AAC Accepts, published online ahead of print on 27 August 2007
Antimicrob. Agents Chemother. doi:10.1128/AAC.00762-07

Synergistic Efficacy of the Combination ST-246 with CMX001 against Orthopoxviruses

Running Title: Combinations of ST-246 and CMX001

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Abstract

The combination of ST-246 and hexadecyloxypropyl (HDP)-cidofovir (CDV) or CMX001 was evaluated for synergistic activity *in vitro* against vaccinia virus (VV) and cowpox virus (CV) and in vivo against CV. In cell culture the combination was highly synergistic against both viruses and the results suggested that combined treatment with these agents might offer superior efficacy in vivo. In animal models, ST-246 was administered orally with or without CMX001 to mice lethally infected with CV. Treatments began 1, 3 or 6 days post infection using lower dosages than previously used for single drug treatment. ST-246 was given at 10, 3 or 1 mg/kg with or without CMX001 at 3, 1 or 0.3 mg/kg to evaluate potential synergistic interactions. Treatment beginning 6 days post viral inoculation with ST-246 alone only increased mean day to death at 10 or 3 mg/kg, but had no effect on survival. CMX001 alone also had no effect on survival. When the combination of the two drugs was begun 6 days after viral infection using various dosages of the two, a synergistic reduction in mortality was observed. No evidence of increased toxicity was noted with the combination either in vitro or in vivo. These results indicate that combinations of ST-246 and CMX001 are synergistic both in vitro and in vivo and suggest that combination therapy using ST-246 and CMX001 for treatment of orthopoxvirus disease in humans or animals may provide an additional benefit over the use of the two drugs by themselves.

Introduction

Previous studies have shown that both ST-246 and CMX001 are effective in preventing mortality of mice infected intranasally with lethal doses of cowpox virus (CV), vaccinia virus (VV) or ectromelia virus (ECTV) (4, 20, 22, 29). While those and other preclinical studies paved

the way for each antiviral compound to move into Phase I clinical trials, evaluation of efficacy using combinations of these two agents has not been performed previously. Since these two drug candidates are the most likely ones to be used in the event of an orthopoxvirus outbreak, it is logical to assume that they might also be used in combination. The advantage to the use of the combination would be to reduce drug dosages thereby lowering the potential risk of toxicity and also to reduce the development of drug resistance. Increased potency of the combined therapy may also make delayed treatment more effective. Drug resistant viruses, such as cidofovir (CDV) resistant isolates, or intentional genetic manipulation to create drug resistant variants by bioterrorists is certainly feasible. A single point mutation in E9L polymerase can confer resistance to CDV, although the CDV resistant isolates also become less virulent in animals (1, 14, 27). Also, a single amino acid change in the VO61 gene of cowpox resulted in resistance to ST-246 (29). Genetic manipulation of orthopoxviruses may overcome vaccine induced immunity as was reported when IL-4 inserts in ectromelia became lethal to mice vaccinated against mousepox or mice genetically resistant to lethal disease (7, 10, 16, 24). An orally available combination approach to smallpox therapy which provides antivirals with differing mechanisms of action could alleviate many of these concerns and may also result in improved efficacy.

Several studies evaluating ST-246 for activity against orthopoxviruses have demonstrated excellent *in vitro* and in vivo efficacy (20, 29). When evaluated *in vitro* against VV, CV, ECTV, monkeypox, camelpox and variola viruses, ST-246 inhibited virus replication by 50% (EC_{50}) at a concentration of ≤ 0.07 µM. In animal models using lethal infections with ECTV, VV or CV, ST-246 was reported to be non-toxic and highly effective in preventing or reducing mortality, even when treatments were delayed up to 72 h post viral inoculation (20, 29). ST-246 was also evaluated in the non-lethal mouse tail lesion model using intravenous VV. When ST-246 was

administered orally twice a day at 15 or 50 mg/kg for 5 days, the tail lesions were significantly reduced (29). Most recently, an infant was given ST-246 as an FDA authorized emergency treatment for eczema vaccinatum which developed after exposure to the parent's pre-deployment military smallpox immunization (Pro-med 20070318.0947).

Several studies evaluating CMX001 for activity against orthopoxviruses have also demonstrated excellent *in vitro* and in vivo efficacy as well (4, 11, 12, 22, 28). CMX001 has entered into Phase I clinical trials based on its performance in murine and primate models of orthopoxvirus disease (17). While this compound does provide the benefit of oral bioavailability that CDV does not offer, the mechanism of action is still an inhibition of DNA polymerase. CMX001 is converted to CDV, the efficacy of which has been well established (3, 21, 26). One major advantage, however, is that CMX001 given orally does not result in the nephrotoxicity seen with CDV (5, 15).

