saúde, Universidade Federal do Rio de Janeiro.

2.8. F13L sequencing

For F13L sequencing, DNA samples from CTGV and VACV-IOC were used as templates in PCR assays containing primers F13fwd Ext (5′-TAC GCA ATA TCT CAA TAG TTT CAT AAT TG-3′) and F13rev Ext (5′-ATT GAC TGA GTT AGG TTG TG ATT CG-3′) (Damaso et al., 2000). Amplicons were purified using the Illustra GFX PCR DNA and Gel Band Purification kit (GE Healthcare, São Paulo, Brazil) and sequenced by the Genomics Unit of the Instituto de Biofisica Carlos Chagas Filho-UFRJ. GenBank accession numbers for CTGV and VACV-IOC are **JX024889** and **JX024890**, respectively. Multiple alignment of the predicted amino acid sequences of F13L orthologs from different orthopoxviruses was generated by BioEdit v. 5.0.9. Virus species and Genbank accession numbers are as follows: VACV-WR (**NC_006998**); Cowpox-Brighton Red (CPXV-BR; **NC_003663**); vaccinia virus-Lister (VACV-lst; **AY678276**); VACV-MVA (**U94848**); VACV-LC16m8 (**AY678275**); VACV-Copenhagen (VACV-Cop; **M35027**); monkeypox virus-Liberia 1970 (MPXV-LBR70; **DQ011156**); horsepoxvirus MNR-76 (HSPV; **DQ792504**); variola virus-Garcia 1966 (VARV-GAR66; **Y16780**); VARV-Banglsdesh-1974 (VARV-BGL74; **DQ441422**); ectromelia virus-Naval (ECTV-NAV; (PBR, 2012)); taterapox virus (TATV; **NC_008291**); camelpox virus (CMLV; **AY009089**).

2.9. Construction of VACV recombinant virus

VACV-WR was used to construct a virus recombinant containing the D217N amino acid substitution in the F13L gene. Site directed mutagenesis was performed using the QuikChange II Site-Directed Mutagenesis Kit (Stratagene, CA) with primers F13L-D21N-F (5′-TTG GGA TAT TCT AGA AAT CTA GAT ACC GAT-3′) and F13L-D217N-R (5′-ATC GGT ATC TAG ATT TCT AGA ATA TCC CAA-3′) and plasmid pWR-F13L, that contains the F13L gene from VACV-WR cloned into plasmid pCR2.1. The DNA from the recombinant plasmid was sequenced to confirm the presence of the D217N mutation. The F13L gene containing the D217N mutation and flanking DNA was PCR amplified using a Platinum PCR SuperMix High Fidelity PCR kit (Life Technologies, OR) and recombinant plasmid DNA with primers, CB129 (5′-GCG ATA TAG CCG ATG ATA TTC-3′) and Vac3981 (5′-CAT CCA TCC AAA TAA CCC TAG-3′). The PCR assay conditions were 30 cycles at 94 °C for 20 s, 55 °C for 20 s, and 68 °C for 2 min and the resulting PCR amplicon was purified using a PCR purification kit (Qiagen, CA). BSC-40 cells were seeded into 6-well plates containing 7.5×10^4 cells/well in 2 ml of growth media and the next day were infected with 0.1 PFU/cell of vvWR-GFP-F13L which contains the GFP gene in place of the F13L coding sequences (Chen et al., 2009). Following infection, the cells were transfected with 500 ng of the PCR product encoding the mutated F13L gene using lipofectamine with Opti-MEM media (Life Technologies, OR). The next day the cells were collected by scraping into 0.5 ml PBS and lysed by repeated freeze–thaw cycles and −80 °C and 37 °C, respectively. The virus suspension was centrifuged at 1000*g* for 10 min at 4 °C to remove cell debris. The virus suspension was titered by plaque assay on fresh BSC-40 monolayers using a 1% methylcellulose overlay. Plaques identified by microscopy that did not exhibit green fluorescence were isolated and expanded in BSC-40 monolayers seeded in a 24-well plate. The virus from these plaque isolates were purified by two additional rounds of plaque isolation. The F13L gene from the final plaque isolates were amplified by PCR and sequenced to confirm the presence of the D217N amino acid change.

2.10. Statistical evaluations

Data presented in this work were expressed as mean ± SD (standard deviation). The results of one test group were compared to another group and analyzed statistically with unpaired Student's *t*-test. The results of more than two sets of measurements in one experiment were analyzed statistically by one-way analysis of variance (ANOVA) followed by Dunnett's and Tukey's Multiple Comparison Tests. A p value <0.05 was considered statistically significant. All analyses were performed using Prism v 5.01 (GraphPad Software, Inc.).

3. Results

3.1. Replication of CTGV, VACV-WR and VACV-IOC in different cell lines

Previous results from our group indicated that CTGV has an overall lower dissemination rate and yield production in cell culture when compared to other VACV strains (Damaso et al., 2000; Jesus et al., 2009a). Therefore, we first evaluated the growth rates of CTGV and two other VACV strains in two different cell lines before further testing the antiviral effect of ST-246 in these cell types. We observed that in RK-13, BSC-40 and BHK-21, CTGV produced less infectious particles than VACV-WR at 24 and 48 h post-infection ($p < 0.01$)

