Antiviral Research 88 (2010) 287–295

Contents lists available at ScienceDirect

Antiviral Research

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

Inhibition of poxvirus growth by Terameprocol, a methylated derivative of nordihydroguaiaretic acid

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ARTICLE INFO

Article history:
Received 31 December 2009
Received in revised form 5 August 2010
Accepted 23 September 2010

Keywords:
Poxvirus
Cowpox virus
Vaccinia virus
Terameprocol
Antiviral agent
Cell-to-cell spread

ABSTRACT

Terameprocol (TMP) is a methylated derivative of nordihydroguaiaretic acid, a phenolic antioxidant originally derived from creosote bush extracts. TMP has previously been shown to have antiviral and anti-inflammatory activities, and has been proven safe in phase I clinical trials conducted to evaluate TMP as both a topical and parenteral therapeutic. In the current study, we examined the ability of TMP to inhibit poxvirus growth in vitro, and found that TMP potently inhibited the growth of both cowpox virus and vaccinia virus in a variety of cell lines. TMP treatment was highly effective at reducing infectious virus yield in multi-step virus growth assays, but it did not substantially inhibit the synthesis of infectious progeny viruses in individual infected cells. These contrasting results showed that TMP inhibits poxvirus growth in vitro by preventing the efficient spread of virus particles from cell to cell. The canonical mechanism of poxvirus cell-to-cell spread requires morphogenesis of cell-associated, enveloped virions. The virions then trigger the formation of actin tails to project them from the cell surface. The number of actin tails present at the surface of poxvirus-infected cells was reduced dramatically by treatment with TMP. Whether TMP inhibits poxvirus morphogenesis, or subsequent events required for actin tail formation, remains to be determined. The results of this study, together with the clinical safety record of TMP, support further evaluation of TMP as a poxvirus therapeutic.

1. Introduction

Widespread public vaccination against smallpox was discontinued following the 1980 declaration by the World Health Organization that the disease had been eradicated. Indeed, vaccination in some countries, including the United States, was halted even earlier. As a result, it is likely that the majority of the current population has no protection against this disease, and that herd immunity has been lost. This has caused concern that smallpox may now constitute a dangerous biological weapon, and as such, the Centers for Disease Control and Prevention has identified the causative agent, variola virus, as amongst the highest potential bioterrorism threats. Another concern is the prospect of increased exposure to monkeypox virus due to human encroachment, globalization, or weaponized use (Parker et al., 2007). Although the currently licensed smallpox vaccine provides protection against both smallpox and monkeypox (Edghill-Smith et al., 2005), the development of a diverse arsenal of poxvirus antiviral agents is paramount for preparedness in the event of a potential outbreak. If exposure of the general public were to occur, poxvirus antiviral agents would provide a crucial complement to vaccination (Neyts and De Clercq, 2003).

Poxvirus replication occurs in the cytoplasm of host cells and results in the formation of four distinct types of infectious virus particles. Intracellular mature virus (IMV) constitutes the primary infectious virion type produced in poxvirus-infected cells (Roberts and Smith, 2008). IMV particles are already enveloped, but some subsequently become wrapped with additional cellular membranes, obtained from either the *trans*-Golgi network or early endosomes, to form so-called intracellular enveloped virus (IEV) (Schmelz et al., 1994; Tooze et al., 1993). IEV particles are transported via microtubules to the surface of the cell (Sanderson et al., 2000). At the surface, the outer IEV membrane fuses with the plasma membrane. Particles that remain associated with the cell are termed cell-associated enveloped virus (CEV), while those that are released are termed extracellular enveloped virus (EEV) (Smith and Law, 2004; Smith et al., 2002, 2003). CEV and EEV particles are distinct only in whether or not they are associated with the cell, and in their pathogenic functions. CEV particles participate in the canonical mechanism of poxvirus cell-to-cell spread. At the surface of infected cells, CEV particles induce the formation of actin-filled projections ("actin tails") that propel them outward toward adjacent cells (Blasco and Moss, 1992; Cudmore et al., 1995). If an adjacent cell is already infected, the CEV particles then trigger the

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doi:10.1016/j.antiviral.2010.09.017

formation of new actin tails on that cell, which blocks superinfection and contributes to the rapid expansion of plaques formed by poxviruses in vitro (Doceul et al., 2010). In contrast, EEV particles are involved in long distance virus spread and systemic dissemination (Payne, 1980). The complex morphogenesis of poxviruses provides additional targets for antiviral compounds beyond the replicative portion of the virus life cycle.

The FDA has approved only one investigational new drug for use against poxviruses. The drug, cidofovir (CDV), is a nucleoside analog that effectively inhibits the replication of many DNA viruses (De Clercq, 2003). Unfortunately, poxviruses with resistance to CDV have been isolated (Smee et al., 2002), demonstrating the need for novel antivirals that target other aspects of poxvirus growth. One recently described compound, ST-246, is readily bioavailable following oral administration, is effective against both variola virus and monkeypox virus in nonhuman primates, and has recently been evaluated in a phase I clinical trial (Huggins et al., 2009; Jordan et al., 2008; Yang et al., 2005). Unlike CDV, ST-246 does not inhibit poxvirus replication, but rather interacts with a specific viral protein to prevent the formation of IEV. However, resistance to ST-246 conferred by a single point mutation has been described (Yang et al., 2005). Thus, there is a need to develop, or identify, additional antiviral agents. Many such investigations have been described, but given the difficulties associated with the clinical trials process, the continued identification of putative compounds is warranted.

Many of the medicinal effects associated with extracts prepared from the creosote bush, *Larrea tridentata*, have been attributed to the phenolic antioxidant nordihydroguaiaretic acid (NDGA), and related molecules (Arteaga et al., 2005). Previous studies have shown that methylated derivatives of NDGA can inhibit replication, and Tat-regulated transactivation, of human immunodeficiency virus (HIV) in vitro (Gnabre et al., 1995). Screening of eight different methylated derivatives of NDGA for increased ability to inhibit HIV transactivation identified tetra-O-methyl NDGA, also known as M4N, EM-1421, or Terameprocol (TMP), as having the highest activity (Hwu et al., 1998). The structure of TMP is shown in Fig. 1. Subsequently, TMP has also been shown to block the replication of herpes simplex viruses (HSV), and to prevent gene expression from the human papillomavirus type 16 P_{97} early promoter (Chen et al., 1998; Craigo et al., 2000). Based on this spectrum of antiviral activities, TMP has been evaluated in phase I/II clinical trials for use as a vaginal microbicide, and has been well tolerated (Khanna et al., 2008; Khanna et al., 2007). TMP is also under evaluation as a cancer therapeutic, and multiple phase I clinical trials have addressed safety and maximum tolerated dose following intravenous infusion in human volunteers (www.Clinicaltrials.gov, accessed 12/10/2009).

In the current study, we examined the ability of TMP to inhibit poxvirus growth in vitro. Our data show that TMP potently inhibits the growth of both cowpox virus (CPXV) and vaccinia virus (VACV) in a variety of cell lines. Mechanistic studies revealed that TMP modestly reduced virus protein expression and DNA synthesis, but had little effect on the accumulation of infectious progeny virus in individual infected cells. However, TMP dramatically inhibited CPXV cell-to-cell spread. The number of CPXV-induced actin tails at the surface of infected cells was reduced, confirming that TMP interfered with the canonical mechanism of poxvirus cell-to-cell spread. Whether TMP treatment prevents the production of CEV, or interferes directly with subsequent events required for actin tail formation, remains to be established. The results of this study, when considered in combination with the established clinical safety of TMP, support the further evaluation of TMP as a poxvirus therapeutic.

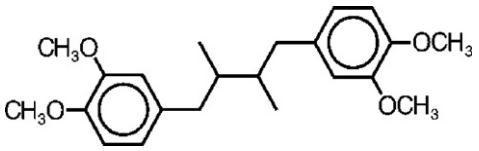

Fig. 1. Chemical structure of TMP.