The current studies are unique in assessing combination therapy for orthopoxvirus diseases. Delayed treatment may be the most important determining factor for the selection of antiviral compounds to pursue in light of the anticipated response time following bioterror events. If confirmed release of smallpox were to occur, detection may be accomplished in a matter of hours, but analysis of the susceptibility of the isolates to antiviral drugs may take days to determine (6, 25). Therefore, combination therapy would be highly useful to improve the likelihood of providing an effective therapeutic approach. The combination would also be expected to improve therapeutic efficacy since these compounds act by different mechanisms. The results of these additional studies using delayed combination treatment may add valuable insights into the utility of combination therapy for orthopoxvirus infections in animals and humans.

Materials and Methods

Cells and Viruses. CV, strain Brighton (CV-BR), and VV, strain Copenhagen (VV-COP), were kindly provided by John W. Huggins, Ph. D. (Department of Viral Therapeutics, Virology Division, U.S. Army Medical Research Institute of Infectious Disease, Frederick, Maryland). VV, strain WR (VV-WR), was obtained from American Type Culture Collection (ATCC, Manassas, Virginia). Stock virus pools were propagated in Vero cells that were also obtained from ATCC. Human foreskin fibroblast (HFF) cells prepared as primary cultures from freshly obtained newborn human foreskins were used in the i*n vitro* susceptibility assays for single drug evaluations performed as described previously (11).

Briefly, to determine efficacy, HFF cells seeded in 6 well plates 2 days prior to use were infected with either VV-COP, VV-WR or CV by the addition of 20-30 plaque forming units (PFU) per well. After a 1 h incubation period, various concentrations of drug were added to triplicate wells and plates incubated at 37º C for 3 days. After incubation, cell monolayers were stained with neutral red for approximately 5-6 h, viral plaques were enumerated, and the concentration which reduced viral replication by 50% (effective concentration or EC_{50}) was determined. For toxicity, the 50% cytotoxic concentration (CC_{50}) was evaluated using confluent non-dividing HFF cells seeded at 2.5 x 10^4 cells/well in 96 well plates, incubated with various concentrations of drug for 7 days at 37ºC and the cell monolayers then stained with neutral red.

In Vitro Combination Assays. Low passage (4-10) HFF cells were added to 96 well plates at a concentration of 2.5 x 10^4 cells/well in Minimal Essential Medium (MEM) containing 10% fetal bovine serum (FBS) and standard concentrations of L-glutamine, penicillin and gentamicin.

The plates were incubated for 24 h at 37ºC in a CO_2 incubator. On the day of the assay, incubation medium was aspirated and 100 µl of MEM containing 2% FBS was added to each well. Six plates (4 for antiviral and 2 for cytotoxicity evaluations) were required for each combination assay. ST-246 was prepared as a 10 ml stock at 6 times the final desired concentration. Addition and dilutions of ST-246 to the combination plates were performed using the BioMek liquid handling system. CMX001 dilutions were prepared in a separate 96 well plate and the dilutions were added to the combination plates by the BioMek. The cells were infected with either VV-Copenhagen or CV-Brighton at 1000 PFU per well for antiviral determinations or medium added to the toxicity plates. After incubation at 37ºC for 7 days, CellTiter-Glo® reagent (Promega, Madison, Wisconsin) was added directly to each well for the VV assays, and read using a Clarity luminometer to measure luminescence. For assays against CV, well contents were aspirated and the cells were stained with a neutral red solution for 1 h. The stain was aspirated and the cell monolayer washed once with PBS. Solubilizing solution (200 µl/well of 50% ETOH/1% glacial acetic acid in H_2O) was added and plate sealers applied to each plate. The plates were placed on a rotary shaker for 15 min and the optical densities read at 540 nm on a Bio-tek plate reader. Results from combination antiviral and cytotoxicity studies were evaluated using the MacSynergy II dose-effect analysis program for multiple drug interactions to determine efficacy of single versus combined antiviral treatments (19).

Mice. Female BALB/c mice, 3-4 weeks of age, were obtained from Charles River Laboratories (CRL, Raleigh, North Carolina) and were utilized in a systemic infection with CV. Mice were housed in microisolator cages and utilized at 15 mice per group. Mice were obtained, housed, utilized and euthanized according to policies of USDA and the Association for Assessment and Accreditation for Laboratory Animal Care, International. All animal procedures were approved

by the University of Alabama at Birmingham, Institutional Animal Care and Use Committee prior to the initiation of studies.

Experimental Inoculations. Systemic CV infections were initiated by intranasal (i.n.) inoculation of BALB/c mice as described previously (21). Mice were anesthetized using ketamine-xylazine and infected with an approximate LD_{90} of CV-BR (9×10^5/mouse) using a micropipettor and a total volume of 40 µl per animal.

