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# Synergistic Efficacy of the Combination of ST-246 with CMX001 against Orthopoxviruses[▽]

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**The combination of ST-246 and hexadecyloxypropyl-cidofovir or CMX001 was evaluated for synergistic activity in vitro against vaccinia virus 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. For animal models, ST-246 was administered orally with or without CMX001 to mice lethally infected with CV. Treatments began 1, 3, or 6 days postinfection using lower dosages than previously used for single-drug treatment. ST-246 was given at 10, 3, or 1 mg/kg of body weight 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 the 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.**

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 by bioterrorists to create drug-resistant variants 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 interleukin 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% (50% effective concentration [$EC_{50}$]) at a concentration of $\leq 0.07$ μM. With animal models using lethal infections with ECTV, VV, or CV, ST-246 was reported to be nontoxic 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 with the nonlethal mouse tail lesion model using intravenous VV. When ST-246 was administered orally twice a day at 15 or 50 mg/kg of body weight 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 predeployment military smallpox immunization (20a).

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 been entered into phase I clinical trials based on its performance with 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).

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TABLE 1. Cytotoxicity and efficacy of ST-246 or CMX001 against VV or CV in HFF cells

| Compound | Result with virus strain[a] | | | | | |
|---|---|---|---|---|---|---|
| | VV-COP | | | VV-WR | CV-BR | |
| | $CC_{50}$ (μM) | $EC_{50}$ (μM) | SI | $EC_{50}$ (μM) SI | $EC_{50}$ (μM) | SI |
| 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 means from two or more assays ± standard deviations. SI, selectivity index ($CC_{50}/EC_{50}$); $CC_{50}$, concentration causing cytotoxic effect on 50% of uninfected confluent cells; $EC_{50}$, effective concentration that reduces plaque formation by 50%.

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 susceptibilities 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 (Department of Viral Therapeutics, Virology Division, U.S. Army Medical Research Institute of Infectious Disease, Frederick, MD). VV, strain WR (VV-WR), was obtained from American Type Culture Collection (Manassas, VA). 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 in vitro susceptibility assays for single-drug evaluations, performed as described previously (11).

Briefly, to determine efficacy, HFF cells seeded in six-well plates 2 days prior to use were infected with either VV-COP, VV-WR, or CV by the addition of 20 to 30 PFU per well. After a 1-h incubation period, various concentrations of drug were added to triplicate wells and plates incubated at 37°C for 3 days. After incubation, cell monolayers were stained with neutral red for approximately 5 to 6 h, viral plaques were enumerated, and the $EC_{50}$ was determined. For toxicity, the 50% cytotoxic concentration was evaluated using confluent nondividing HFF cells seeded at $2.5 \times 10^4$ cells/well in 96-well plates and incubated with various concentrations of drug for 7 days at 37°C; the cell monolayers were then stained with neutral red.

**In vitro combination assays.** Low-passage (4 to 10) HFF cells were added to 96-well plates at a concentration of $2.5 \times 10^4$ cells/well in minimal essential medium containing 10% fetal bovine serum and standard concentrations of L-glutamine, penicillin, and gentamicin.

The plates were incubated for 24 h at 37°C in a $CO_2$ incubator. On the day of the assay, incubation medium was aspirated and 100 μl of minimal essential medium containing 2% fetal bovine serum was added to each well. Six plates (four for antiviral efficacy and two for cytotoxicity evaluations) were required for each combination assay. ST-246 was prepared as a 10-ml stock at six times the final desired concentration. Addition and dilutions of ST-246 in 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-COP or CV-BR at 1,000 PFU per well for antiviral determinations or medium added to the toxicity plates. After incubation at 37°C for 7 days, CellTiter-Glo reagent (Promega, Madison, WI) 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 phosphate-buffered saline. Solubilizing solution (200 μl/well of 50% ethyl alcohol–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 synergy analysis program for multiple drug interactions to determine efficacies of single versus combined antiviral treatments (19).

**Mice.** Female BALB/c mice, 3 to 4 weeks of age, were obtained from Charles River Laboratories (Raleigh, NC) 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 the U.S. Department of Agriculture 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 90% lethal dose of CV-BR ($9 \times 10^5$PFU/mouse) using a micropipettor and a total volume of 40 μl per animal.