2. Materials and methods

2.1. Reagents and antibodies

All fetal bovine serum (FBS), cell culture media, and cell culture reagents were purchased from Invitrogen Corporation (Carlsbad, CA). Ascites fluid containing murine monoclonal antibody (Ab) TW2.3, specific to the VACV early gene product E3L (Yuwen et al., 1993), was a generous gift from Dr. Jack Bennink (National Institutes of Health, Bethesda, MD). Rabbit polyclonal Ab specific to the CPXV A-type inclusion (ATI) protein has been previously described (Patel et al., 1986) and was a gift from Dr. David Pickup (Duke University, Durham, NC). Murine monoclonal Ab specific to β-actin, and tetramethylrhodamine B isothiocyanate (TRITC) conjugated phalloidin were purchased from Sigma–Aldrich (St. Louis, MO). Alexa Fluor® 488 conjugated goat anti-mouse IgG Abs were obtained from Invitrogen Corporation (Carlsbad, CA). Secondary Abs for use with the Odyssey Infrared Imaging System, goat anti-mouse IRDye® 680 and goat anti-rabbit IRDye® 800CW, were purchased from Li-Cor Biosciences (Lincoln, NE). All chemicals, including dimethyl sulphoxide (DMSO) and cytosine arabinoside (AraC) were purchased from Sigma–Aldrich (St. Louis, MO). TMP in DMSO solvent was obtained from Erimos Pharmaceuticals (Raleigh, NC).

2.2. Cell lines

Cell lines were obtained from the American Type Culture Collection (ATCC) (Manassas, VA). 143B cells (ATCC CRL-8303) were cultured in Minimum Essential Medium (MEM) supplemented with 5% FBS. BS-C-1, 293, HEP-G2, and A431 cells (ATCC CCL-26, CRL-1573, HB-8065, and CRL-1555, respectively) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS. CCD-1138SK cells (ATCC CRL-2707) were cultured in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 10% FBS. C3HA murine fibroblasts (Gooding, 1979) were cultured in DMEM supplemented with 1 mM sodium pyruvate and 5% FBS. All cell lines were maintained at 37 °C in a 5% CO_2 atmosphere.

2.3. Viruses

Viruses employed in this study were CPXV strain Brighton Red (ATCC VR-302) and VACV strain Western Reserve (ATCC VR-1354). Virus titers were determined by plaque assays in 143B cells.

2.4. CPXV plaque-reduction assay

143B cells or BS-C-1 cells were plated to near confluence in 6-well tissue culture plates. The cells were then mock-infected with 200 μL of serum-free medium, or infected with approximately 200 plaque forming units (PFU) of CPXV in 200 μL of serum-free medium per well. The virus was allowed to adsorb for 45 minutes (min) with shaking every 10 min. The inoculum was removed and replaced with cell culture medium supplemented with 3.125 μM, 6.25 μM, or 12.5 μM TMP, or the solvent control (0.1% DMSO). Plaques were allowed to develop for 30 hours (h). The resultant plaques were visualized by negative staining with 0.1% crystal violet in 20% ethanol solution. Plaque size (mm^2) was measured using Bio-Rad GelDoc XR plaque analysis software (Bio-Rad Laboratories, Hercules, CA).

2.5. Virus growth assays and determination of virus yields

Low- and high-multiplicity of infection (MOI) growth assays were conducted to determine the effects of TMP treatment on poxvirus growth. For low-MOI growth assays, cells were pretreated with 25 μM TMP or solvent control (0.1% DMSO) for 1 h, unless otherwise stated. The treated cells were then infected with CPXV or VACV at 0.01 PFU/cell in serum-free medium containing 25 μM TMP or 0.1% DMSO. Virus was allowed to adsorb for 1 h with shaking every 15 min. Following adsorption, incubations were continued in cell-appropriate growth media containing 25 μM TMP or 0.1% DMSO. Total virus yield was collected at 5 h, 12 h, 24 h, and 48 h after infection for CPXV growth assays, and at 48 h after infection for VACV growth assays. Total virus yield was collected by freeze–thaw lysis of infected cells and culture media, followed by further disruption by sonication. Virus titers were measured with standard plaque assays on 143B cells.

For CPXV high-MOI growth assays, cells were pretreated with 25 μM TMP or solvent control (0.1% DMSO) for 1 h or 24 h as indicated. The treated cells were then infected with CPXV at 5 PFU/cell in serum-free medium containing 25 μM TMP, 10 μg/mL AraC, or 0.1% DMSO. Virus was allowed to adsorb for 1 h with shaking every 15 min. Following adsorption, incubations were continued in cell-appropriate growth media containing 25 μM TMP, 10 μg/mL AraC or 0.1% DMSO. Total virus yield was determined 24 h after infection by freeze–thaw lysis of infected cells and culture media, followed by further disruption by sonication, and measurement of virus titer by standard plaque assays on 143B cells.

2.6. Quantitative immunoblotting

BS-C-1 cells were infected with CPXV at 5 PFU/cell and cultured in growth medium containing 25 μM TMP, 10 μg/mL AraC, or solvent control (0.1% DMSO). Cell monolayers were solubilized in lysis buffer (50 mM HEPES (pH 7.4), 1 mM EGTA, 1 mM EDTA, 0.2 mM sodium orthovanadate, 2 mM PMSF, 5% β-mercaptoethanol and 0.5% SDS) and collected by scraping at the indicated times post-infection. Total protein concentration of the lysates was determined using the Bio-Rad DC protein assay (Bio-Rad Laboratories). Equal amounts of total protein (5 μg) were loaded onto 10% polyacrylamide Tris–glycine–SDS gels and separated by electrophoresis on a Novex MiniCell System (Invitrogen Corporation). Proteins were transferred to Immobilon-FL PVDF membranes (Millipore, Bedford, MA) and blocked overnight in Odyssey Blocking Buffer (Li-Cor Biosciences). The primary Abs (anti-ATI, and anti-β-actin) were diluted in 0.1% Tween-20 Odyssey Blocking Buffer and incubated with membrane for 1 h at room temperature. Membranes were washed extensively in PBS containing 0.1% Tween-20. Secondary Abs specifically designed for use with the Li-Cor Odyssey system were diluted in 0.1% Tween-20 Odyssey Blocking buffer and incubated for 45 min at room temperature, followed by extensive washing with PBS containing 0.1% Tween-20. Band visualization and quantification were completed on a Li-Cor Odyssey scanning system running Odyssey 2.1 software (Li-Cor Biosciences). The integrated intensity of each specific band of interest was measured and normalized to the respective β-actin control band to account for any minor variations in total protein loads.

2.7. Isolation and quantification of viral DNA

BS-C-1 cells were infected with CPXV at 5 PFU/cell and cultured in growth medium containing 25 μM TMP, 10 μg/mL AraC, or solvent control (0.1% DMSO). Cells were harvested by scraping at 6 h and 12 h post-infection. Total cellular and viral DNA was isolated using the QIAmp DNA Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. CPXV DNA synthesis was measured by real-time PCR amplification of a portion of the CPXV CP77 gene using the primers 5′-GGA AAA TGA GGA GGT GGC TCT CGA TG-3′ and 5′-GAT AAT CGT CAT TTT GTG ATG GAT ATT CTT-3′ in a Bio-Rad iCycler running MyIQ System Software Version 1.1.410. A portion of the host cellular GAPDH gene was amplified with the primers 5′-GGA TTT GGT CGT ATT GGG CG-3′ and 5′-TGG AAG ATG GTG ATG GGA TTT C-3′ for use as a DNA load control. All primers were purchased from Integrated DNA Technologies (Coralville, IA). Reactions were carried out in 25 μL volume, using Quantace SensiMix *Plus* SYBR® & Fluorescein (Quantace, London, UK) with 5 ng of the isolated DNA per reaction. Copy number was established by comparison to a standard curve that was generated using DNA templates amplified with the use of cognate primer sets for the CP77 gene and GAPDH gene. Thermal cycling was performed as follows: 1.5 min at 95 °C, followed by 50 cycles each of 95 °C for 0.5 min, 59 °C for 0.5 min, and 72 °C for 2 min, followed by melt-curve data collection and analysis. Final RT-PCR data are expressed as the ratio of CP77 copy number per 100 copies of GAPDH. The experiment was conducted twice, and all reactions were performed in triplicate for each experiment.