Antiviral Treatments. Cidofovir (CDV, Vistide® Gilead Sciences, Inc., Foster City, California) was diluted in sterile saline to yield the desired dosages within a 0.1 ml volume. It was administered intraperitoneally (i.p.) once daily for 5 days as the positive control. ST-246 or 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 synthesized and supplied by SIGA Technologies (Corvallis, Oregon). It was suspended in aqueous 0.75% methylcellulose (Sigma, St. Louis, Missouri) containing 1% Tween 80 (Sigma) to yield the desired dosages of 10, 3 or 1 mg/kg within a 0.2 ml volume for CV infections. ST-246 was administered by oral gavage once daily for 5 days beginning 1, 3 or 6 days post viral inoculation. CMX001 was synthesized and supplied by Chimerix Inc. (Durham, North Carolina). It was suspended in water to yield the desired dosages of 3, 1 or 0.3 mg/kg within a 0.2 ml volume and was administered by oral gavage once daily for 5 days beginning 1, 3 or 6 days post viral inoculation. Depending on experimental protocol, antiviral compounds were either administered individually with at least a 10-11 hour interval between doses, or antiviral compounds were mixed together and administered once daily as a dual suspension.

Statistical Evaluation. Groups of mice treated with antivirals were compared to vehicle treated groups for statistical evaluation. Mortality rates were analyzed by Fisher's exact test. The mean

day of death data were analyzed by Mann-Whitney U rank sum which compares the median values non-parametrically. A p-value of 0.05 or less was considered significant.

Results

ST-246 and CMX001 synergistically inhibit orthopoxvirus replication in vitro. Both ST-246 and CMX001 are potent inhibitors of orthopoxvirus replication when used as single agents. Both drugs exhibited submicromolar EC_{50} values against VV-COP, VV-WR and CV and were relatively non toxic (**Table 1**). ST-246 and CMX001 have different mechanisms of action and were hypothesized to inhibit viral replication in a synergistic manner. The antiviral activity was determined against VV in a CellTiter-Glo® assay using combinations of these drugs and synergistic interactions were characterized by standard methods (18, 19). In this assay, both drugs were effective when used individually, and combinations of the drugs were even more effective. An initial analysis plotted the line of EC_{50} values at all of the combinations of concentrations to yield an isobologram (**Fig. 1A**). This analysis demonstrated that the addition of very low concentrations of ST-246 lowered EC_{50} of CMX001 by more than 100 fold. These interactions were also analyzed in a synergy plot (**Fig. 1B**), that identified a broad range of concentrations that resulted in statistically significant synergistic interactions and the volume of synergy produced by the combination was >300 $\mu M^2\%$ at the 95% confidence level. This effect was repeatable and represents a very strong synergistic effect. A simultaneous evaluation of cytotoxicity using these drugs did not reveal any synergistic toxicity (data not shown).

Synergistic antiviral activity against CV was evaluated in a neutral red up-take assay because the CellTiter-Glo® assay did not work as well for this virus (data not shown). The neutral red assay performed well with CV, although the variance was slightly higher than that observed using CellTiter-Glo® assays with VV. Combinations of the two drugs were also plotted

as an isobologram and the results indicated that concentrations of ST-246 above about 3.3 μM decreased the EC_{50} of CMX001 by an order of magnitude (**Fig. 1C**). These interactions were further explored with a synergy plot and identified synergistic interactions at CMX001 concentrations similar to those identified in VV, although more ST-246 was required to produce the effect (**Fig. 1D**). This analysis calculated the volume of synergy that was >100 $\mu M^2\%$ at 95% confidence. In this case, the lower volume of synergy was attributable to the increased variance of the assay and did not reflect a reduced synergistic effect against this virus. Concurrent cytotoxicity assays did not detect any synergistic toxicity and is consistent with results observed with VV.

The most intense synergistic interactions against both viruses were observed at concentrations of CMX001 ranging between 0.04 and 0.004 μM and occurred at multiple concentrations of ST-246. This effect is best illustrated by dose-response curves for ST-246 with and without the addition of CMX001. In VV infected cells, the addition of 0.01 μM CMX001 significantly improved the efficacy of ST-246 at many concentrations, even though it did not impact viral replication detectably when used individually (**Fig. 2A**). A similar effect was observed in CV infected cells where the addition of 0.04 μM CMX001 significantly improved the efficacy of ST-246, although the effect was more modest than that observed with VV (**Fig. 2B**). The effect of addition of ST-246 to CMX001 on the replication of VV (**Fig. 2C**) and CV (**Fig. 2D**) resulted in a similar but less pronounced inhibition. These data taken together suggest strongly that combinations of ST-246 and CMX001 inhibit the replication of orthopoxvirus replication in a strongly synergistic manner.