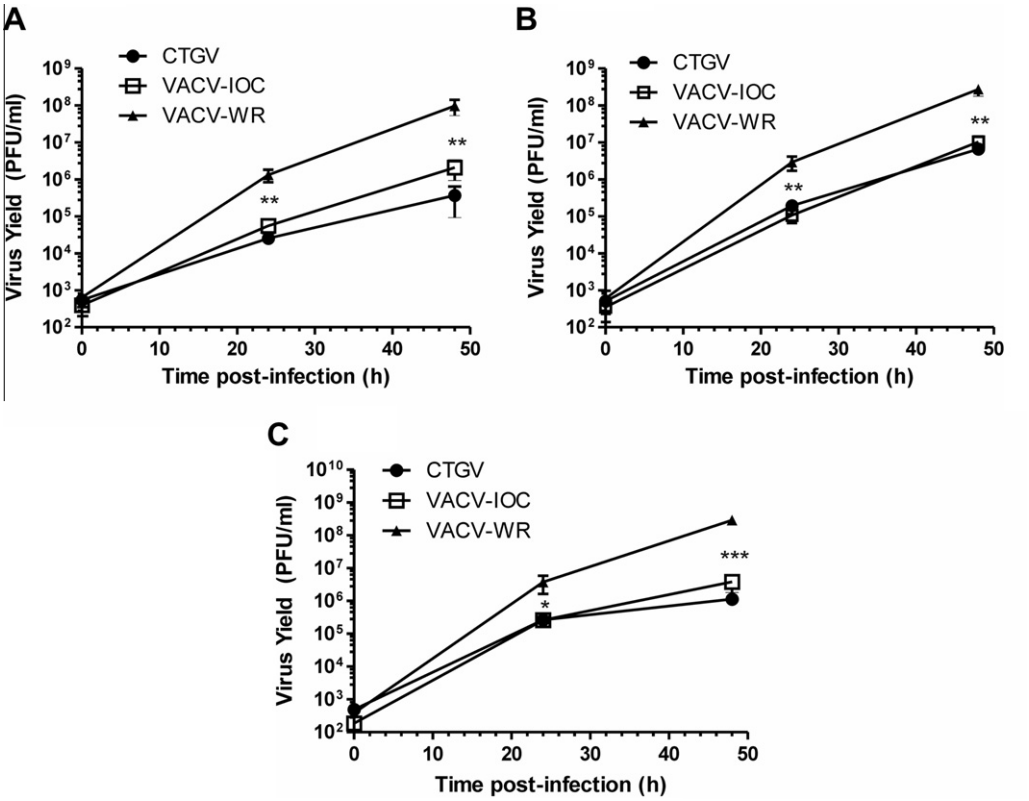

Fig. 1. Virus replication in RK-13, BSC-40 and BHK-21 cells. RK-13 (A), BSC-40 (B) and BHK-21 (C) cells were infected with CTGV, VACV-WR or VACV-IOC (MOI = 0.001). At zero, 24 and 48 h post-infection, the monolayers were harvested for virus titration in BSC-40 cells. The values (±SD) represent the mean of 3 independent assays. Asterisks: $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$ (One-way Anova followed by Donnett's and Tukey's tests) when comparing CTGV or VACV-IOC with VACV-WR. Differences in yield between CTGV and VACV-IOC were not statically significant.

(Fig. 1A–C). VACV-IOC showed similar growth kinetics as CTGV in all cell lines tested ($p > 0.05$). Despite the lower rates of replication, both CTGV and VACV-IOC were able to develop their replicative cycle and produce virus particles over the course of infection in these cells. All subsequent experiments were done in BSC-40 cells.

3.2. ST-246 inhibits CTGV replication in vitro

ST-246 has been previously evaluated for toxicity to BSC-40 cells (Yang et al., 2005). MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)-based assays confirmed that the drug was not toxic to the monolayers revealing that 97.3 ± 13.94% of the cells were viable after 48 h in the presence of 100 μM ST-246 ($p > 0.05$; Student's *t*-test) (data not shown).

To evaluate the antiviral effect of ST-246 on the replication of CTGV, we analyzed the formation of virus plaques in the presence of the drug for 48 h. As shown in Fig. 2A, ST-246 inhibited CTGV plaque formation at 48 h post-infection and this effect appeared to be more dramatic than that observed for VACV strains IOC and WR. Similar effects on plaque formation were observed at 96 h post-infection ($p < 0.001$; one-way Anova followed by Tukey's tests) or when RK-13 or BHK-21 cells were infected with these viruses ($p < 0.01$; one-way Anova followed by Tukey's tests) (data not shown). We extended the concentration range of ST-246 and included other orthopoxviruses in the assay (Fig. 2B). The antiviral effect of ST-246 was dose-dependent for all viruses tested, but CTGV was significantly more susceptible to the effect of ST-246 than other orthopoxviruses. At 0.02 μM ST-246, a 95.1% reduction of virus plaque formation was measured for CTGV whereas an inhibition of <40% in plaque formation was measured for all other orthopoxviruses at this concentration ($p < 0.001$). The EC_{50} values obtained in infected BSC-40 cells are shown in Table 1. These values confirmed that ST-246 was more potent at inhibiting CTGV replication when compared with other orthopoxviruses ($p < 0.001$). Based on the EC_{50} and CC_{50} values, the resulting selective index (SI; CC_{50}/EC_{50}) was estimated to be >11,600 for CTGV and >1800 for VACV-WR.

CTGV was isolated in 1999, and during the past decade there have been numerous reports of outbreaks of CTGV-like infections in several states of Brazil (Damaso et al., 2007; Medaglia et al., 2009; Megid et al., 2008; Nagasse-Sugahara et al., 2004). To investigate the response profile of different CTGV isolates to ST-246, we selected 15 clinical samples collected in three states of Brazil from 2000 to 2008, which were PCR-confirmed as CTGV (Damaso et al., 2007). The virus samples were tested for the formation of virus plaques in the presence of different concentrations of ST-246. As observed in Fig. 2C, similar dose–response curves were observed for all CTGV isolates ($p > 0.05$). These data confirmed the increased susceptibility of all CTGV isolates to ST-246 when compared to VACV-IOC ($p < 0.01$).

Viral plaques formed during CTGV infection at 48 h post-infection in the absence of compound were smaller than those formed by VACV-WR ($p < 0.001$; Student's *t*-test) (Fig. 2A). In the presence of ST-246, the plaque size was further reduced, consistent with reports by others (Smith et al., 2009; Yang et al., 2005). To better visualize plaque formation, we infected BSC-40 cells with recombinant CTGV and VACV-WR expressing the β-galactosidase gene under control of a viral early/late promoter in the presence of increasing concentrations of ST-246. The average plaque numbers obtained in untreated monolayers were similar between CTGV and VACV-WR infections ($p > 0.05$; Student's *t*-test). In the presence of ST-246, we observed a dramatic reduction in