**Antiviral treatments.** CDV (Vistide Gilead Sciences, Inc., Foster City, CA) 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, OR). It was suspended in aqueous 0.75% methylcellulose (Sigma, St. Louis, MO) containing 1% Tween 80 (Sigma) to yield the desired dosage 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, NC). 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- to 11-h interval between doses or 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 using the Mann-Whitney U rank sum test, which compares the median values nonparametrically. 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 $EC_{50}$s against VV-COP, VV-WRm and CV and were relatively nontoxic (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

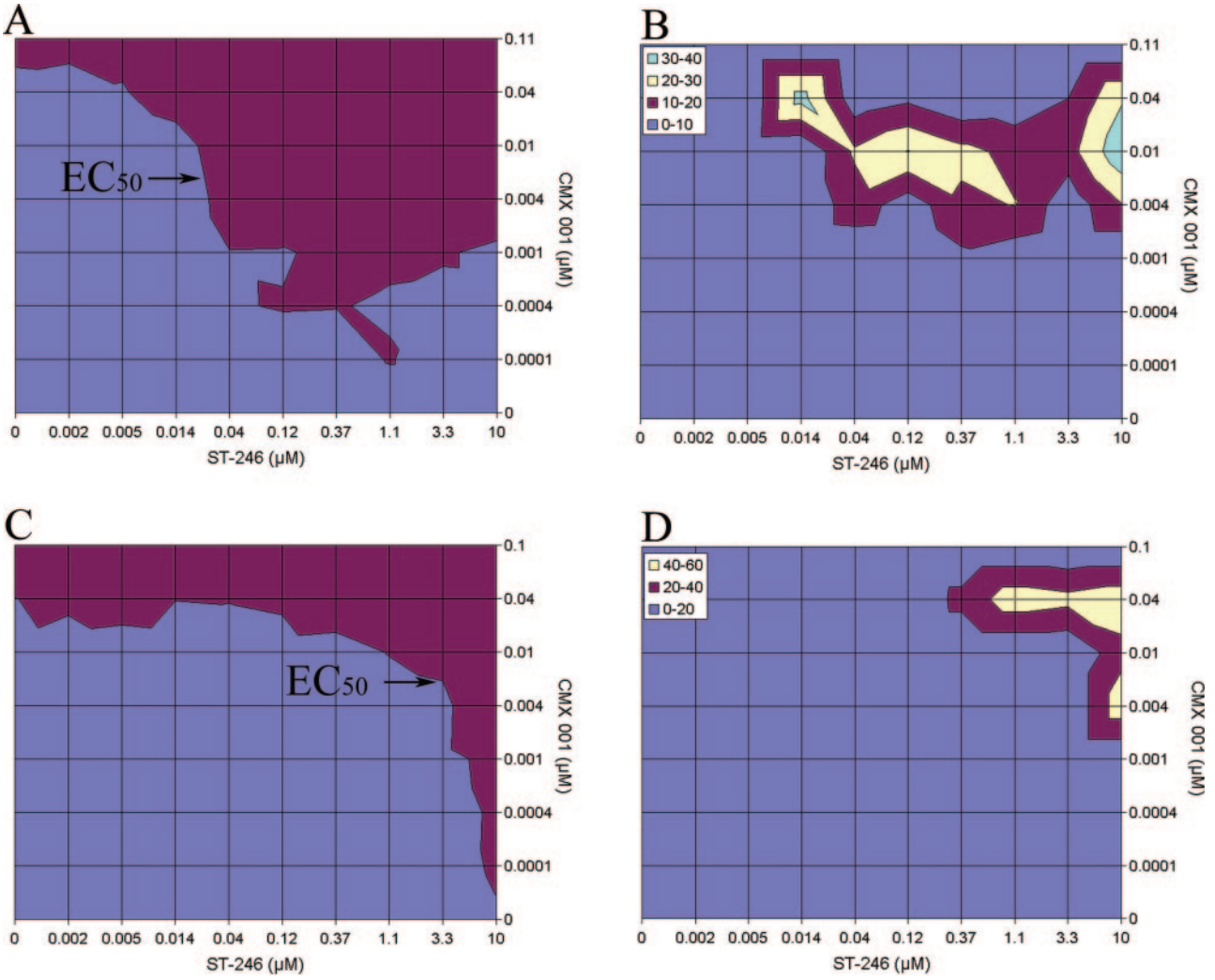


FIG. 1. Effects of combinations of CMX001 and ST-246 against vaccinia virus and cowpox virus. (A) Inhibition of vaccinia virus replication was evaluated in a CellTiter-Glo assay with a matrix of drug concentrations, and an isobologram depicts $EC_{50}$s for each drug combination. (B) A synergy plot (19) represents greater-than-expected inhibition, with increasing synergistic intensity represented by maroon, yellow, and green regions, respectively. 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. (C) Efficacy of this drug combination was also determined against cowpox virus in a neutral red assay, and the $EC_{50}$ isobologram is shown. (D) A synergy plot identified several combinations of concentrations where synergistic interactions occurred, shown at the 65% confidence level. This analysis calculated the volume of synergy at 106 $\mu M^2\%$ at the 95% confidence level.

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 $EC_{50}$s 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 the $EC_{50}$ of CMX001 by more than 100-fold. These interactions were also analyzed in a synergy plot (Fig. 1B), which 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 uptake 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 $\mu M$ decreased the $EC_{50}$ of CMX001 by an order of magnitude (Fig. 1C). These interactions were further explored with a synergy plot, identifying synergistic interactions at CMX001 concentrations similar to those identified for 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