Effect of ST-246 and CMX001 combination therapy on mortality of mice inoculated with CV. Results from the *in vitro* studies demonstrated that combinations of ST-246 and

CMX001 synergistically inhibited replication of CV infections and suggested this drug combination might offer improved efficacy in animal models. This hypothesis was tested in a series of experiments in mice with a systemic lethal CV infection. In the first experiment, ST-246 was administered orally to CV-infected mice for 5 days in the mornings using 10, 3 or 1 mg/kg once daily beginning 1 day after CV inoculation. CMX001 was administered orally to CV-infected mice for 5 days in the evenings using 3, 1 or 0.3 mg/kg once daily beginning 1 day after CV inoculation. Mortality was reduced significantly ($P<0.001$) using only 5 days of treatment with ST-246 alone at the 3 mg/kg dosage (**Table 2**). Administration of 10 mg/kg did not reduce mortality significantly in this particular experiment, but the mean day of death was increased ($P = 0.001$). The lowest dose did not affect the course of infection significantly. CMX001 also significantly reduced mortality at 3 or 1 mg/kg when given as a single therapy ($P<0.001$), while the lowest dose of 0.3 mg/kg of CMX001 was ineffective in reducing mortality. Combination therapy with of ST-246 and CMX001 administered in the morning and evening, respectively, was also highly effective and significantly reduced mortality in 8 of the 9 treatment groups. Only the group receiving the lowest dose of both drugs failed to respond to the treatment. These data were encouraging since the combination appeared to be effective and no adverse reactions to the combined therapy were observed. However, the efficacy of each agent used by itself precluded the evaluation of an enhanced response with the combinations

In the next experiment, treatment was delayed until 3 days after infection in an attempt to establish conditions under which monotherapy was ineffective. We reasoned that this might improve chances of confirming improved efficacy with combined therapy. In these experiments, ST-246 and CMX001 were administered orally to CV-infected mice for 5 days at 10, 3 or 1 mg/kg once daily beginning 3 days after CV inoculation. Combined therapy was given orally

once daily as a mixed suspension. Mortality was reduced significantly (P<0.001) using only 5 days of treatment with ST-246 alone at the 10 or 3 mg/kg dosages, but not the 1 mg/kg dose (**Table 3**). A statistically significant (P<0.05) increase in the mean day of death was also achieved with at all doses of the drug. CMX001 significantly reduced mortality at 3 mg/kg when given as a single therapy, while lower doses of 1 or 0.3 mg/kg of CMX001 were not effective in reducing mortality. All doses of this drug increased the mean day to death (P=0.01). When combinations of ST-246 and CMX001 were utilized 7 of the 9 treatment groups exhibited reduced mortality (P < 0.001). The group of animals that received 1 mg/kg of both compounds was interesting since the significant reduction of mortality was not observed with the same dose of these drugs used singly. These data were intriguing since they provided the first indication that the combined therapy with these drugs might offer improved efficacy in the animal model.

To extend and confirm these results, therapy was delayed for 6 days following a lethal infection in an attempt to increase further the stringency of this model. As a control, each of the drugs alone were administered orally to CV-infected mice for 5 days at 10, 3 or 1 mg/kg once daily beginning 6 days after CV inoculation. Combined therapy was also given as an oral suspension for 5 days starting 6 days after infection. In this experiment, neither ST-246 nor CMX001 given alone significantly reduced mortality at any dose (**Table 4**). In contrast, mortality was reduced significantly in three groups treated with a combination and two of the groups were highly significant (P < 0.001). This was interesting because no mice survived when these doses of drugs were administered as single agents. The reduced mortality observed in animals given 3 mg/kg/day of ST-246 and 1 mg/kg/day of CMX001 was particularly informative. Calculations using assumptions of either Bliss independence or Loewe additivity (9) demonstrated statistically significant survival over groups receiving the drugs as single agents at three fold higher

concentrations. We conclude that combinations ST-246 and CMX001 protect mice synergistically from a lethal CV infection.

Discussion

Experience with therapy for HIV has shown the superiority of multi-drug over single drug therapy and is the standard of care. This approach should also work in therapies for other viral infections, particularly if the drugs in the combination act via different mechanisms. We applied these principles to the therapy of orthopoxvirus infections. Combinations of ST-246 and CMX001 appear to be logical candidates given their impressive preclinical efficacy when used individually and their distinctly different mechanisms of action. The results obtained in the experiments reported here clearly demonstrated that the two combined are strongly synergistic against VV and CV in vitro. This was anticipated, since CMX001 inhibits DNA polymerase resulting in reduced viral replication and ST-246 inhibits extracellular virus production through inhibition of secondary envelopment involving the major envelope protein (29). These results were encouraging and suggested that this combination might offer improved efficacy in animal models.

