Vaccinia virus, which is closely related to variola and monkeypox viruses, belongs to the Poxviridae family of double-stranded DNA viruses. Poxviruses replicate exclusively in the cytoplasm of infected cells in discrete locations termed viral factories, which have been shown to be free of host cell organelles (23). Since these viruses do not enter the nucleus, they must encode the proteins necessary for viral gene transcription and genome replication. Poxvirus transcription is cascade-like in nature, with early gene products directing intermediate transcription and intermediate gene products directing late transcription (5).

Smallpox, the most devastating disease caused by poxvirus infection, was eradicated nearly a quarter of a century ago. However, poxviruses still represent a significant public health issue due to the threat of bioterrorism and the increasing emergence of monkeypox. Numerous strategies targeted at inhibiting poxvirus replication have been described (6, 7, 10, 11, 12). Currently, there is no licensed therapeutic agent to treat smallpox (21). While a smallpox vaccine is available, its side effects of immunization, such as postvaccinial encephalitis, are not acceptable to many (19). Therefore, the discovery of a safe, potent, and selective antipoxvirus drug remains crucial. Vaccinia virus is the virus of choice for the study of pathogenic human orthopoxviruses, as it is safe to work with and shares over 90% genomic sequence homology with variola virus and monkeypox virus. The most extensively studied small molecules for human poxvirus infection are nucleoside/nucleotide analogues. These compounds owe their inhibitory effect to their ability to inhibit enzymes essential for vaccinia virus replication or to incorporate into the viral genome, respectively. One such nucleotide analogue, cidofovir, has been shown to be effective against vaccinia virus, cowpox virus, camelpox virus, monkeypox virus, and molluscum contagiosum virus (10). The antibiotic distamycin A has also been demonstrated to be effective against vaccinia virus by inhibiting postreplicative transcription and replication. However, this compound is too toxic to be used in humans (3, 6).

Aurintricarboxylic acid (ATA) has been shown to inhibit the replication of viruses from several different families, including human immunodeficiency virus, vesicular stomatitis virus, and the coronavirus causing severe acute respiratory syndrome. This study characterizes the inhibitory effect of ATA on vaccinia virus replication in HeLa, Huh7, and AD293 cells. Vaccinia virus replication is significantly abrogated upon ATA treatment, which is associated with the inhibition of early viral gene transcription. This inhibitory effect may be attributed to two findings. First, ATA blocks the phosphorylation of extracellular signal-regulated kinase 1/2, an event shown to be essential for vaccinia virus replication. Second, ATA inhibits the phosphatase activity of the viral enzyme H1L, which is required to initiate viral transcription. Thus, ATA inhibits vaccinia virus replication by targeting both cellular and viral factors essential for the early stage of replication.
FIG. 1. Effect of ATA on viral replication. Huh7 (A), HeLa (B), and AD293 (C) cells were infected with vaccinia virus (WR) at an MOI of 5. AD293 cells (D) were infected with Ad-enhanced green fluorescent protein at an MOI of 0.1. Cells were treated with the indicated concentrations of ATA. Plaque assays for vaccinia virus were performed in triplicate on BSC-1 cells 24 hpi. Plaque assays for Ad were performed in triplicate on AD293 cells and were counted by immunostaining 24 hpi. (E) The CC<sub>50</sub> values in Huh7 (■), HeLa (○), and AD293 (▲) cells were determined. Cells were treated for 24 h with the indicated concentrations of ATA (µg/ml) and then washed three times with PBS, followed by the addition of WST-1 reagent, and the absorbance was measured at 450 nm. All experiments were performed in triplicate. Error bars represent the standard errors of the means.
stage of vaccinia virus replication. Treatment of HeLa cells with ATA completely abolished extracellular signal-regulated kinase (ERK) phosphorylation. Activation of the ERK cascade in the early stages of vaccinia virus infection is essential for viral replication (1). We also show that ATA can inhibit the activity of the vaccinia virus phosphatase H1L in vitro. H1L plays an essential role in initiating viral early gene transcription and has also been implicated in evasion of the host immune response (19, 24).