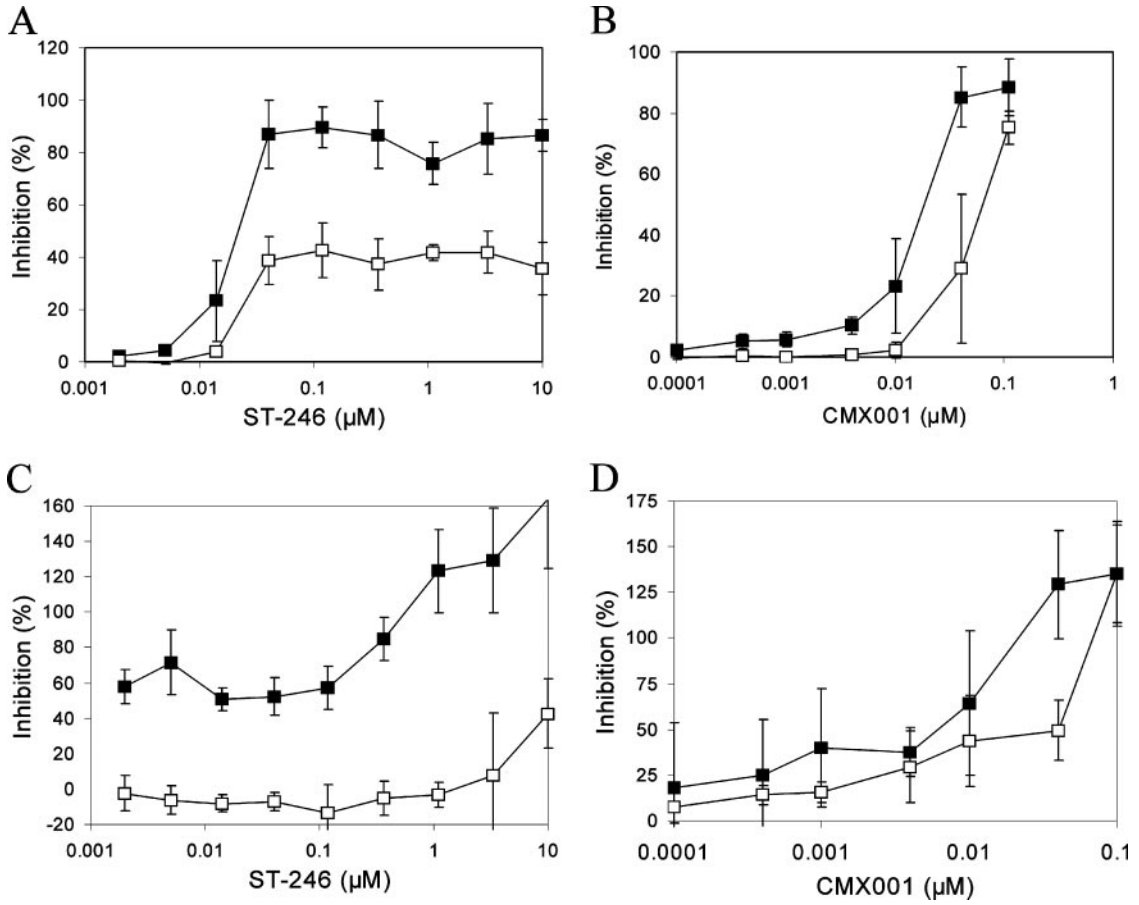


FIG. 2. Dose-response curves for vaccinia virus or cowpox virus in the presence of ST-246 and CMX001 alone or in combination. Dose-response curves against VV are shown for ST-246 alone (open symbols) or in the presence of 0.01 μM CMX001 (filled symbols) (A) with standard deviations shown or CMX001 alone (open symbols) or in the presence of 0.014 μM ST-246 (filled symbols) (B). Dose-response curves against CV are shown for ST-246 alone (open symbols) or in the presence of 0.04 μM CMX001 (filled symbols) (C) with standard deviations shown or CMX001 alone (open symbols) or in the presence of 3.3 μM ST-246 (filled symbols) (D).

effect against this virus. Concurrent cytotoxicity assays did not detect any synergistic toxicity, 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 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 with animal models. This hypothesis was tested in a series of experiments with 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

TABLE 2. Effect of oral combination treatment with ST-246 and CMX001 on mortality of BALB/c mice inoculated i.n. with CV-BR

| Treatment[a] | No. of mice killed/no. tested (%) | *P* value for mortality | MDD[b] | *P* value for MDD |
|---|---|---|---|---|
| Vehicle, day 1 (a.m.) + water (p.m.) | 14/15 (93) | | 9.6 ± 1.3 | |
| CDV, day 1 (a.m.) | | | | |
| 15 mg/kg | 0/15 (0) | <0.001 | | |
| ST-246, day 1 (a.m.) | | | | |
| 10 mg/kg | 11/15 (73) | NS[c] | 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 (p.m.) | | | | |
| 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 (a.m.) + CMX001 (p.m.) | | | | |
| 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 orally in 0.2-ml doses. CMX001 was provided by Chimerix Inc. and was suspended in sterile water and given orally 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 5 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 compared to result for vehicle control.

dose of 0.3 mg/kg of CMX001 was ineffective at 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 eight of the nine 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 using 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 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, seven of the nine 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 