Previous reports describe the successful therapy of lethal CV infections with 100, 30 or 10 mg/kg/day doses of ST-246 administered 72 h following a lethal dose of CV (20). Similarly, CMX001 has been reported to be active as a single therapy at 12.5 mg/kg in BALB/c mice when administered 72 h following a lethal infection with CV (22). Results presented here, demonstrate that combinations of ST-246 and CMX001 at doses of 1 and 3 mg/kg/day are effective in reducing mortality, even if therapy is delayed 6 days after a lethal CV infection. These results are significant since the drug combination offers improved efficacy over the drugs used singly. They

also suggest that lower concentrations of the compounds could be administered with a similar therapeutic effect, and thus minimize the potential for adverse events. In this regard, no synergistic cytotoxicity was observed *in vitro* with combinations of these agents, and no adverse effects were observed in mice treated with these drugs.

Another significant advantage of combination therapy is the increased efficacy even when treatment is delayed. In response to a confirmed release, people will likely be infected for a significant period of time before treatments become distributed or vaccinations are made readily available. The combined therapy should also reduce the emergence of drug resistance and can provide a measure of protection to strains resistant to one of the agents. This first report of combined therapies using ST-246 with CMX001 will be followed by further investigations into other combinations of two or possibly three compounds *in vitro* and in vivo. Combinations of therapies which affect viral replication using different mechanisms, even those compounds which may not seem highly efficacious alone, may prove beneficial with this approach (18, 23).

Acknowledgments

This work was supported by N01-AI-30049, NO1-AI-15439, 5-UO1-AI057233 and 1 U54 AI057157-01. Excellent technical assistance was kindly provided by Deborah J. Collins, Terri L. Rice, Robin Conley and Shalisa Sanders. Earl R. Kern has an equity interest and serves as a consultant to Chimerix, Inc. The terms of this arrangement has been reviewed and approved by the University of Alabama at Birmingham in accordance with their conflict of interest policies.

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Figure 1.

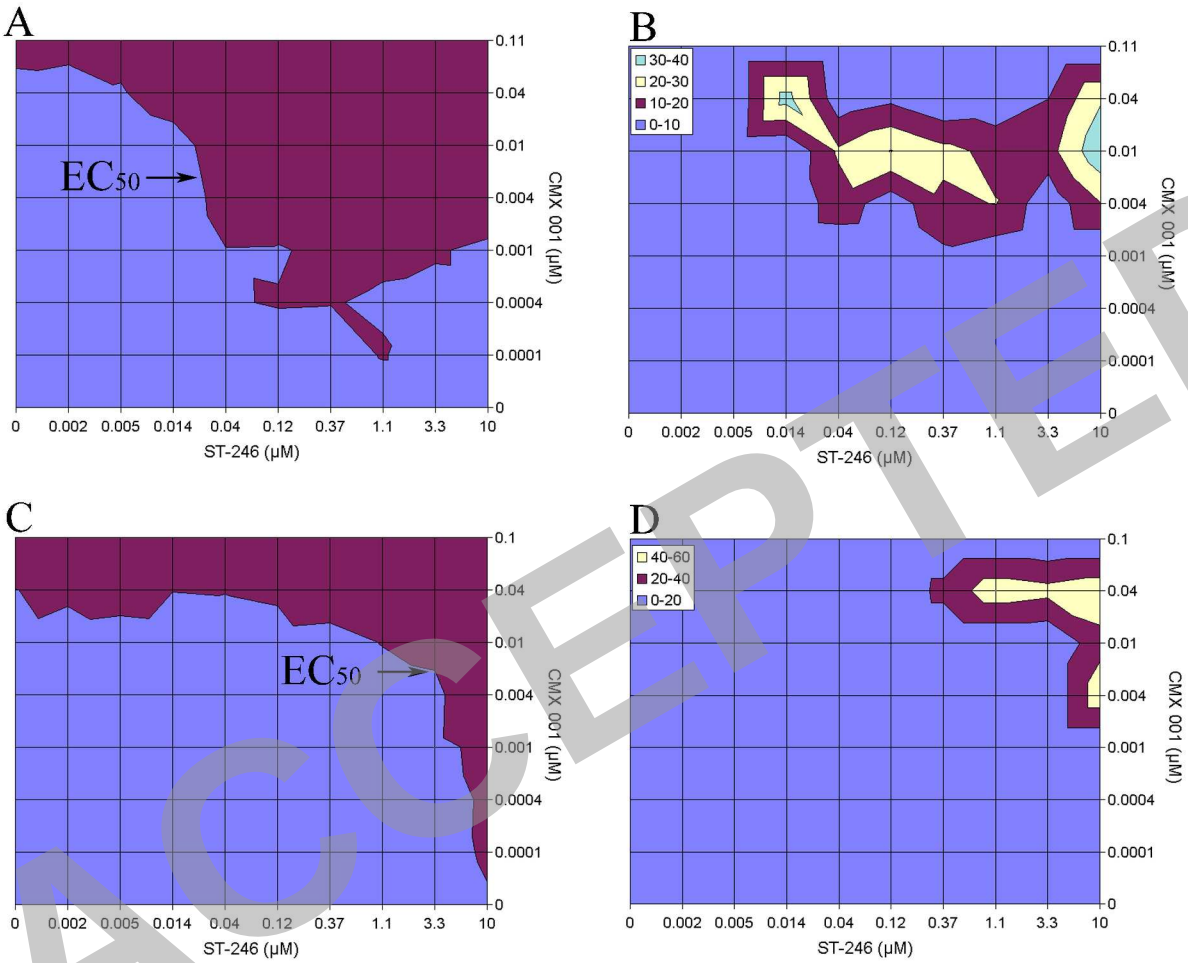

Figure 2.