2.8. Immunofluorescence microscopy

For evaluation of actin tail formation, 8-well glass chamber slides (Nalge Nunc International, Naperville, IL) were seeded with 5×10^4 BS-C-1 cells and incubated at 37 °C for approximately 8 h to allow cell attachment. Cells were then mock-infected or infected with CPXV at 5 PFU/cell in TMP- or DMSO-treated serum free medium and incubated for 1 h at 37 °C to allow virus adsorption. The inoculum was then removed and incubations were continued in 2.5% FBS DMEM with 25 μM TMP or 0.1% DMSO. At 14 h post-infection, the cells were fixed in 4% formaldehyde PBS for 20 min at room temperature and washed twice in 3% BSA PBS. Cells were then permeabilized with 0.1% Triton X-100 PBS for 5 min, washed twice in 3% BSA PBS, and incubated with TRITC–phalloidin and anti-E3L Ab (TW2.3) in 0.3% BSA for 1 h. Cells were then washed again twice in 3% BSA PBS and incubated with Alexa Fluor® 488 conjugated goat anti-mouse Abs in 0.3% BSA PBS for 30 min, washed twice in 3% BSA PBS, and mounted in anti-fade slide mount containing 4′,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA). For evaluation of virus spread mechanisms at the leading edge of CPXV plaques, 8-well chamber slides were seeded to near confluence with BS-C-1 cells or C3HA cells in 2.5% FBS DMEM. Cultures were then infected with CPXV at 0.001 PFU/cell in 2.5% FBS DMEM, and plaques were allowed to develop for 20 h. The slides were then prepared and immunostained as described above. Microscopy was conducted on a Zeiss Axioskop 2 Plus at 1000-fold magnification and images were captured using Spot Advanced software (Diagnostic Instruments, Sterling Heights, MI).

2.9. Statistical analysis

Data are presented as standard error of the mean (SEM). Group analysis of variance was compared by Kruskal–Wallis test followed by Dunn's multiple comparison test of log-transformed data. Proportion analysis was conducted using Fisher's exact test. All statistical analyses were performed using GraphPad Prism (version 5.01) software (GraphPad Software, La Jolla, CA).

3. Results

3.1. TMP reduces the size of plaques formed by CPXV

We first evaluated the ability of TMP to interfere with poxvirus growth by performing CPXV plaque-reduction assays. The formation of a plaque requires the combination of virus replication, virus

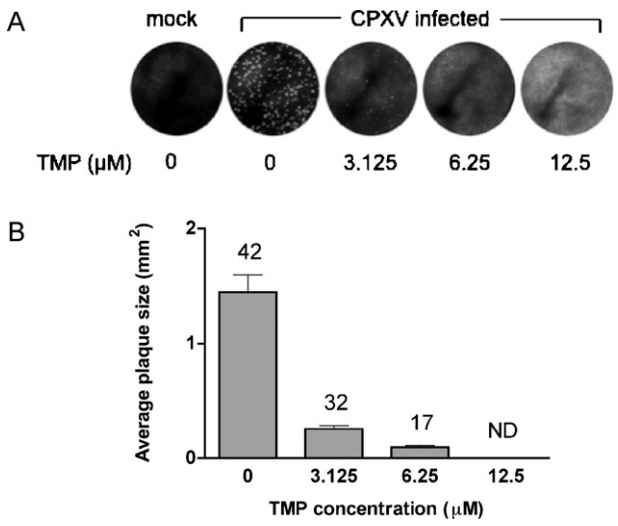

Fig. 2. TMP reduces the size of plaques formed by CPXV. Near-confluent monolayers of 143B cells grown in 6-well tissue culture plates were mock-infected (200 μL of serum free medium), or infected with approximately 200 PFU/well CPXV. Plaques were allowed to develop in cell growth medium supplemented with the indicated concentrations of TMP, or with the solvent control, 0.1% DMSO (0 μM TMP). (A) Cell monolayers were stained with 0.1% crystal violet in a 20% ethanol solution at 30 h post-infection. Well images are shown. (B) Plaque size (mm^2) was determined using Bio-Rad Gel-Doc XR plaque analysis software. Data are means and SEM for each test condition. Plaque density (number of plaques/cm^2) at each tested concentration of TMP is shown above the columns; N.D., none detected.

cell-to-cell spread, and virus-induced cell lysis. Thus, effects on plaque size can be used to screen for antiviral activity directed at any of these processes. Plaque-reduction assays were conducted on confluent monolayers of 143B cells in 6-well plates that were infected with approximately 200 PFU of CPXV per well. Plaques were allowed to develop in culture medium containing 2-fold increasing concentrations of TMP (3.125 μM, 6.25 μM, 12.5 μM) or the solvent control (0.1% DMSO), and the resultant plaques were detected by negative staining with crystal violet. Data from a representative experiment are shown in Fig. 2A, and average plaque sizes for each condition are presented in Fig. 2B. The overall number of plaques formed, both visible to the naked eye, and visible only by microscopy, also was progressively reduced by increasing concentrations of TMP (Fig. 2, and data not shown). However, individual infected cells cannot be detected by the plaque assay, so a reduction in the number of CPXV infection centers established in the culture cannot necessarily be inferred from these results.

These data clearly show that TMP treatment caused a dramatic, dose-dependent decrease in CPXV plaque size, and also reduced the total number of plaques that could be detected. At the highest tested concentration of TMP (12.5 μM) plaques were no longer detected. Results similar to those obtained with monolayers of 143B cells also were obtained from CPXV plaque-reduction assays conducted with BS-C-1 cells (data not shown). Thus, TMP was able to inhibit the growth of CPXV in a dose-dependent manner.

3.2. TMP only has minimal effect on synthesis of CPXV proteins, DNA, and infectious virus particles

We next sought to determine whether TMP treatment inhibited virus growth in a cell-autonomous manner. For this, we investigated the effect of TMP on the yield of infectious progeny virus obtained from one-step (high-MOI) growth assays. BS-C-1 cells are a standard cell line for in vitro propagation of poxviruses, and therefore were used for this experiment, as well as for subsequent experiments to elucidate the mechanism of the antiviral activity of TMP against CPXV. For these, and all subsequent experiments, TMP was used at a concentration of 25 μM, as pharmacokinetic and tissue distribution studies have shown that TMP can be detected at, or above, this concentration in the plasma, intestines, and spleens of mice following repeated oral administration (Park et al., 2005). We evaluated the dose-dependent toxicity of TMP by trypan blue dye-exclusion of solvent control- (0.1% DMSO) and TMP-treated BS-C-1 cells over a 48 h incubation. We observed no apparent reduction of cell growth, or increase in cell death, when BS-C-1 cells were cultured in media supplemented with 25 μM TMP (data not shown).

For one-step growth assays, BS-C-1 cells were treated with DMSO, TMP, or the nucleoside analog AraC, a well-characterized inhibitor of poxvirus DNA replication, and were infected with CPXV at 5 PFU/cell. Infected cells and culture medium were combined and collected at 24 h post-infection, and virus titers were determined by standard plaque assays. As indicated in Fig. 3A, TMP treatment did not cause a significant reduction of CPXV yield. In marked contrast, and as expected, treatment of BS-C-1 cells with the positive-control compound, AraC, a potent inhibitor of poxvirus DNA replication, resulted in a significant ($P < 0.05$), near three-log (99.9%), decrease in viral yield compared to solvent control-treated cells.