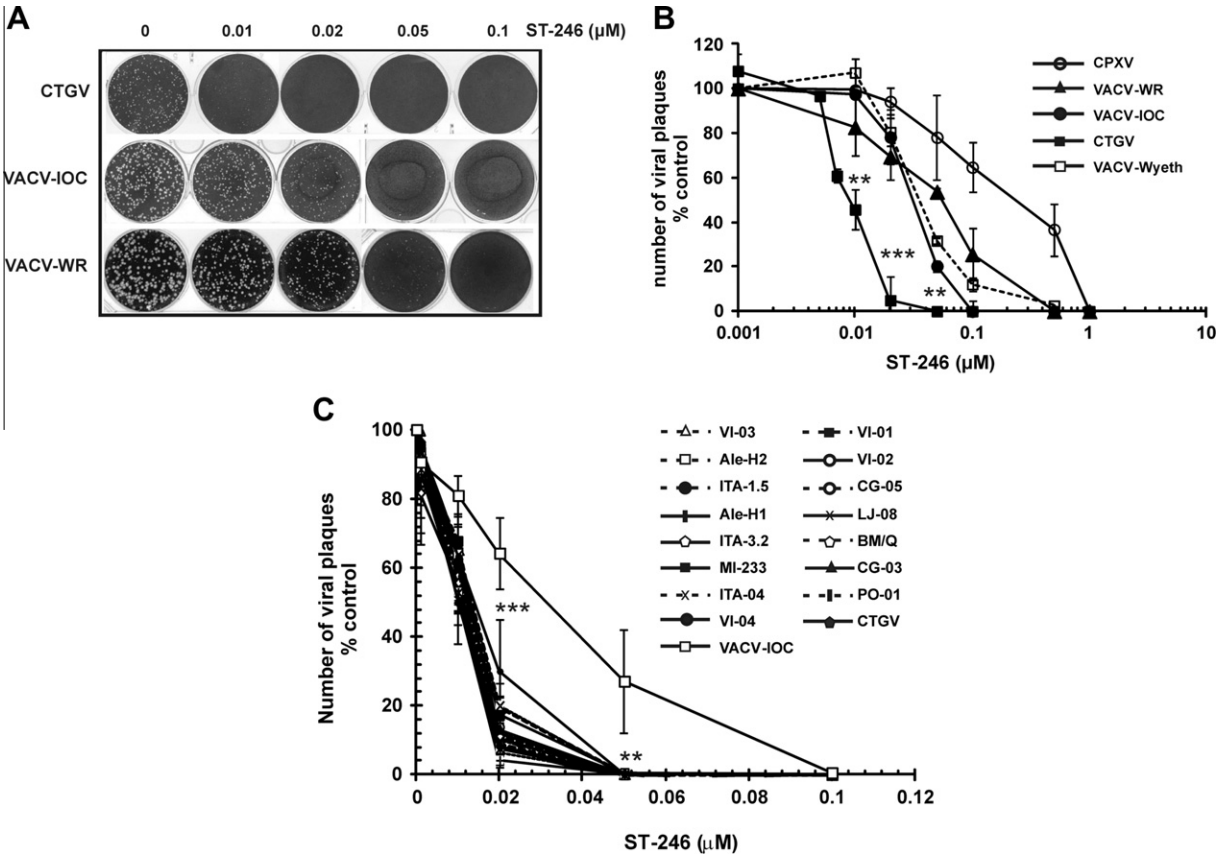

Fig. 2. Effect of ST-246 on virus plaque formation. BSC-40 cells were infected with approximately 200 PFU of CTGV, VACV-IOC or VACV-WR (A) in addition to the orthopoxviruses cowpox, VACV-Wyeth (B) or several clinical isolates of CTGV (C), in the absence or presence of the indicated concentrations of ST-246. Viral plaques were visualized by crystal violet staining after 48 h and manually counted. The values (±SD) represent the mean of at least 3 independent assays. (A) The average numbers of viral plaques in untreated cells were 264 ± 38.5 for VACV-WR, 244.8 ± 46.84 for VACV-IOC and 215.28 ± 56.60 for CTGV ($p > 0.05$; one-way Anova followed by Tukey's tests). (B and C) Asterisks: $^{**}p < 0.01$ or $^{***}p < 0.001$ (One-way Anova followed by Donnett's and Tukey's tests) when comparing CTGV and the other orthopoxviruses tested (B) or each isolate of CTGV and VACV-IOC in the indicated concentration (C).

Table 1
EC_{50} and SI values of ST-246 for distinct orthopoxviruses.

Virus	EC_{50} (μM)[a]	CC_{50} (μM)	Selective Index (SI)[b]
Cowpox	0.33 ± 0.025	>100	>303.03
VACV-WR	0.055 ± 0.003	>100	>1,818.18
VACV-Wyeth	0.046 ± 0.002	>100	>2,173.91
VACV-IOC	0.034 ± 0.006	>100	>2,941.17
CTGV	0.0086 ± 0.001[c]	>100	>11,627.9

[a] The EC_{50} for each virus was estimated based on values obtained in Fig. 1B ± standard deviations.
[b] The selective index for each virus was determined as the ratio CC_{50}/EC_{50}. The values represent the mean of at least 3 independent assays.
[c] $p < 0.001$ (one-way Anova followed by Donnett's and Tukey's tests) relative to other orthopoxviruses tested.

CTGV plaque size at 0.01 and 0.02 μM ($p < 0.001$; Student's tests), whereas VACV-WR plaques were only slightly affected at these concentrations ($p > 0.05$; Student's tests) (Fig. 3A). We also measured β-galactosidase activity in infected cells as a direct evidence of virus replication in the sites of plaque formation (Fig. 3B). In the presence of ST-246, the enzyme activity in CTGV-infected cells was significantly reduced compared to VACV-WR infected cells, with a maximal difference of nearly 8-fold at 0.02 μM ($p < 0.001$). Taken together these results confirmed the increased susceptibility of CTGV to ST-246 when compared with other orthopoxviruses.

3.3. ST-246 inhibits the production of extracellular CTGV

ST-246 is reported to inhibit virus egress from infected cells, reducing the production of extracellular viruses and the subsequent spread of infection (Duraffour et al., 2007; Yang et al., 2005). To evaluate the effect of ST-246 on the production of extracellular particles of CTGV, we performed a comet tail reduction assay. Comet tails are formed by secondary viral plaques generated due to the efficient spread of extracellular virus at long distances from the primary site of replication (primary plaques). In untreated cells, CTGV formed smaller comet tails compared to VACV-WR (Fig. 4A). In the presence of increasing concentrations of ST-246, comet tails were reduced for both viruses, demonstrating the clear effect of ST-246 on extracellular virus production. Nevertheless, in CTGV-infected cells, comet tails were barely detected at 0.015 μM ST-246 whereas VACV-WR still generated small comets and primary plaques at 0.05 μM. This result suggested that the production of extracellular particles in CTGV-infected cells was more susceptible to the effect of ST-246 than in cells infected with VACV-WR.