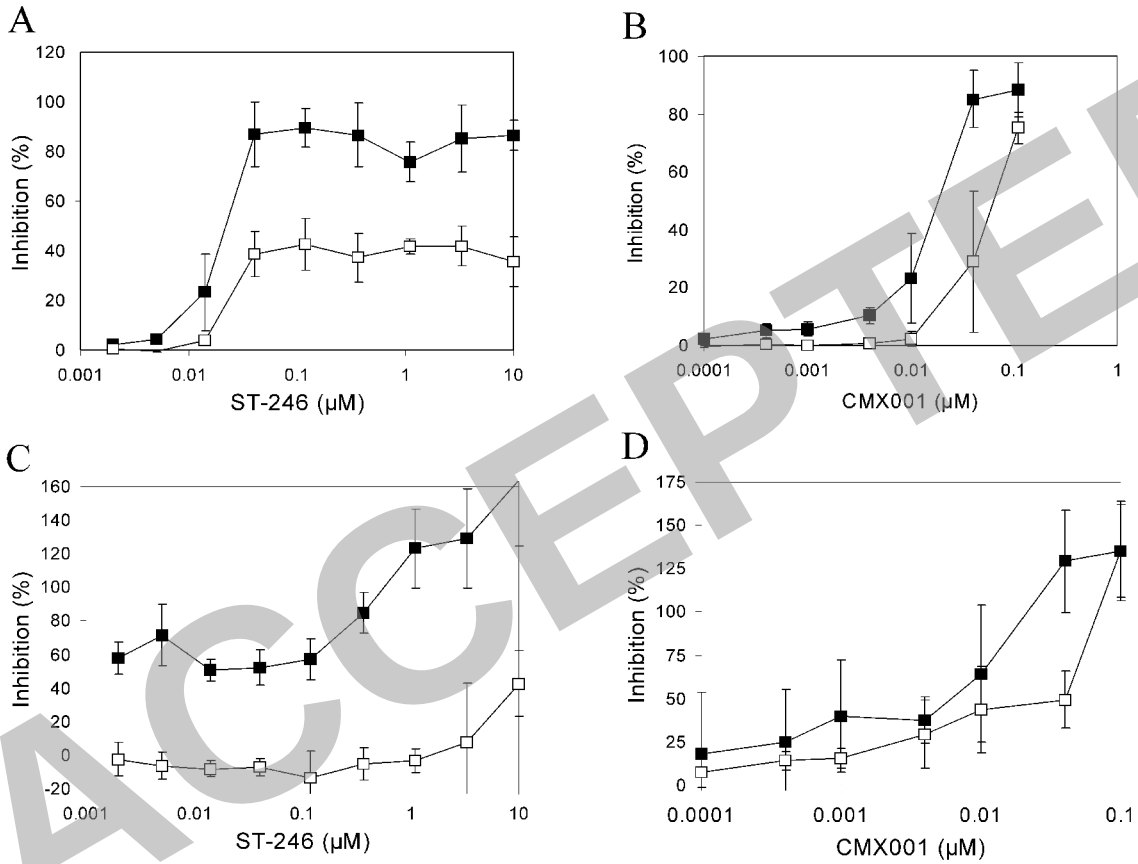

Figure Legends.

Figure 1.

Effect of combinations of CMX001 and ST-246 against vaccinia virus and cowpox virus. Inhibition of vaccinia virus replication was evaluated in a CellTiter-Glo® assay with a matrix of drug concentrations and an isobologram depicts EC_{50} values at each drug combination (A). A synergy plot (19) is also shown that represents greater than expected inhibition with increasing synergistic intensity represented by maroon, yellow and green regions, respectively (B). This analysis determined that combinations of ST-246 and CMX001 were strongly synergistic with volumes of 326 $\mu M^2\%$ at the 95% confidence level. Efficacy of this drug combination was also determined against cowpox virus in a neutral red assay and the EC_{50} isobologram is shown (C). A synergy plot also identified several combinations of concentrations where synergistic interactions occurred and are shown at the 65% confidence level (D). This analysis calculated the volume of synergy at 106 $\mu M^2\%$ at the 95% confidence level.