with 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 using 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 for two of the groups, results 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 ST-246 and 1-mg/kg/day CMX001 was particularly informative. Calculations using assumptions of either Bliss independence or Loewe additivity (9) demonstrated statistically significant survival over that of groups receiving the drugs as single agents at threefold-higher concentrations. We conclude that combinations of ST-246 and CMX001 protect mice synergistically from a lethal CV infection.

## DISCUSSION

Experience with therapy for human immunodeficiency virus has shown the superiority of multidrug therapy over single-drug therapy and is the standard of care. This ap-

TABLE 3. Effect of oral combination treatment with ST-246 and CMX001 on mortality of BALB/c mice inoculated i.n. with CV-BR

| Treatment[a] | No. of mice killed/no. tested (%) | *P* value for mortality | MDD[b] | *P* value for MDD |
|---|---|---|---|---|
| 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[c] | 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 was 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 5 days beginning 3 days after viral inoculation.
[b] MDD, mean day of death.
[c] NS, not significant compared to result for vehicle control.

proach 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 therapy for orthopoxvirus infections. Combinations of ST-246 and CMX001 appear to be logical candidates given the impressive preclinical efficacy of these drugs when used individually and their distinctly different mechanisms of action. The results obtained in the experiments reported here

TABLE 4. Effect of oral combination treatment with ST-246 and CMX001 on mortality of BALB/c mice inoculated i.n. with CV-BR

| Treatment[a] | No. of mice killed/no. tested (%) | *P* value for mortality | MDD[b] | *P* value for MDD |
|---|---|---|---|---|
| Vehicle, day 6 | 15/15 (100) | | 10.9 ± 0.6 | |
| CDV, day 6 | | | | |
| 25 mg/kg | 12/15 (80) | NS[c] | 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 was 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 5 days beginning 6 days after viral inoculation.
[b] MDD, mean day of death.
[c] NS, not significant compared to result for vehicle control.