It is important to note that comet tails were visualized in CTGV-infected cells after 4 days of infection, whereas VACV-WR comets were better visualized after 3 days because of the difference in the sizes of virus plaques. Therefore, to measure the effect of ST-246 after similar period of treatment and infection, BSC-40 cells were infected in the presence of different concentrations of ST-246, and cell medium was harvested after 48 h. Medium samples were first depleted of contaminating IMV released from lysed cells

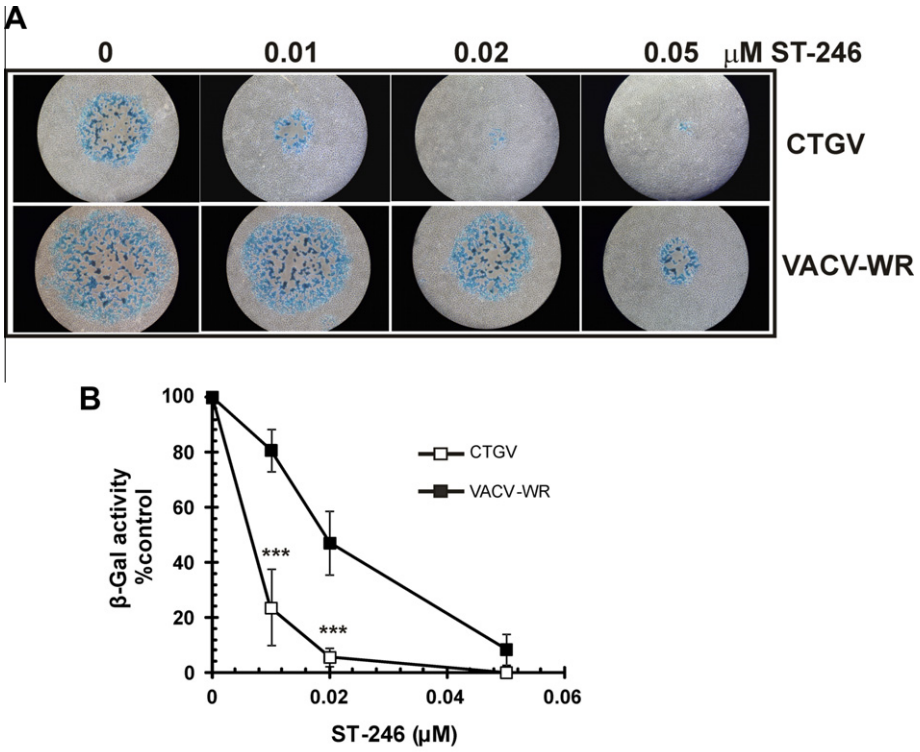

Fig. 3. Effect of ST-246 on β-galactosidase expression under control of a viral promoter. BSC-40 monolayers were infected with 200 PFU of recombinant CTGV and VACV-WR expressing β-galactosidase in the presence of the indicated concentrations of ST-246. (A) After 48 h, the cells were fixed and incubated with X-Gal for visualization of blue plaques, as described. Representative images are shown. (B) Alternatively, the monolayers were collected in PBS and processed for detection of β-galactosidase in the presence of ONPG, as described. The values (±SD) represent the mean of four independent assays. Asterisks: $p < 0.001$ (Student's *t*-tests) when comparing CTGV to VACV-WR at the indicated concentration of ST-246. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

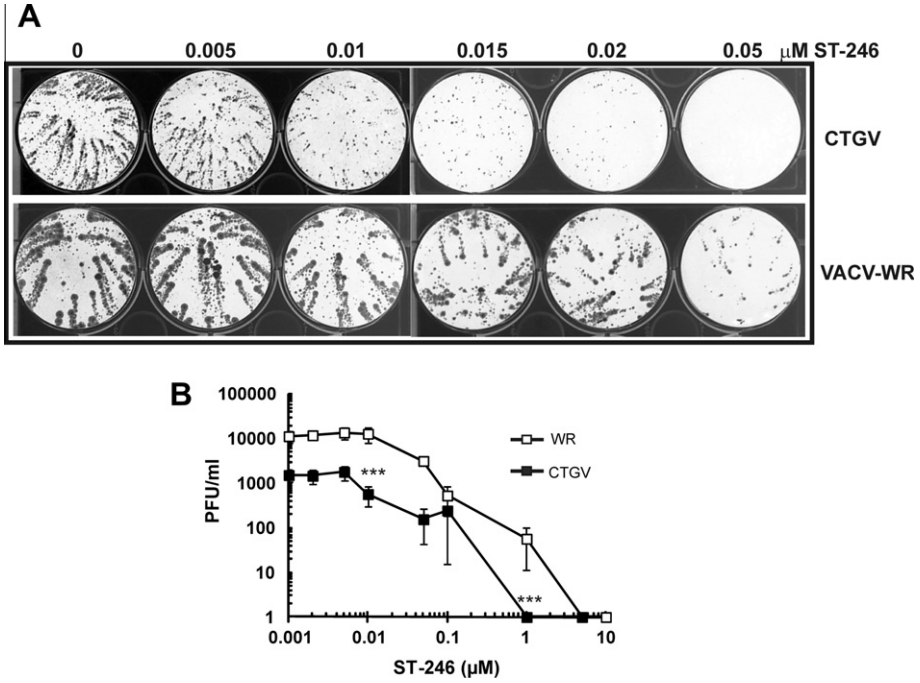

Fig. 4. Production of extracellular virus in the presence of ST-246. (A) Comet tail assay: BSC-40 cells were infected with 50 PFU of CTGV or VACV-WR in the presence of the indicated concentration of ST-246. The plates were placed under inclination of 5° during the incubation period. After 48 h, the cells were stained with crystal violet for visualization of virus plaques and comet tails. The negative image of a representative assay is shown. (B) Yields of extracellular virus: BSC-40 cells were infected with CTGV or VACV-WR (1000 PFU) either in the absence or presence of the indicated concentrations of ST-246, and the yields of extracellular virus in medium after 48 h was determined by plaque assay. The values represent the mean (±SD) of three independent assays titrated in duplicate. Asterisks: $p < 0.001$ (Student's *t*-tests) when comparing CTGV to VACV-WR at the same concentrations of ST-246.

Table 2
Data on the formation of lesions following animal infection and treatment with ST-246.