Figure 2.

Dose response curves for vaccinia virus or cowpox virus in the presence of ST-246 and CMX001 alone or in combination. Dose-response curves against VV are shown for (A) ST-246 alone (open symbols) and in the presence of 0.01 μM CMX001 (filled symbols) with standard deviations shown, and (B) CMX001 alone (open symbols) and in the presence of 0.014 μM ST-246 (filled symbols). Dose-response curves against CV are shown for (C) ST-246 alone (open symbols) and in the presence of 0.04 μM CMX001 (filled symbols) with standard deviations shown, and (D) CMX001 alone (open symbols) and in the presence of 3.3 μM ST-246 (filled symbols).

Table 1: Cytotoxicity and efficacy of ST-246 or CMX001 against VV or CV in human foreskin fibroblast (HFF) cells

		Vaccinia Copenhagen		Vaccinia WR		Cowpox Brighton	
Compound	CC_{50} (µM)[a]	EC_{50} (µM)[a]	SI[b]	EC_{50} (µM)[a]	SI[b]	EC_{50} (µM)[a]	SI[b]
ST-246	>100 ± 0	0.05 ± 0.02	>2000	0.1±0.05	>1000	0.48 ± 0.01	>208
CMX001	42±25	0.14 ± 0.09	300	0.13 ± 0.01	323	0.24 ± 0.1	175
CDV	>317 ± 0	29.2 ± 14	>10.9	33±13	>9.6	41.1 ± 4.2	>7.7

a. Values are the mean of 2 or more assays ± standard deviation.
b. Selectivity Index (SI) = CC_{50}/EC_{50}
CC_{50} (concentration causing cytotoxic effect on 50% of uninfected confluent cells)
EC_{50} (effective concentration that reduce plaque formation by 50%)

Table 2:Effect of Oral Combination Treatment with ST-246 and CMX001 on Mortality of BALB/c Mice Inoculated Intranasally with Cowpox, Brighton

Treatment[a]	Mortality Number	Percent	P-value	MDD[b]	P-value
Vehicle Day 1(am) + water (pm)	14/15	93	---	9.6±1.3	---
CDV Day 1 (am)					
15 mg/kg	0/15	0	<0.001	---	---
ST-246 Day 1 (am)					
10 mg/kg	11/15	73	NS	13.2±2.8	0.001
3 mg/kg	3/15	20	<0.001	11.3±1.2	0.06
1 mg/kg	15/15	100	NS	9.4±1.0	NS
CMX001 Day 1 (pm)					
3 mg/kg	0/15	0	<0.001	---	---
1 mg/kg	3/15	20	<0.001	13.7±5.5	NS
0.3 mg/kg	11/15	73	NS	11.7±2.6	0.05
ST-246 Day 1 (am) + CMX001 (pm)					
ST-246 10 mg/kg + CMX 3 mg/kg	0/15	0	<0.001	---	---
ST-246 10 mg/kg + CMX 1 mg/kg	0/15	0	<0.001	---	---
ST-246 10 mg/kg + CMX 0.3 mg/kg	4/15	27	<0.001	13.3±2.6	<0.01
ST-246 3 mg/kg + CMX 3 mg/kg	1/15	7	<0.001	7.0±00	NS
ST-246 3 mg/kg + CMX 1 mg/kg	2/15	13	<0.001	13.0±1.4	<0.05
ST-246 3 mg/kg + CMX 0.3 mg/kg	8/15	53	<0.05	13.8±4.3	0.05
ST-246 1 mg/kg + CMX 3 mg/kg	4/15	27	<0.001	11.8±3.6	NS
ST-246 1 mg/kg + CMX 1 mg/kg	7/15	47	0.01	7.1±1.4	0.001
ST-246 1 mg/kg + CMX 0.3 mg/kg	15/15	100	NS	11.1±4.3	NS

a. ST-246 was provided by SIGA Technologies in vehicle of 0.75% methylcellulose with 1% Tween 80 and given p.o. in 0.2 ml doses. CMX001 was provided by Chimerix Inc. and suspended in sterile water and given p.o. in 0.2 ml doses. CDV was prepared in sterile saline and given i.p. in 0.1 ml doses. Animals were treated daily for five days beginning 1 day after viral inoculation except for CDV which was dosed once daily as usual.

b. MDD = Mean Day of Death.

c. NS = Not significant when compared to the vehicle control.