To confirm that TMP treatment had little effect on CPXV in individual infected cells, we also evaluated viral protein expression and DNA synthesis. Quantitative immunoblot analysis was used to measure the accumulation of the CPXV A-type inclusion (ATI) protein in infected cells. Poxvirus gene expression is temporally regulated, and the expression of the CPXV ATI protein is restricted to the late stage of replication, after the onset of viral DNA synthesis (Patel and Pickup, 1987). Therefore, the accumulation of the late stage-restricted ATI protein can serve as an indicator of unperturbed expression of all temporal classes of poxvirus genes. We applied quantitative immunoblotting to CPXV-infected BS-C-1 cell lysates, and compared the accumulation of the ATI protein in DMSO-treated (solvent control) cells to that in TMP-treated cells, and AraC-treated cells. As expected, the data shown in Fig. 3B indicate that treatment with the DNA synthesis inhibitor AraC caused a significant reduction in the accumulation of the ATI protein when compared to control-treated cells at 12 h and 24 h post-infection. ATI protein levels were also reduced in TMP-treated cells; however, the data clearly demonstrate that TMP does not inhibit late-stage viral protein accumulation to the extent seen for AraC, a bona fide inhibitor of poxvirus DNA replication.

We next investigated directly whether poxvirus DNA synthesis was reduced in TMP-treated BS-C-1 cells. Viral DNA synthesis was measured by real-time PCR quantification of the CPXV CP77 gene, normalized to host-cell GAPDH copy number. As shown in Fig. 3C, TMP-treated cells contained fewer copies of the CPXV CP77 gene at 6 h and 12 h post-infection compared to cells treated with DMSO, indicating that TMP treatment reduced viral DNA synthesis. However, as described for viral protein accumulation, TMP had a much smaller effect on viral DNA synthesis than the positive-control compound, AraC.

Taken together, the results of these experiments show that treatment of CPXV-infected cells with TMP can modestly reduce both viral protein expression and DNA synthesis, but these effects are not sufficient to prevent virus replication, or the synthesis of new infectious virions at near normal levels. These results implicate TMP-dependent inhibition of virus spread from infected cells to non-infected cells, or the inhibition of infected cell lysis, as the cause of the potent antiviral effects that were observed in plaque-reduction assays.

3.3. TMP inhibits cell-to-cell spread of CPXV in BS-C-1 cells

Multi-step (low-MOI) growth assays, together with drug-pretreatment in one-step growth assays, were used to test directly for the ability of TMP to inhibit the cell-to-cell spread of CPXV. In

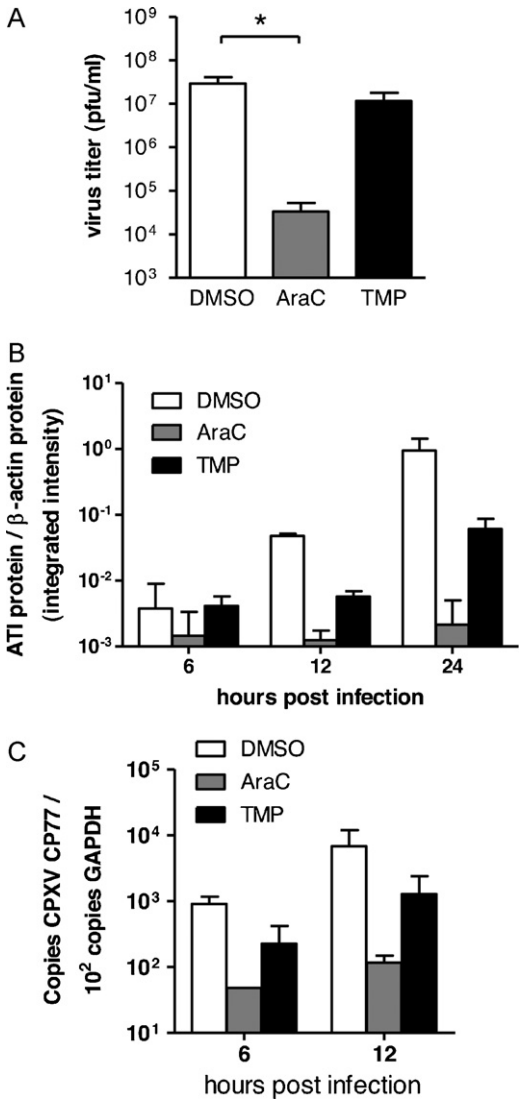

Fig. 3. TMP only has minimal effect on synthesis of CPXV proteins, DNA, and infectious virus particles. (A) BS-C-1 cells were infected with CPXV at 5 PFU/cell and cultured in cell growth medium containing 25 μM TMP, 10 μg/mL AraC, or solvent control (0.1% DMSO). Total virus yield (PFU/mL) was determined at 24 h after infection by freeze–thaw lysis of cells and culture media, and standard plaque assay on 143B cells. Data are means and SEM from five (DMSO and TMP treated) or three (AraC) independent experiments. Differences indicated (*) are significant ($P < 0.05$ by Kruskal–Wallis test with Dunn's multiple comparison test of log-transformed data). (B) BS-C-1 cells were infected with CPXV at 5 PFU/cell, and cultured in growth medium containing 25 μM TMP, 10 μg/mL AraC, or solvent control (0.1% DMSO). Cell lysates were prepared 6, 12, or 24 h after infection. Lysates (5 μg) were resolved by SDS-PAGE and immunoblotted to detect the CPXV ATI protein and host-cell β-actin. The near infrared fluorescence intensity (integrated intensity) of each ATI-specific band was measured with the LiCor Odyssey system, and was normalized to respective β-actin controls. Protein quantification data are means and SEM from two independent experiments. (C) BS-C-1 cells were infected and treated as described above. Total cellular and viral DNA was collected at 6 and 12 h post-infection. Real-time PCR amplification of a portion of the CPXV CP77 gene and the host-cell GAPDH gene was conducted. Copy number was established by comparisons of PCR amplification data to appropriate standard curves. Data are expressed as the ratio of CPXV CP77 copies per 100 copies of GAPDH. Data are means and SEM from two independent experiments. ATI protein levels (B), and CP77 copy number (C), in AraC-treated cells were significantly reduced ($P < 0.1$ by Kruskal–Wallis test with Dunn's multiple comparison test of log-transformed data) when compared to DMSO-treated controls.

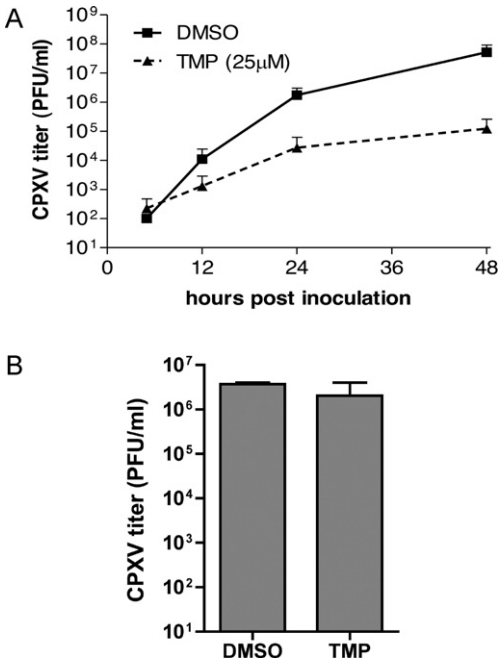

Fig. 4. TMP inhibits cell-to-cell spread of CPXV. (A) BS-C-1 cells were pre-treated with 25 μM TMP or solvent control (0.1% DMSO) for 1 h. The cells were then infected with CPXV at 0.01 PFU/cell, and cultured in cell growth medium containing 25 μM TMP or 0.1% DMSO. Total virus yield was determined at 5, 12, 24, and 48 h after infection by freeze–thaw lysis of cells and culture media, and virus titer (PFU/mL) was determined by standard plaque assay on 143B cells. Data are means and SEM from two independent experiments. (B) BS-C-1 cells were pretreated with 25 μM TMP or solvent control (0.1% DMSO) for 24 h. The treated cells were then infected with CPXV at 5 PFU/cell and cultured in cell growth medium containing 25 μM TMP, or 0.1% DMSO. Total virus yield (PFU/mL) was determined at 24 h after infection by freeze–thaw lysis of cells and culture media, and standard plaque assay on 143B cells. Data are means and SEM from two independent experiments.

multi-step growth assays, BS-C-1 cells were treated with DMSO or TMP, and were infected with CPXV at 0.01 PFU/cell. Infected cells and culture medium were combined and collected at 5, 12, 24, or 48 h after infection, and virus titers were determined by standard plaque assays. As shown in Fig. 4A, TMP treatment caused a dramatic reduction in CPXV titer in multi-step growth assays when compared to solvent control-treated cells. By 48 h post-infection, TMP-treated cells exhibited a reduction in virus yield of nearly three logs (99.8%). In multi-step growth assays, as well as in plaque assays, most cells in the culture might be incubated for many hours in the presence of TMP prior to becoming infected with virus. Thus, it was important to confirm that pretreatment of uninfected cells with TMP for an extended period prior to infection with CPXV could not account for its effectiveness in these assays. As a control, one-step growth experiments were conducted in which BS-C-1 cells were pretreated with TMP for 24 h prior to infection with CPXV, instead of the standard pretreatment of 1 h used in all other experiments. As shown in Fig. 4B, 24 h pretreatment with TMP had no cell-autonomous effect on virus yield. In contrast, after 24 h in a multi-step growth assay TMP treatment reduced the yield of CPXV by about two logs (Fig. 4A).