Groups	No. animals with primary lesions[a] (infection rate)	Primary lesion area ± SD (cm^2)[b]	Primary lesions[c] Appearance/ Scab fall-off	Severity of primary lesions[d]	No. animals with secondary lesions[e] (mean number of lesions ± SD)
VACV-WR vehicle	16/16 (100%)	0.42 ± 0.039	4–5 days/16–19 days	+++++	16/16 (8.19 ± 6.804)
VACV-WR 50 mg/kg ST-246	12/12 (100%)	0.41 ± 0.028	4–5 days/16–19 days	++++	0/12 (0)
VACV-WR 100 mg/kg ST-246	12/12 (100%)	0.43 ± 0.039	4–5 days/12–14 days	+++	0/12 (0)
CTGV vehicle	19/19 (100%)	0.42 ± 0.042	4–5 days/12–15 days	++	2/19 (0.16 ± 0.501)[f]
CTGV 50 mg/kg ST-246	3/12 (25%)	0.40 ± 0.025	4–5 days/9–11 days	±	0/12 (0)
CTGV 100 mg/kg ST-246	0/12 (0%)	–	–	_	0/12 (0)

[a] Number of animals that developed primary lesions/number of infected animals.
[b] The differences between scab areas were not statistically significant ($p > 0.7$; one-way Anova using Tukey's test).
[c] Days post-infection when primary lesions were first noticed and the scabs peeled off.
[d] The following clinical signs in the site of inoculation were scored to evaluate the severity of lesions: redness, swollen area, involvement of deeper tissues.
[e] Number of animals that developed secondary lesions/number of infected animals.
[f] $p < 0.0001$ (Student's test) relative to VACV-WR (vehicle).

by incubation with anti-A28 neutralizing antibody and were subsequently titrated on BSC-40 cells. As shown in Fig. 4B, ST-246 inhibited the production of infectious extracellular CTGV in a dose–response way. Extracellular yield was inhibited by nearly 64% at 0.01 μM ST-246, whereas a decrease of approximately 4% was detected for VACV-WR at this dose ($p < 0.001$). At 1 μM, the yield of extracellular CTGV dropped 3 logs when compared with a 2-log reduction for VACV-WR ($p < 0.001$).

3.4. ST-246 inhibits the formation of lesions in mice infected with CTGV

We next investigated the antiviral effect of ST-246 on the replication of CTGV *in vivo*. To determine the best route of infection for producing measurable disease in mice, we tested intravenous injection into the tail vein, intranasal inoculation and scarification on the tail. Intravenous inoculations of BALB/c mice with up 5×10^4 PFU of CTGV by the tail vein did not generate lesions on the tail in contrast to inoculation with 5×10^3 PFU of VACV-WR, which produced visible lesions by day 13 post-infection (data not shown). Similarly, intranasal inoculation of mice with 10^5 or 5×10^7 PFU of CTGV did not produce clinical signs of disease in mice such as weight loss (weight was measured daily for 21 days), ruffled fur or lethargy (Reis, Moussatche and Damaso, unpublished data) whereas intranasal inoculation of mice with 10^5 PFU of VACV-WR produced measurable clinical disease with symptoms that have been used by others to describe disease severity (Alcami and Smith, 1996; Bray et al., 2000). In addition, CTGV yields were not detected in lungs, liver or spleen of mice after intranasal infections with 10^5 PFU suggesting that virus replication was localized to the site of infection and rapidly cleared by the host (Reis, Moussatche and Damaso, unpublished data). Nevertheless, the tail scarification model produced detectable lesions at the site of inoculation with CTGV and it was selected for further evaluation of ST-246.

Mice were infected with 1×10^6 PFU of CTGV or VACV-WR by scarification of the skin on the base of the tail. At 4 h post-infection, the vehicle or 10, 25, 50 or 100 mg/kg ST-246 was administered by oral gavage. Drug treatment continued every 24 h for 7 days. Vehicle-treated animals infected with either virus developed primary lesions of similar extent on the scarified area after 4–5 days post-infection (Table 2). However, lesions resulting from VACV-WR infection were more severe and appeared to affect deeper tissues than those developed in mice infected with CTGV (Table 2), as determined by visual inspection at 7 and 9 days post-infection (Fig. 5A and F; details in Fig. 5K and M). In addition, infection with VACV-WR generated secondary lesions (satellite lesions) on the tail, which were rarely observed during CTGV infection (Table 2) (Fig. 5A and K, arrows). Treatment with different doses of ST-246 had no effect on the extent of lesion formation (Table 2) and only minor effects on the severity of primary lesions produced by VACV-WR (Fig. 5A–E). Animals administered 100 mg/kg of ST-246, had less severe lesions that resolved sooner relative to vehicle-treated animals. (Table 2) (Fig. 5A, E, K and L). Nevertheless, as indicated in Table 2, the generation of satellite lesions by VACV-WR was completely inhibited in animals treated with ST-246 (Fig. 5E; details in Fig. 5L). On the other hand, the primary lesions produced by CTGV infection were greatly reduced in severity by ST-246 treatment (Fig. 5F–J; details in Fig. 5M and N). At 25 mg/kg of ST-246, the lesions on the tail were less severe than those in vehicle-treated mice (Fig. 5H), and were not visible in animals treated with 100 mg/kg (Table 2) (Fig. 5J and N). Similar results were observed when mice were evaluated up to 20 days post-infection (data not shown). The infection of mice with 1×10^8 PFU of CTGV slightly increased the severity of the lesions, but did not produce satellite lesions on the tail (Fig. 5O). Treatment with ST-246 at 100 mg/kg also prevented primary lesion development with this elevated virus dose (Fig. 5P).

To quantify the production of virus at the site of inoculation after treatment with ST-246, the animals were euthanized at 5 days post-infection, and the primary lesions were excised and processed for virus titration. Skin areas adjacent to the primary lesion were not removed because CTGV rarely induced satellite lesions along the tail in contrast to VACV-WR infection, which produced measurable satellite lesions on the tail (Table 2). As observed in Fig. 6, CTGV yields in the primary lesion were significantly reduced after treatment with 50 and 100 mg/kg ST-246. The production of infectious particles was inhibited by 96.9 ± 9.77% and 98.4 ± 4.07%, respectively ($p < 0.001$). On the contrary, no significant effect was observed in the production of VACV-WR in the primary lesion ($p > 0.05$). This result confirmed the increased efficacy of ST-246 against CTGV infection *in vivo*.