Table 3: Effect of Oral Combination Treatment with ST-246 and CMX001 on Mortality of BALB/c Mice Inoculated Intranasally with Cowpox, Brighton

Treatment[a]	Mortality				
	Number	Percent	P-value	MDD[b]	P-value
Vehicle Day 3	15/15	100	---	9.9±0.7	---
CDV Day 3					
15 mg/kg	0/15	0	<0.001	---	---
ST-246 Day 3					
10 mg/kg	2/15	13	<0.001	15.5±2.1	<0.05
3 mg/kg	5/15	33	<0.001	15.2±3.7	<0.001
1 mg/kg	15/15	100	NS	14.4±3.4	<0.001
CMX001 Day 3					
3 mg/kg	4/15	27	<0.001	16.3±3.1	<0.01
1 mg/kg	14/14	100	NS	12.0±3.0	0.01
0.3 mg/kg	13/15	87	NS	12.8±3.4	0.01
ST-246 + CMX001 Day 3					
ST-246 10 mg/kg + CMX 3 mg/kg	0/15	0	<0.001	--	--
ST-246 10 mg/kg + CMX 1 mg/kg	0/15	0	<0.001	--	--
ST-246 10 mg/kg + CMX 0.3 mg/kg	15/15	100	NS	9.1±1.1	0.06
ST-246 3 mg/kg + CMX 3 mg/kg	0/15	0	<0.001	---	---
ST-246 3 mg/kg + CMX 1 mg/kg	4/15	27	<0.001	13.0±2.9	0.07
ST-246 3 mg/kg + CMX 0.3 mg/kg	2/15	13	<0.001	14.0±4.2	<0.05
ST-246 1 mg/kg + CMX 3 mg/kg	0/15	0	<0.001	---	---
ST-246 1 mg/kg + CMX 1 mg/kg	2/15	13	<0.001	9.5±4.9	NS
ST-246 1 mg/kg + CMX 0.3 mg/kg	15/15	100	NS	9.3±1.7	NS

a. ST-246 was provided by SIGA Technologies in vehicle of 0.75% methylcellulose with 1% Tween 80 and given p.o. in 0.2 ml doses. CMX001 was provided by Chimerix Inc. and suspended in sterile water and given p.o. in 0.2 ml doses. CMX001 was weighed and suspended in with ST-246 and given p.o. in 0.2 ml doses. CDV was prepared in sterile saline and given i.p. in 0.1 ml doses. Animals were treated once daily for five days beginning 3 days after viral inoculation.

b. MDD = Mean Day of Death.

c. NS = Not significant when compared to the vehicle control.

Table 4: Effect of Oral Combination Treatment with ST-246 and CMX001 on Mortality of BALB/c Mice Inoculated Intranasally with Cowpox, Brighton

Treatment[a]	Mortality				
	Number	Percent	P-value	MDD[b]	P-value
Vehicle Day 6	15/15	100	---	10.9±0.6	---
CDV Day 6					
25 mg/kg	12/15	80	NS	11.5±3.5	NS
15 mg/kg	9/15	60	0.01	12.8±4.1	NS
5 mg/kg	14/15	93	NS	11.2±3.2	NS
ST-246 Day 6					
10 mg/kg	15/15	100	NS	13.5±2.0	0.001
3 mg/kg	12/15	80	NS	13.5±2.4	0.001
1 mg/kg	15/15	100	NS	9.5±0.5	<0.001
CMX001 Day 6					
3 mg/kg	15/15	100	NS	9.9±0.9	0.001
1 mg/kg	15/15	100	NS	9.9±1.2	0.001
0.3 mg/kg	15/15	100	NS	10.0±0.8	<0.01
ST-246 + CMX -001 Day 6					
ST-246 10 mg/kg + CMX 3 mg/kg	1/15	7	<0.001	11.0±0	NS
ST-246 10 mg/kg + CMX 1 mg/kg	12/15	80	NS	13.3±3.7	NS
ST-246 10 mg/kg + CMX 0.3 mg/kg	15/15	100	NS	11.3±1.6	NS
ST-246 3 mg/kg + CMX 3 mg/kg	12/15	80	NS	12.4±3.9	NS
ST-246 3 mg/kg + CMX 1 mg/kg	9/15	60	0.01	11.7±2.1	NS
ST-246 3 mg/kg + CMX 0.3 mg/kg	15/15	100	NS	12.4±1.8	<0.01
ST-246 1 mg/kg + CMX 3 mg/kg	6/15	40	<0.001	11.8±1.5	NS
ST-246 1 mg/kg + CMX 1 mg/kg	15/15	100	NS	9.9±1.0	<0.01
ST-246 1 mg/kg + CMX 0.3 mg/kg	14/15	93	NS	10.5±1.3	NS

a. ST-246 was provided by SIGA Technologies in vehicle of 0.75% methylcellulose with 1% Tween 80 and given p.o. in 0.2 ml doses. CMX001 was provided by Chimerix Inc. and suspended in sterile water and given p.o. in 0.2 ml doses. CDV was prepared in sterile saline and given i.p. in 0.1 ml doses. Animals were treated daily for five days beginning 6 days after viral inoculation.
b. MDD = Mean Day of Death.
c. NS = Not significant when compared to the vehicle control.