**ATA inhibits replication of vaccinia virus.** The effect of ATA on vaccinia virus replication was studied using several cell lines including HeLa, Huh7, AD293, RK13, Vero, 3T3, and BHK21 cells. Here, we show data for ATA inhibition of vaccinia virus replication in Huh7 (Fig. 1A), HeLa (Fig. 1B), and AD293 (Fig. 1C) cells. Cells were cultured in Dulbecco’s modified Eagle medium (Gibco), supplemented with 10% fetal calf serum and 1% penicillin-streptomycin, at 37°C and 5% CO₂. Confluent cells in 6-well plates were infected with vaccinia virus (WR strain) at a multiplicity of infection (MOI) of 5 in the presence of serial dilutions of ATA at 0, 25, 50, 100, 200, and 400 μg/ml. Plaque assays on BSC-1 cells were used to determine the level of viral replication 24 h postinfection (hpi). ATA blocked vaccinia virus replication in all cell lines tested. Virus yield decreased with increasing quantities of ATA, indicating a dose-dependent inhibition of viral replication. At 400 μg/ml, viral replication was almost completely abolished, as virus titers remained near the baseline level (data not shown). The lowest concentration of 25 μg/ml still inhibited vaccinia virus replication approximately fourfold in Huh7 cells. We found that ATA inhibited the replication of vaccinia virus strain Copenhagen (Cop) to a comparable degree. Moreover, ATA was found to inhibit another orthopoxvirus, ectromelia virus (data not shown).

To exclude the possibility that the inhibition of vaccinia virus replication is due to general cytotoxicity, we studied the effect of ATA on adenovirus (Ad) replication. Confluent AD293 cells in 6-well plates were infected with Ad-enhanced green fluorescent protein (Clonetech) at an MOI of 0.1. The Ad infection was preformed under multicycle conditions, since infection at a high MOI in the presence of ATA will kill most cells within 8 h, while the single-step replication of vaccinia virus in the presence of ATA was titrated 24 hpi. Thus, performing the Ad infection at a lower MOI with ATA makes the experiment comparable to that performed with vaccinia virus in that it allows the examination of the possible long-term cytotoxicity of ATA. Viral replication was found to be slightly enhanced when Ad-infected AD293 cells were treated with ATA (Fig. 1D). Furthermore, we determined the 50% cytotoxicity (CC₅₀) of ATA in Huh7, HeLa, and AD293 cells to be 1,250, 1,905, and 1,025 μg/ml, respectively (Fig. 1E). Assays were performed using the Cell Proliferation WST-1 reagent (Roche).

**ATA inhibits vaccinia virus gene transcription.** To further investigate the mechanism of inhibition, the levels of early, intermediate, and late viral gene transcripts were measured by reverse transcription-PCR (RT-PCR). Confluent HeLa cells were infected with vaccinia virus strain WR at an MOI of 5 and treated with or without 400 μg/ml ATA. Cells were collected at the indicated times, and RNA was extracted using the RNeasy Mini kit (QIAGEN). RT-PCR was performed on 750 ng of total RNA using the Advantage RT-For-PCR kit (Clonetech). The level of viral mRNA was determined for the D12L (early), A1L (intermediate), and A7L (late) genes. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels were also measured as a control. ATA inhibited early, intermediate, and late viral gene transcription throughout the time course (Fig. 2). The level of GAPDH remained relatively constant. At late times, the GAPDH level was strongly reduced in cells infected with vaccinia virus in the absence of ATA. However, the level of GAPDH transcript in ATA-treated cells was maintained at the same level as that of the uninfected control.