3.5. Sequence analysis of the F13L ortholog in CTGV

The protein F13 (p37) is encoded by F13L gene and has been mapped as the target of ST-246 in distinct orthopoxviruses (Chen et al., 2009; Duraffour et al., 2008; Yang et al., 2005). Analysis of

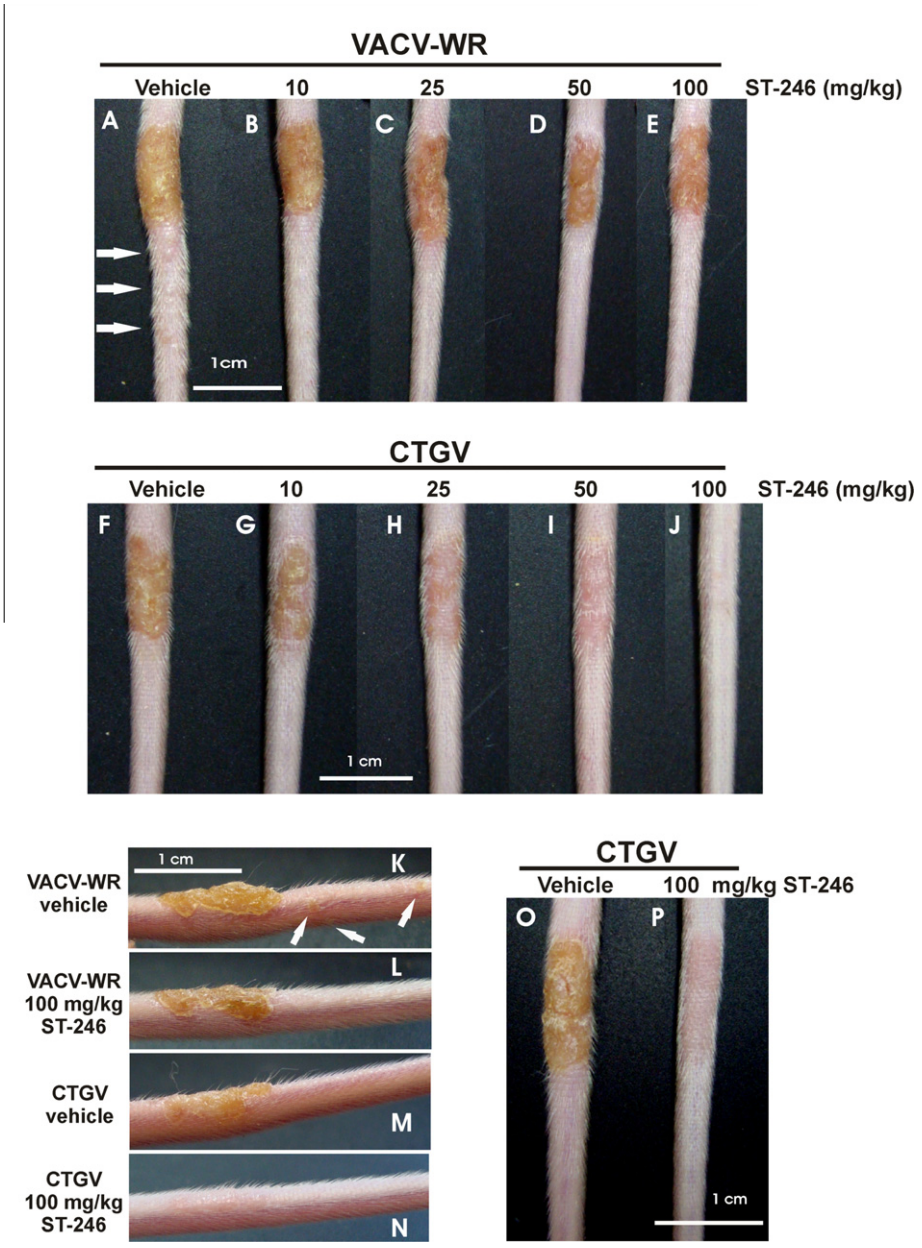

Fig. 5. ST-246 inhibits tail lesions induced by CTGV infection in mice. Female BALB/c mice ($n \geqslant 5$) were infected with 1×10^6 PFU of purified CTGV or VACV-WR (A–N) or 1×10^8 PFU of CTGV (O and P) by tail scarification. ST-246 treatment with the indicated doses was initiated 4 h post-infection by oral gavage and was repeated every 24 h for 7 days. Representative images of animals at 7 (A–J; N and O) and 9 days post-infection (K–N) are shown. Arrows point to secondary lesions.

the nucleotide sequence of F13L ortholog in CTGV revealed 4 silent mutations and 1 missense substitution, which led to the insertion of an asparagine replacing an aspartic acid in residue 217 of the protein (D217N) (Fig. 7A). Based on the predicted amino acid sequence, F13 expressed by CTGV preserved the sites of palmitoylation, the HKD phospholipase motif involved in F13 function, the YPPL motif required for efficient release of extracellular virus, and the G residue in position 277 involved in resistance to ST-246. Nevertheless, the substitution D217N was specific to F13L ortholog of CTGV and was not found in any other *Orthopoxvirus* (Fig. 7A).

To investigate whether the D217N polymorphism in F13L gene accounted for the increased susceptibility of CTGV to ST-246, recombinant VACV-WR were constructed expressing the F13 protein containing the D217N amino acid substitution. The susceptibility to ST-246 was evaluated by three different assays to measure the effects on CPE, number of viral plaques and yield in the presence of increasing concentrations of ST-246. As shown in Fig. 7B, two isolates (#B and #C) of recombinant viruses expressing mutated F13L were slightly less susceptible to ST-246 than VACV-WR expressing WT F13L by CPE-reduction assays. This was confirmed by analysis of the EC_{50} values obtained from at least three independent experiments ($p < 0.01$ for mutant #B and $p < 0.001$ for mutant #C) (Table 3). Nevertheless, analysis of virus plaque formation in the presence of ST-246 (Fig. 7C) and yield-reduction assays (Table 3) indicated that both mutant viruses and wild-type VACV-WR were equally susceptible to ST-246. Differences in the EC_{50} values for virus yield and inhibitory values for plaque number and virus yield at 0.05 μM ST-246 were not statistically significant ($p > 0.05$) (Table 3). Overall, these results suggest that the D217N