Taken together, the results of these experiments show that TMP reduces plaque size and virus titer in multi-step growth assays by inhibiting the cell-to-cell spread of CPXV.

3.4. TMP treatment prevents the CPXV-induced formation of actin tails in infected cells

Poxvirus infection normally results in the formation of four different types of infectious virions: IMV, IEV, CEV, and EEV. Of these,

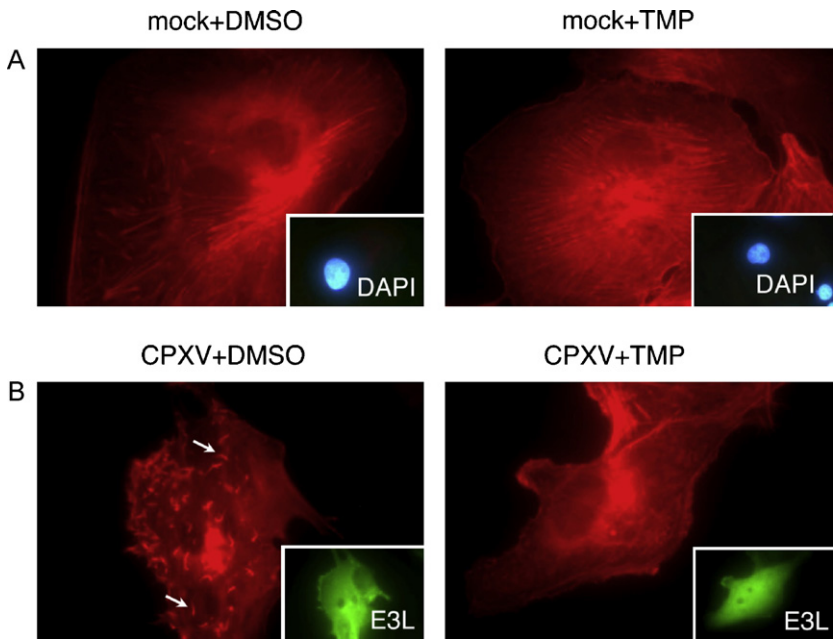

Fig. 5. TMP treatment prevents the formation of actin tails by CPXV-infected cells. BS-C-1 cells were mock-infected (A), or infected with CPXV at 5 PFU/cell (B), and were then cultured in 2.5% FBS DMEM containing 25 μM TMP or solvent control (0.1% DMSO) for 14 h. Cells were then fixed, permeabilized, and immunostained with anti-E3L mAb and AlexaFluor® 488 secondary Ab to detect viral antigen, TRITC-conjugated phalloidin to detect F-actin, and DAPI staining to visualize nucleic acids. (A) Representative fields of phalloidin-stained mock infected cells treated with DMSO or TMP as indicated. DAPI staining is shown in the figure insets. (B) Representative fields of phalloidin-stained CPXV-infected cells treated with DMSO or TMP as indicated. White arrows identify examples of actin tails. E3L immunostaining is shown in the figure insets.

the CEV form is thought to be the most important for efficient cell-to-cell spread (Blasco and Moss, 1992). During poxvirus cell-to-cell movement by the canonical mechanism, CEV induces the formation of actin tails that project the virus particles outward from the surface of infected cells to potentially penetrate, and infect, neighboring cells (Cudmore et al., 1995). Thus, the formation of actin tails at the surface of poxvirus-infected cells provides an indication that cell-to-cell movement is proceeding normally. Accordingly, we evaluated the effect of TMP treatment on actin tail formation in CPXV-infected BS-C-1 cells using fluorescence microscopy. To visualize cellular actin and actin tails, the cells were fixed, permeabilized, and stained with TRITC-labeled phalloidin. That the cells were infected with CPXV was confirmed by immunostaining for a viral non-structural protein (E3L). As shown in Fig. 5A, normal actin central-spanning fibers are apparent in both TMP- and DMSO-treated control cells, indicating that TMP treatment does not disturb the distribution and organization of actin filaments in uninfected cells. As expected, no actin tails were present at the surface of mock-infected BS-C-1 cells. However, actin tails were readily discernable, and found in copious amounts, at the surface of CPXV-infected BS-C-1 cells that were treated with DMSO solvent control (Fig. 5B, left panel). In striking contrast, cells that were infected with CPXV but treated with 25 μM TMP lacked actin tails (Fig. 5B, right panel). Comparisons of ten random microscopy fields per condition indicated that 75% (91 of 121) of CPXV-infected BS-C-1 cells in wells treated with DMSO were positive for the presence of actin tails, while actin tails were present on only 4.5% (4 of 88) of CPXV-infected BS-C-1 cells in wells treated with TMP. Thus, TMP treatment caused a significant reduction in the formation of actin tails by CPXV-infected BS-C-1 cells ($P < 0.0001$ by Fisher's exact test). In comparatively, we observed no difference in the expression of the viral non-structural protein, E3L, between CPXV-infected cells treated with DMSO or TMP. These results demonstrate that TMP interferes with the cell-to-cell spread of infectious virions via the canonical mechanism. However, whether the effect of TMP is to prevent the production of CEV, or to interfere directly with the formation of actin tails, cannot be ascertained.

3.5. TMP inhibits poxvirus growth in a variety of cell lines

We next evaluated whether TMP could effectively inhibit CPXV growth in a panel of different cell lines chosen to represent biologically relevant tissues from mice and humans. Multi-step growth experiments were conducted in 293 human kidney cells (Fig. 6A), C3HA mouse kidney cells (Fig. 6B), Hep-G2 human liver cells (Fig. 6C), A431 human skin cells (Fig. 6D), and CCD-1138SK human skin cells (Fig. 6E). As described for BS-C-1 cells, none of the tested cell types exhibited any loss of cell growth, or increase in cell death, when cultured in media supplemented with 25 μM TMP (data not shown). For each cell type, the virus yield at 5, 12, 24 and 48 h post-infection was determined, and the percent inhibition of CPXV growth in TMP-treated cells compared to DMSO-treated cells after 48 h was calculated, and is indicated in the lower-right portion of each graph (Fig. 6). As shown in Fig. 6A–E, TMP inhibited CPXV growth in all cell lines tested, with efficacies of inhibition ranging from 30% to over 99.9% at 48 h post-infection.

To determine whether TMP exhibits antiviral activity against other poxviruses, we conducted multi-step growth assays with VACV in the same panel of cell lines. The VACV growth experiments were performed similarly to those described above for CPXV, except that total virus preparations were collected at 48 h post-infection only, and each experiment was conducted in duplicate. As shown in Fig. 6F, TMP also inhibited VACV growth in all cell types tested. And, as seen for CPXV, the efficacies of VACV growth inhibition by TMP ranged from 30%, to over 99.9%.