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 with animal models.

Previous reports describe successful therapy for 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-drug 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 until 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, thus minimizing 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 against 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).

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## REFERENCES

1. **Andrei, G., D. B. Gammon, P. Fiten, E. De Clercq, G. Oppendakker, R. Snoeck, and D. H. Evans.** 2006. Cidofovir resistance in vaccinia virus is linked to diminished virulence in mice. J. Virol. **80:**9391–9401.
2. Reference deleted.
3. **Bray, M., M. Martinez, D. F. Smee, D. Kefauver, E. Thompson, and J. W. Huggins.** 2000. Cidofovir protects mice against lethal aerosol or intranasal cowpox virus challenge. J. Infect. Dis. **181:**10–19.
4. **Buller, R. M., G. Owens, J. Schriewer, L. Melman, J. R. Beadle, and K. Y. Hostetler.** 2004. Efficacy of oral active ether lipid analogs of cidofovir in a lethal mousepox model. Virology **318:**474–481.
5. **Ciesla, S. L., J. Trahan, W. B. Wan, J. R. Beadle, K. A. Aldern, G. R. Painter, and K. Y. Hostetler.** 2003. Esterification of cidofovir with alkoxyalkanols increases oral bioavailability and diminishes accumulation in the kidney. Antivir. Res. **59:**163–171.
6. **Fedele, C. G., A. Negredo, F. Molero, M. P. Sanchez-Seco, and A. Tenorio.** 2006. Use of internally controlled real-time PCR for detection of variola virus and other orthopoxviruses infecting humans. J. Clin. Microbiol. **44:**4464–4470.
7. **Finkel, E.** 2001. Engineered mouse virus spurs bioweapon fears. Science **291:**585.
8. Reference deleted.
9. **Greco, W. R., G. Bravo, and J. C. Parsons.** 1995. The search for synergy: a critical review from a response surface perspective. Pharmacol. Rev. **47:**331–385.
10. **Jackson, R. J., A. J. Ramsay, C. D. Christensen, S. Beaton, D. F. Hall, and I. A. Ramshaw.** 2001. Expression of mouse interleukin-4 by a recombinant ectromelia virus suppresses cytolytic lymphocyte responses and overcomes genetic resistance to mousepox. J. Virol. **75:**1205–1210.
11. **Keith, K. A., M. J. Hitchcock, W. A. Lee, A. Holý, and E. R. Kern.** 2003. Evaluation of nucleoside phosphonates and their analogs and prodrugs for inhibition of orthopoxvirus replication. Antimicrob. Agents Chemother. **47:**2193–2198.
12. **Kern, E. R.** 2003. *In vitro* activity of potential anti-poxviral agents. Antivir. Res. **57:**35–40.
13. Reference deleted.
14. **Kornbluth, R. S., D. F. Smee, R. W. Sidwell, V. Snarsky, D. H. Evans, and K. Y. Hostetler.** 2006. Mutations in the E9L polymerase gene of cidofovir-resistant vaccinia virus strain WR are associated with the drug resistance phenotype. Antimicrob. Agents Chemother. **50:**4038–4043.
15. **Lacy, S. A., M. J. Hitchcock, W. A. Lee, P. Tellier, and K. C. Cundy.** 1998. Effect of oral probenicid on the chronic toxicity and pharmacokinetics of intravenous cidofovir in cynomolgus monkeys. Toxicol. Sci. **44:**97–106.
16. **Müllbacher, A., and M. Lobigs.** 2001. Creation of killer poxvirus could have been predicted. J. Virol. **75:**8353–8355.
17. **Painter, G. R., and K. Y. Hostetler.** 2004. Design and development of oral drugs for the prophylaxis and treatment of smallpox infection. Trends Biotechnol. **8:**423–427.
18. **Prichard, M. N., K. A. Keith, D. C. Quenelle, and E. R. Kern.** 2006. Activity and mechanism of action of *N*-methanocarbathymidine against herpesvirus and orthopoxvirus infections. Antimicrob. Agents Chemother. **50:**1336–1341.
19. **Prichard, M. N., and C. Shipman, Jr.** 1990. A three dimensional model to analyze drug-drug interactions. Antivir. Res. **14:**181–205.
20. **Quenelle, D. C., R. M. Buller, S. Parker, K. A. Keith, D. E. Hruby, R. Jordan, and E. R. Kern.** 2007. Efficacy of delayed treatment with ST-246 given orally against systemic orthopox infections in mice. Antimicrob. Agents Chemother. **51:**689–695.