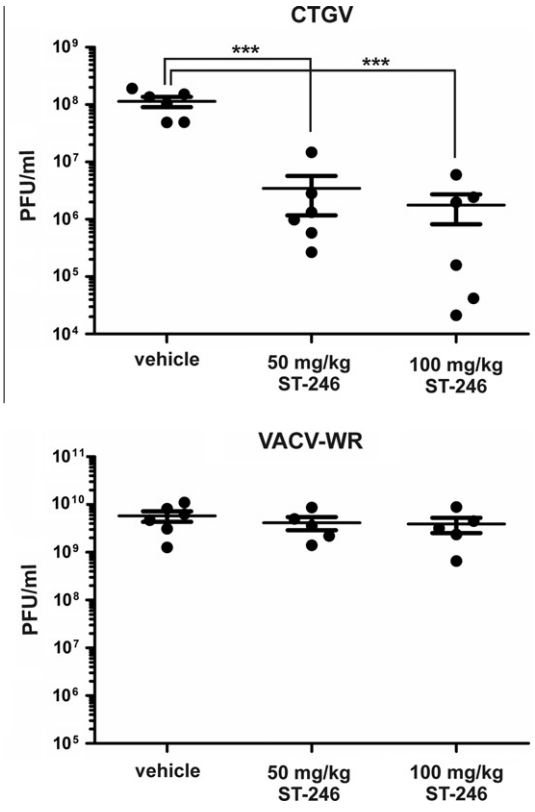

Fig. 6. Effect of ST-246 on the production of infectious particles in primary lesions after tail scarification. BALB/c mice ($n = 6$) were infected with 1×10^6 PFU of purified CTGV or VACV-WR by tail scarification. ST-246 at 50 or 100 mg/kg was administered by oral gavage for 5 days when mice were euthanized for removal of the primary lesions. Virus yields were determined by plaque assay of the tissue extracts and normalized by protein concentration. Asterisks: $p < 0.001$ (one-way Anova followed by Donnett's and Tukey's tests).

polymorphism was probably not involved in the increased susceptibility of CTGV to ST-246.

4. Discussion

The pustulovesicular disease caused by Cantagalo virus in dairy cows and dairy workers was initially detected in Rio de Janeiro state and neighboring states of Southeastern Brazil (Damaso et al., 2000, 2007; Nagasse-Sugahara et al., 2004). Recent reports show that CTGV infection has spread to distant regions, including the Amazon region, with an increasing number of human cases (Medaglia et al., 2009; Quixabeira-Santos et al., 2011). This emerging viral zoonosis has significant agricultural and occupational impacts, and the implementation of an effective antiviral therapy associated with an efficient surveillance of animal trade would certainly help to reduce these effects.

The results obtained in this study demonstrate that ST-246 has potent antiviral activity against CTGV replication. The EC_{50} values found for CTGV in plaque-reduction assays were significantly lower than the values obtained for other VACV strains and cowpox virus. Similar dose–response curves were observed for different field isolates of CTGV collected during outbreaks in different states of Brazil from 2000 to 2008, indicating that the increased susceptibility to ST-246 is a well-preserved genetic feature of this field strain of VACV. All clinical isolates share the small-plaque phenotype observed for CTGV reference isolate CM-01 (data not shown), which is clearly in line with the poor spread of CTGV infection in cell culture. This inefficient dissemination of CTGV could be evaluated not only by the reduced size of the CTGV plaques, but also by the diminished formation of comet tails during CTGV infection and lower rates of virus replication when compared with those produced by VACV-WR. Under these circumstances, production of intracellular and extracellular CTGV particles was nearly 1 log lower than VACV-WR yields.

Poor dissemination of CTGV infection was also observed *in vivo*. Tail scarification assays produced less severe primary lesions and few satellite lesions were rarely detected along the tail in contrast to the infection with VACV-WR. CTGV doses 100 times higher than those of VACV-WR did not increase virus dissemination. In these *in vivo* assays, ST-246 was clearly more effective in inhibiting CTGV replication than it was for VACV-WR. Doses of ST-246 above 25 mg/kg efficiently inhibited the dissemination of VACV-WR to secondary sites of replication on the tail (satellite lesions), but had mild effect on the severity of the primary lesions. Nevertheless, a significant reduction of the primary lesions generated by CTGV was observed in animals treated with $\geqslant$25 mg/kg ST-246. At 100 mg/kg, ST-246 prevented the formation of CTGV lesions. Titration of virus yields at the site of the primary lesions confirmed these visual observations.

F13 protein (p37) has been reported to be the target of ST-246 antiviral effect (Duraffour et al., 2008; Yang et al., 2005). This viral protein is located to the TGN/endosomal membranes and is required for the wrapping of intracellular mature virions (MVs) (Blasco and Moss, 1991; Roper and Moss, 1999). It has been shown that ST-246 prevents p37 interaction with endosomal proteins such as Tip47 and Rab9 thus blocking the formation of wrapped virus (WV) (Chen et al., 2009). F13 ortholog from CTGV has a D217N polymorphism not found in p37 from other orthopoxviruses. Nonetheless, we were not able to associate this polymorphism with the increased sensitivity of CTGV to ST-246. The EC_{50} values found for WT VACV-WR in these assays were lower than those previously obtained in Table 1 and may be related to differences in the cell culture conditions and quantification methods as well as time of ST-246 treatment. Moreover, recombinant viruses expressing p37 with D217N did not show an increase in susceptibility to the effect of the drug compared with WT virus. On the contrary, both isolates of recombinant virus were more resistant to the inhibitory effect of ST-246 on CPE-reduction assays or showed similar levels of susceptibility to the drug in yield reduction assays and virus plaque reduction assays. Therefore, the mechanisms underlying the increased susceptibility of CTGV to the effect of ST-246 are still under investigation. However, it is plausible that the increased susceptibility of CTGV to ST-246 could be related to the reduced ability of CTGV to disseminate in cell culture and in animals. Because ST-246 targets the process of virus egress and consequently, virus dissemination, viruses deficient in the process of dissemination could potentially be more affected by ST-246 in successive rounds of virus multiplication.

So far, FK-506, brequinar, cidofovir (CDV) and treazole derivatives have been the only drugs reported to present antiviral activity against CTGV (Jesus et al., 2009b; Jordao et al., 2009; Reis et al., 2006; Schnellrath and Damaso, 2011). Nevertheless, FK-506 and brequinar are immunosuppressive drugs, which is concerning. While systemic CDV administration in humans has been associated with use limiting toxicities, new oral prodrugs of CDV (CMX001) have been developed that appear safe and well tolerated in humans and active against poxvirus infections *in vivo* (Kern et al., 2002; Painter et al., 2012; Quenelle et al., 2004b).