3.6. Predominant mechanisms of poxvirus cell-to-cell spread vary amongst cell types in vitro

Although TMP could inhibit poxvirus growth in all cell lines tested, we noted that its relative effectiveness varied in a cell type-dependent manner. TMP was much less effective at inhibiting poxvirus growth in C3HA cells, and CCD-1138SK cells, than in the other cell lines tested (Fig. 6). Our results with BS-C-1 cells showed a specific effect of TMP on CPXV cell-to-cell spread by the canonical

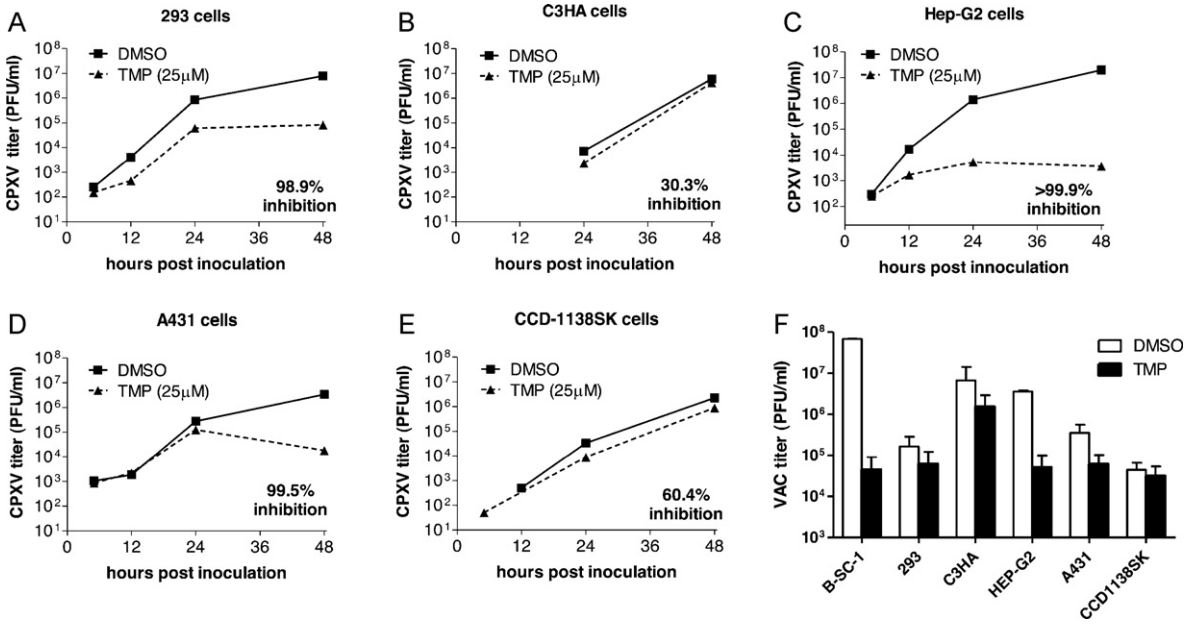

Fig. 6. TMP inhibits poxvirus growth in a variety of cell lines. (A–E) Cells of each type indicated were pretreated with 25 μM TMP or solvent control (0.1% DMSO) for 1 h. The cells were then infected with CPXV at 0.01 PFU/cell, and cultured in cell growth medium containing 25 μM TMP or 0.1% DMSO. Total virus yield (PFU/mL) was determined at 5, 12, 24, and 48 h after infection by freeze–thaw lysis of cells and culture media, followed by disruption by sonication, and standard plaque assay on 143B cells. The percent inhibition of CPXV growth in TMP-treated cells compared to DMSO treated cells at 48 h post-infection is indicated in the lower-right portion of the graph. (F) BS-C-1 cells, 293 cells, C3HA cells, HEP-G2 cells, A431 cells, and CCD-1138SK cells were pre-treated with 25 μM TMP or solvent control (0.1% DMSO) for 1 h, and then infected with VACV at 0.01 PFU/cell and cultured in cell growth medium containing 25 μM TMP or 0.1% DMSO. Total virus yield was determined as described above. Data are means and SEM from two independent experiments.

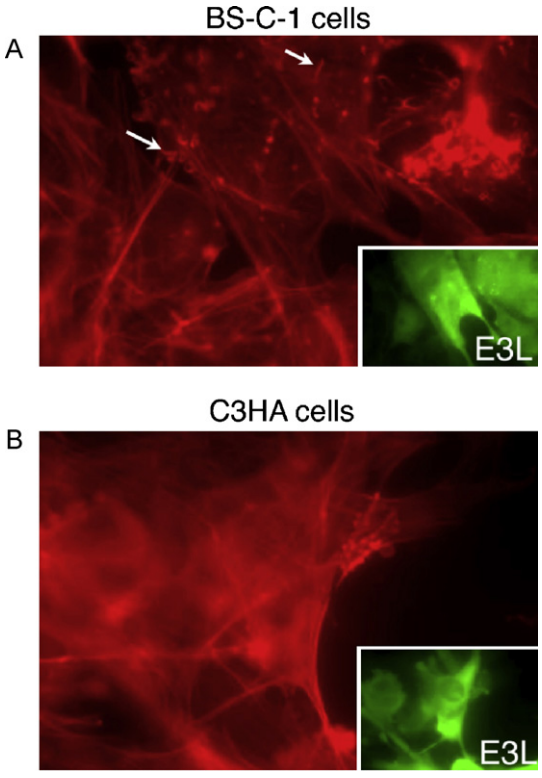

Fig. 7. Mechanisms of virus spread may vary amongst cell types. BS-C-1 or C3HA cells were infected with CPXV at 0.001 PFU/cell and were then cultured in 2.5% FBS DMEM for 20 h. Cells were then fixed, permeabilized, and immunostained with anti-E3L mAb and AlexaFluor® 488 secondary Ab to detect viral antigen, and TRITC conjugated phalloidin to detect F-actin. (A) Representative fields of the leading edge of a CPXV plaque formed on a BS-C-1 cell monolayer. (B) Representative field of the leading edge of a CPXV plaque formed on a C3HA cell monolayer. E3L staining to confirm poxvirus infection is shown in the figure insets.

mechanism that has been described in the literature. Accordingly, we considered that the reduced effectiveness of TMP in C3HA cells and CCD-1138SK cells might be a reflection of poxvirus spread by an alternate mechanism in these cells. To test this hypothesis, we compared actin tail formation during CPXV plaque formation in C3HA cells and BS-C-1 cells. At the leading edge of an expanding plaque, virus spread from infected to uninfected cells is occurring, so we determined the number of actin tails formed on infected cells at this boundary. Monolayers of BS-C-1 and C3HA cells were grown in chamber slides, and were infected with 0.001 PFU CPXV/cell. Plaques were allowed to develop for 20 h, and the slides were then prepared, and immunostained, as previously described. As shown in Fig. 7A, actin tails were readily discernable on CPXV-infected BS-C-1 cells at the leading edge of expanding plaques. In contrast, actin tails were not apparent on CPXV-infected C3HA cells at the leading edge of otherwise similar plaques (Fig. 7B). These results indicate that at least one non-canonical mechanism of poxvirus cell-to-cell spread operates in certain cell lines in vitro. They also suggest that the relative effectiveness of TMP as an antiviral in a given cell type in vitro is determined by the degree of dependence on the canonical mechanism of poxvirus cell-to-cell spread exhibited by that cell type.

4. Discussion

In this report, we have shown that TMP, a methylated derivative of NDGA, has potent antiviral activity against both CPXV and VACV in a variety of cell lines in vitro, and was effective at a concentration found to be attainable in a pharmacokinetic study conducted in mice (Park et al., 2005). Although TMP was highly effective in reducing virus growth in multi-step virus growth assays, as well as in plaque-reduction assays, it did not prevent the synthesis of infectious progeny in one-step growth assays. Furthermore, while TMP had only modest inhibitory effects on viral protein expression and DNA synthesis, it was able to markedly reduce the formation

of virus-induced actin tails at the surface of infected cells. Taken together, the results of this study show that TMP inhibits poxvirus growth in vitro by preventing cell-to-cell spread of poxviruses through the canonical mechanism that involves CEV and actin tails. The antiviral effect of TMP contrasts with that of its parent compound, NDGA, which was found instead to directly inhibit CPXV replication in a cell-autonomous fashion (Palumbo and Buller, 1991).