20a. **ProMED-mail.** 2007. Smallpox vaccine, eczema vaccination—USA. Archive no. 20070318.0947. http://www.promedmail.org/.

21. **Quenelle, D. C., D. J. Collins, and E. R. Kern.** 2003. Efficacy of multiple- or single-dose cidofovir against vaccinia and cowpox infections in mice. Antimicrob. Agents Chemother. **47:**3275–3280.
22. **Quenelle, D. C., D. J. Collins, W. B. Wan, J. R. Beadle, K. Y. Hostetler, and E. R. Kern.** 2004. Oral treatment of cowpox and vaccinia virus infections in mice with ether lipid esters of cidofovir. Antimicrob. Agents Chemother. **48:**404–412.
23. **Quenelle, D. C., K. A. Keith, and E. R. Kern.** 2006. *In vitro* and *in vivo* evaluation of isatin-beta-thiosemicarbazone and marboran against vaccinia and cowpox virus infections. Antivir. Res. **71:**24–30.
24. **Robbins, S. J., R. J. Jackson, F. Fenner, S. Beaton, J. Medveczky, I. A. Ramshaw, and A. J. Ramsay.** 2005. The efficacy of cidofovir treatment of mice infected with ectromelia (mousepox) virus encoding interleukin-4. Antivir. Res. **66:**1–7.
25. **Skottman, T., H. Piiperinan, H. Hyytiäinen, V. Myllys, M. Skurnik, and S. Nikkari.** 2007. Simultaneous real-time PCR detection of *Bacillus anthracis*, *Francisella tularensis* and *Yersinia pestis*. Eur. J. Clin. Microbiol. Infect. Dis. **26:**207–211.
26. **Smee, D. F., K. W. Bailey, and R. W. Sidwell.** 2003. Comparative effects of cidofovir and cyclic HPMPC on lethal cowpox and vaccinia virus respiratory infections in mice. Chemotherapy **49:**126–131.
27. **Smee, D. F., M. K. Wandersee, K. W. Bailey, K. Y. Hostetler, A. Holy, and R. W. Sidwell.** 2005. Characterization and treatment of cidofovir-resistant vaccinia (WR strain) virus infections in cell culture and in mice. Antivir. Chem. Chemother. **16:**203–211.
28. **Smee, D. F., M. Wong, K. W. Bailey, J. R. Beadle, K. Y. Hostetler, and R. W. Sidwell.** 2004. Effects of four antiviral substances on lethal vaccinia virus (IHD strain) respiratory infections in mice. Int. J. Antimicrob. Agents **23:**430–437.
29. **Yang, G., D. C. Pevear, M. H. Davies, M. S. Collett, T. Bailey, S. Rippen, L. Barone, C. Burns, G. Rhodes, S. Tohan, J. W. Huggins, R. O. Baker, R. L. M. Buller, E. Touchette, K. Waller, J. Schriewer, J. Neyts, E. DeClercq, K. Jones, D. Hruby, and R. Jordan.** 2005. An orally bioavailable antipoxvirus compound (ST-246) inhibits extracellular virus formation and protects mice from lethal orthopoxvirus challenge. J. Virol. **79:**13139–13149.