Given the nature of CTGV infections, topical application of antiviral drugs to the teats and udders of infected cattle and use of latex gloves by workers could potentially limit spread of CTGV and reduce disease burden. Topical formulations of CDV have been used for treating cutaneous lesions caused by orthopoxviruses

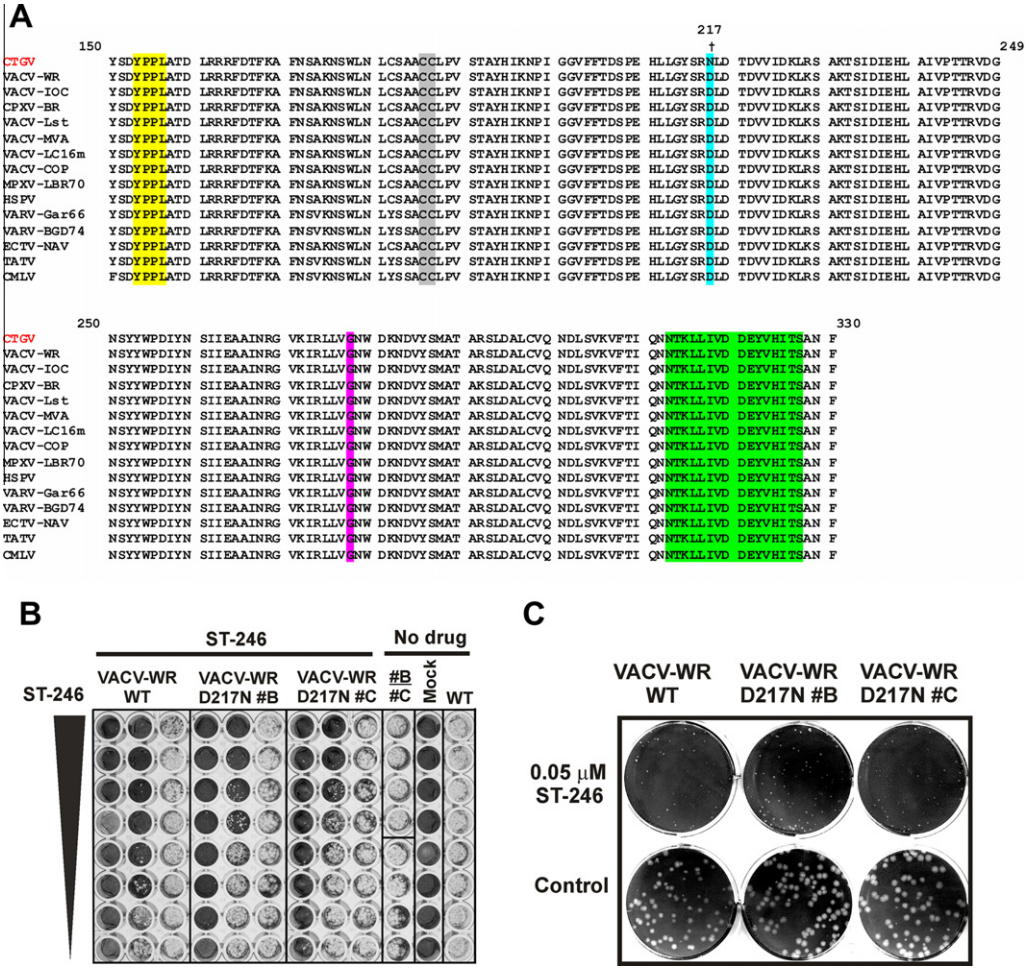

Fig. 7. D217N polymorphism in F13 is not associated with the increased sensitivity of CTGV to ST-246. (A) Multiple alignment of the partial amino acid sequences of F13 orthologs in different orthopoxviruses. The yellow box indicates the YPPL motif; the grey box indicates the palmitoylation site; the blue box indicates the D217N polymorphism present only in F13 sequence from CTGV; the pink box indicate the resistance site for ST-246; the green box indicates the KHD motif. (B) BSC-40 cells were infected with two different isolates (#B and #C) of recombinant VACV-WR expressing the D217 amino acid substitution or the wild-type (WT) VACV-WR in the presence of 24 different concentrations of ST-246 varying from 0.001 to 5 μM. After 72 h post-infection, the cells were stained with crystal violet and CPE was quantified by OD reading. Mock indicates non-infected cells. (C) BSC-40 cells were infected with 100 PFU of isolates #B and # C of VACV-WR or WT virus and were either treated with 0.05 μM ST-246 or with 0.1% DMSO (control). Plaques were stained with crystal violet after 48 h post-infection. Representative images of at least 3 independent assays are shown in (B) and (C). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 3
Effect of ST-246 on the replication of VACV-WR expressing mutated F13.

Virus	CPE-reduction assay		Yield-reduction assay			Plaque-reduction assay
	EC_{50} (μM)[a]	SI[b]	EC_{50} (μM)[a]	SI[b]	% inhibition[a,c]	% inhibition[a,c]
VACV-WR	0.033 ± 0.002	>3,030	0.013 ± 0.002	>7,692	76.34 ± 8.59	40.0 ± 7.21
VACV-WR D217N #B	0.064 ± 0.006[d]	>1,563	0.022 ± 0.012[f]	>4,545	61.97 ± 7.87 [f]	32.3 ± 12.15[f]
VACV-WR D217N #C	0.075 ± 0.038[e]	>1,333	0.021 ± 0.010[f]	>4,762	65.50 ± 1.81 [f]	20.6 ± 14.57[f]

[a] The values represent the mean of at least 3 independent assays ± standard deviations.
[b] The selective index for each virus was determined as the ratio CC_{50}/EC_{50}, where CC_{50} was estimated to be >100 μM.
[c] Values correspond to the % inhibition in the presence of 0.05 μM ST-246.
[d] $p < 0.01$ (one-way Anova followed by Donnett's and Tukey's tests) relative to control group VACV-WR.
[e] $p < 0.001$ (one-way Anova followed by Donnett's and Tukey's tests) relative to control group VACV-WR.
[f] $p > 0.05$ (one-way Anova followed by Donnett's and Tukey's tests) relative to control group VACV-WR.

(Quenelle et al., 2004a). While ST-246 has not been formulated as a topical antiviral it would be interesting to test its efficacy *in vivo* when ST-246 was applied topically on CTGV lesions alone and in combination with CDV.

Acknowledgments

This work was supported by grants from CNPq, IFS, FAPERJ, MAPA and INPeTAm. ESF, MLGM and COB were recipients of

fellowships from Capes and FAPERJ. LCS is recipient of a fellowship from CNPq. CMB, KBC, RJ and DEH are shareholders of SIGA Technologies, Inc.

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