Previous studies have shown that TMP has antiviral activity against HSV and HIV in vitro (Chen et al., 1998; Gnabre et al., 1995). For both these viruses, TMP was shown to inhibit replication by blocking the binding of the host cell transcription factor, Sp1, to viral promoters. Poxvirus genomes are largely devoid of putative Sp1 high-affinity binding sites, and the transcription of poxvirus genes depends on transcriptional activator proteins encoded by the viral genome, although some host-cell nuclear proteins have been identified in association with cytoplasmic virus factories (Oh and Broyles, 2005). When considered in combination with our results indicating that TMP does not prevent the replication of CPXV or VACV in one-step growth assays, it seems unlikely that TMP inhibits poxvirus growth by interfering with Sp1-dependent transcription of viral genes, as described for other types of viruses. It remains possible, however, that TMP might interfere with Sp1-dependent transcription of a gene for a host cellular factor that is required for poxvirus cell-to-cell spread.

In the canonical mechanism of poxvirus cell-to-cell spread, CEV particles are propelled on actin-filled projections ("actin tails") from the surface of infected cells. The production of CEV, via the intermediate IEV particles, is dependent on successful virus replication and subsequent formation of IMV particles within infected cells (Smith and Law, 2004; Smith et al., 2002, 2003). Although infectious, IMV is not sufficient for efficient poxvirus cell-to-cell spread (Blasco and Moss, 1992; Payne, 1980). Our results showing that TMP does not significantly reduce the total yield of infectious virus per cell suggest that IMV is produced normally in TMP-treated cells. Therefore, TMP must inhibit poxvirus cell-to-cell spread at a step subsequent to the formation of IMV.

Our data demonstrate that TMP treatment prevents the formation of actin tails by poxvirus-infected cells. Production of CEV is a prerequisite for actin tail formation (Smith and Law, 2004; Smith et al., 2002, 2003). Intracellular membrane wrapping of IMV creates IEV particles, which are then transported to the plasma membrane where a membrane fusion event occurs, leaving exposed CEV on the surface of the cell (Smith and Law, 2004; Smith et al., 2002, 2003). Although it is chemically dissimilar to TMP, it is noteworthy that the promising antiviral candidate ST-246 inhibits poxvirus morphogenesis, likely by interfering with the function of a viral protein required for IEV formation (Yang et al., 2005). The cellular microtubule network facilitates transport of IMV and IEV particles during the normal morphogenic process (Sanderson et al., 2000). Once at the cell surface, specific viral proteins associated with the CEV particle concentrate at the edge of the plasma membrane and trigger a signal cascade that mimics a tyrosine kinase signaling pathway involved in normal cellular control of actin polymerization (Frischknecht et al., 1999; Reeves et al., 2005). The activation of this process by CEV-associated proteins results in the polymerization of actin filaments, which thereby propel the CEV away from the cell.

The lack of actin tail formation in poxvirus-infected cells treated with TMP suggests two possible ways in which virus cell-to-cell spread may be inhibited. Firstly, TMP may inhibit the morphogenic pathway from IMV, via IEV, to CEV, and thereby prevent actin tail formation indirectly. Secondly, TMP may inhibit actin tail formation through direct interference with the signal transduction, or downstream steps, required for poxvirus-induced actin polymerization, subsequent to the formation of CEV. TMP has not been reported to affect the microtubule network, but its parent compound, NDGA, has been shown to inhibit microtubule-dependent processes (Arasaki et al., 2007; Nakamura et al., 2003; Tagaya et al., 1996). Also, although we found no such effect in cells treated with TMP, in some cell types NDGA has been shown to disrupt the actin cytoskeleton, evident by a loss of central-spanning stress fibers (Seufferlein et al., 2002). Thus, it is conceivable that TMP may perturb microtubule functions, which might inhibit the formation of IEV, or the transport of IEV particles to the surface of the cell, or exert subtle effects on cellular actin that can prevent directly actin tail formation. Alternatively, TMP may interact with a viral protein that is essential either for poxvirus morphogenesis, as is the case for ST-246, or for poxvirus-induced actin polymerization.

In this study, we found that TMP can inhibit the cell-to-cell spread of poxviruses by the canonical mechanism, which involves the formation of actin tails that propel virus particles away from the surface of infected cells. We also determined that poxvirus cell-to-cell spread can occur independently of actin tail formation in certain cell types in vitro, and that TMP was correspondingly less effective at inhibiting poxvirus growth in these specific cell types. The extent of poxvirus growth inhibition mediated by TMP in these cell lines (30.3% in C3HA cells and 60.4% in CCD-1138SK cells) was consistent with the cell-autonomous effect of TMP on virus yield that we observed in BS-C-1 cells (60.9%). Nevertheless, the effect of TMP on poxvirus cell-to-cell spread mediated via actin tails has the most impact on virus yield in vitro. Thus, the success of TMP as a therapeutic will likely depend on the importance of this mechanism of cell-to-cell spread for poxvirus pathogenesis in vivo. In this regard, it is worth noting that the antiviral ST-246, which inhibits poxvirus morphogenesis, and so would also be expected to prevent cell-to-cell spread mediated via actin tails, is effective in animal models (Huggins et al., 2009; Yang et al., 2005). Also, poxvirus mutants that are defective specifically in actin tail formation are highly attenuated in vivo (Parkinson and Smith, 1994), which provides direct evidence for the relative importance of this mechanism of cell-to-cell spread for poxvirus pathogenesis.

The results of this study have identified TMP as a novel antiviral agent that effectively prevents poxvirus growth in a variety of cell lines. When considered in combination with the multiple phase I clinical trials conducted to address dose tolerance and safety, these data suggest that further development of TMP as a poxvirus therapeutic is warranted.

Acknowledgements

The authors thank Rebecca Hansen Curry and Dawn Eads for their expert technical assistance, David Pickup and Jack Bennick for generously providing antibodies, and Mike Sikes for critical review of the manuscript. This work was funded in part by the College of Agriculture and Life Sciences, North Carolina State University, and by a grant from Erimos Pharmaceuticals, LLC.

References

Arasaki, K., Tani, K., Yoshimori, T., Stephens, D.J., Tagaya, M., 2007. Nordihydroguaiaretic acid affects multiple dynein–dynactin functions in interphase and mitotic cells. Mol. Pharmacol. 71, 454–460.

Arteaga, S., Andrade-Cetto, A., Cárdenas, R., 2005. Larrea tridentata (Creosote bush), an abundant plant of Mexican and US-American deserts and its metabolite nordihydroguaiaretic acid. J. Ethnopharmacol. 98, 231–239.

Blasco, R., Moss, B., 1992. Role of cell-associated enveloped vaccinia virus in cell-to-cell spread. J. Virol. 66, 4170–4179.

Chen, H., Teng, L., Li, J.N., Park, R., Mold, D.E., Gnabre, J., Hwu, J.R., Tseng, W.N., Huang, R.C., 1998. Antiviral activities of methylated nordihydroguaiaretic acids. 2. Targeting herpes simplex virus replication by the mutation insensitive transcription inhibitor tetra-O-methyl-NDGA. J. Med. Chem. 41, 3001–3007.

Craigo, J., Callahan, M., Huang, R.C., DeLucia, A.L., 2000. Inhibition of human papillomavirus type 16 gene expression by nordihydroguaiaretic acid plant lignan derivatives. Antiviral Res. 47, 19–28.

Cudmore, S., Cossart, P., Griffiths, G., Way, M., 1995. Actin-based motility of vaccinia virus. Nature 378, 636–638.
De Clercq, E., 2003. Clinical potential of the acyclic nucleoside phosphonates cidofovir, adefovir, and tenofovir in treatment of DNA virus and retrovirus infections. Clin. Microbiol. Rev. 16, 569–596.
Doceul, V., Hollinshead, M., van der Linden, L., Smith, G.L., 2010. Repulsion of superinfecting virions: a mechanism for rapid virus spread. Science 327, 873–876.
Edghill-Smith, Y., Golding, H., Manischewitz, J., King, L.R., Scott, D., Bray, M., Nalca, A., Hooper, J.W., Whitehouse, C.A., Schmitz, J.E., Reimann, K.A., Franchini, G., 2005. Smallpox vaccine-induced antibodies are necessary and sufficient for protection against monkeypox virus. Nat. Med. 11, 740–747.
Frischknecht, F., Moreau, V., Röttger, S., Gonfloni, S., Reckmann, I., Superti-Furga, G., Way, M., 1999. Actin-based motility of vaccinia virus mimics receptor tyrosine kinase signalling. Nature 401, 926–929.
Gnabre, J.N., Brady, J.N., Clanton, D.J., Ito, Y., Dittmer, J., Bates, R.B., Huang, R.C., 1995. Inhibition of human immunodeficiency virus type 1 transcription and replication by DNA sequence-selective plant lignans. Proc. Natl. Acad. Sci. U.S.A. 92, 11239–11243.
Gooding, L.R., 1979. Antibody blockade of lysis by T lymphocyte effectors generated against syngeneic SV40 transformed cells. J. Immunol. 122, 2328–2336.
Huggins, J., Goff, A., Hensley, L., Mucker, E., Shamblin, J., Wlazlowski, C., Johnson, W., Chapman, J., Larsen, T., Twenhafel, N., Karem, K., Damon, I.K., Byrd, C.M., Bolken, T.C., Jordan, R., Hruby, D., 2009. Nonhuman primates are protected from smallpox virus or monkeypox virus challenges by the antiviral drug ST-246. Antimicrob. Agents Chemother. 53, 2620–2625.
Hwu, J.R., Tseng, W.N., Gnabre, J., Giza, P., Huang, R.C., 1998. Antiviral activities of methylated nordihydroguaiaretic acids. 1. Synthesis, structure identification, and inhibition of tat-regulated HIV transactivation. J. Med. Chem. 41, 2994–3000.
Jordan, R., Tien, D., Bolken, T.C., Jones, K.F., Tyavanagimatt, S.R., Strasser, J., Frimm, A., Corrado, M.L., Strome, P.G., Hruby, D.E., 2008. Single-dose safety and pharmacokinetics of ST-246, a novel orthopoxvirus egress inhibitor. Antimicrob. Agents Chemother. 52, 1721–1727.
Khanna, N., Dalby, R., Connor, A., Church, A., Stern, J., Frazer, N., 2008. Phase I clinical trial of repeat dose terameprocol vaginal ointment in healthy female volunteers. Sex. Transm. Dis. 35, 577–582.
Khanna, N., Dalby, R., Tan, M., Arnold, S., Stern, J., Frazer, N., 2007. Phase I/II clinical safety studies of terameprocol vaginal ointment. Gynecol. Oncol. 107, 554–562.
Nakamura, M., Nakazawa, J., Usui, T., Osada, H., Kono, Y., Takatsuki, A., 2003. Nordihydroguaiaretic acid, of a new family of microtubule-stabilizing agents, shows effects differentiated from paclitaxel. Biosci. Biotechnol. Biochem. 67, 151–157.
Neyts, J., De Clercq, E., 2003. Therapy and short-term prophylaxis of poxvirus infections: historical background and perspectives. Antiviral Res. 57, 25–33.
Oh, J., Broyles, S.S., 2005. Host cell nuclear proteins are recruited to cytoplasmic vaccinia virus replication complexes. J. Virol. 79, 12852–12860.
Palumbo, G.J., Buller, R.M., 1991. Inhibitors of the lipoxygenase pathway specifically block orthopoxvirus replication. Virology 180, 457–463.
Park, R., Chang, C.C., Liang, Y.C., Chung, Y., Henry, R.A., Lin, E., Mold, D.E., Huang, R.C., 2005. Systemic treatment with tetra-O-methyl nordihydroguaiaretic acid suppresses the growth of human xenograft tumors. Clin. Cancer Res. 11, 4601–4609.
Parker, S., Nuara, A., Buller, R.M., Schultz, D.A., 2007. Human monkeypox: an emerging zoonotic disease. Future Microbiol. 2, 17–34.
Parkinson, J.E., Smith, G.L., 1994. Vaccinia virus gene A36R encodes a M(r) 43–50 K protein on the surface of extracellular enveloped virus. Virology 204, 376–390.
Patel, D.D., Pickup, D.J., 1987. Messenger RNAs of a strongly-expressed late gene of cowpox virus contain 5′-terminal poly(A) sequences. EMBO J. 6, 3787–3794.
Patel, D.D., Pickup, D.J., Joklik, W.K., 1986. Isolation of cowpox virus A-type inclusions and characterization of their major protein component. Virology 149, 174–189.
Payne, L.G., 1980. Significance of extracellular enveloped virus in the in vitro and in vivo dissemination of vaccinia. J. Gen. Virol. 50, 89–100.
Reeves, P.M., Bommarius, B., Lebeis, S., McNulty, S., Christensen, J., Swimm, A., Chahroudi, A., Chavan, R., Feinberg, M.B., Veach, D., Bornmann, W., Sherman, M., Kalman, D., 2005. Disabling poxvirus pathogenesis by inhibition of Abl-family tyrosine kinases. Nat. Med. 11, 731–739.
Roberts, K.L., Smith, G.L., 2008. Vaccinia virus morphogenesis and dissemination. Trends Microbiol. 16, 472–479.
Sanderson, C.M., Hollinshead, M., Smith, G.L., 2000. The vaccinia virus A27L protein is needed for the microtubule-dependent transport of intracellular mature virus particles. J. Gen. Virol. 81, 47–58.
Schmelz, M., Sodeik, B., Ericsson, M., Wolffe, E.J., Shida, H., Hiller, G., Griffiths, G., 1994. Assembly of vaccinia virus: the second wrapping cisterna is derived from the trans Golgi network. J. Virol. 68, 130–147.
Seufferlein, T., Seckl, M.J., Schwarz, E., Beil, M., v Wichert, G., Baust, H., Lührs, H., Schmid, R.M., Adler, G., 2002. Mechanisms of nordihydroguaiaretic acid-induced growth inhibition and apoptosis in human cancer cells. Br. J. Cancer 86, 1188–1196.
Smee, D.F., Sidwell, R.W., Kefauver, D., Bray, M., Huggins, J.W., 2002. Characterization of wild-type and cidofovir-resistant strains of camelpox, cowpox, monkeypox, and vaccinia viruses. Antimicrob. Agents Chemother. 46, 1329–1335.
Smith, G.L., Law, M., 2004. The exit of vaccinia virus from infected cells. Virus Res. 106, 189–197.
Smith, G.L., Vanderplasschen, A., Law, M., 2002. The formation and function of extracellular enveloped vaccinia virus. J. Gen. Virol. 83, 2915–2931.
Smith, G.L., Murphy, B.J., Law, M., 2003. Vaccinia virus motility. Annu. Rev. Microbiol. 57, 323–342.
Tagaya, M., Henomatsu, N., Yoshimori, T., Yamamoto, A., Tashiro, Y., Mizushima, S., 1996. Inhibition of vesicle-mediated protein transport by nordihydroguaiaretic acid. J. Biochem. 119, 863–869.
Tooze, J., Hollinshead, M., Reis, B., Radsak, K., Kern, H., 1993. Progeny vaccinia and human cytomegalovirus particles utilize early endosomal cisternae for their envelopes. Eur. J. Cell Biol. 60, 163–178.
Yang, G., Pevear, D.C., Davies, M.H., Collett, M.S., Bailey, T., Rippen, S., Barone, L., Burns, C., Rhodes, G., Tohan, S., Huggins, J.W., Baker, R.O., Buller, R.L., Touchette, E., Waller, K., Schriewer, J., Neyts, J., DeClercq, E., Jones, K., Hruby, D., Jordan, R., 2005. An orally bioavailable antipoxvirus compound (ST-246) inhibits extracellular virus formation and protects mice from lethal orthopoxvirus Challenge. J. Virol. 79, 13139–13149.
Yuwen, H., Cox, J.H., Yewdell, J.W., Bennink, J.R., Moss, B., 1993. Nuclear localization of a double-stranded RNA-binding protein encoded by the vaccinia virus E3L gene. Virology 195, 732–744.