**ATA is required at the early stage of vaccinia virus replication for effective inhibition.** To further determine which stage of replication is blocked by ATA, we infected HeLa cells with vaccinia virus (Cop) at an MOI of 5 and treated the cells at 0, 1, 3, and 6 hpi with ATA (400 μg/ml). The level of viral replication was determined 24 hpi using plaque assays on BSC-1 cells. ATA inhibited vaccinia virus replication only when added at the start of infection (Fig. 3A, bar 2). When ATA was added even 1 hpi (Fig. 3A, bar 3), viral titers approached those of untreated controls.

To investigate whether ATA can inhibit virus entry into the cell, we performed two experiments. First, HeLa cells were preincubated with or without ATA for 1 h and then washed with phosphate-buffered saline (PBS) and infected with vaccinia virus. Second, vaccinia virus was preincubated with or without ATA and then titrated on BSC-1 cells. Viral replication was unaffected in each experiment, indicating that ATA had no inhibitory effect when applied in this manner (data not shown).

**ATA inhibits ERK phosphorylation.** ATA has been shown to alter the activity of cellular signaling pathways (4, 8, 14, 27, 28). Since the activation of ERK signaling within the first hour of vaccinia virus infection is a necessary event for replication (1), we investigated the effect of ATA on this signaling cascade. Confluent HeLa cells in 6-well plates were treated with ATA (400 μg/ml) and collected at 10 min, 30 min, and 1, 3, 6, and 8 h posttreatment. Protein samples were separated on Criterion XT precast gels (Bio-Rad) and transferred onto Hybond-C nitrocellulose membranes (Amersham Bioscience). Membranes were probed with antibodies for phosphorylated and nonphosphorylated ERK (Cell Signaling Technologies) and β-actin (Sigma) and developed with Western Lightning Chemi-
ATA inhibits the H1L phosphatase. ATA has previously been shown to inhibit dual-specificity phosphatases, including those from both eukaryotic and prokaryotic sources (9, 18). H1L plays an essential role in initiating early viral gene transcription (19). To further characterize the mechanism behind the inhibition of vaccinia virus replication by ATA, we performed enzyme inhibition assays to determine if ATA could inhibit the H1L phosphatase. The H1L coding sequence was amplified by PCR from vaccinia virus WR genomic DNA. The N-terminal primer was 5′-TTTTATCAAA TAACTTCTTAATTGAGCTCGCCT-3′. The C-terminal primer was 5′-TTTTATCAAA TAATATATTCTTAATTGAGCTCGCCT-3′. The PCR product was sequenced for fidelity and ligated into pGEX-6P-3 (a gift from M. Carpenter, National Microbiology, Canada). The enzyme was expressed in Escherichia coli cells and purified by Abgent (San Diego, CA).

Previous studies have shown that the glutathione S-transferase-tagged H1L fusion protein and H1L alone have nearly identical activities (13). Thus, the tyrosine phosphatase activity of H1L was determined using purified glutathione S-transferase-tagged H1L. Assays were first performed to determine the maximum velocity ($V_{\text{max}}$) and the Michaelis constant ($K_m$) of H1L towards the experimental substrate para-nitrophenylphosphate (pNPP). The $K_m$ value is required to perform 50% inhibitory concentration (IC$_{50}$) assays. Reactions were performed at 37°C with 2.5 μg of H1L in a solution containing 25 mM HEPES, 50 mM NaCl, 5 mM dithiothreitol, 2.5 mM EDTA, and 25 μg of bovine serum albumin. The reaction product was quantified by measuring the absorbance at 405 nm using a SpectroMax Plus spectrophotometer (Molecular Devices). The resulting data were fit to a Lineweaver-Burk plot, with a $V_{\text{max}}$ of 0.1 (absolute units) and a $K_m$ of 1.00 mM pNPP (data not shown).

To determine the extent to which ATA inhibits the tyrosine phosphatase activity of H1L, IC$_{50}$ assays were performed. Reaction conditions were identical to those described above, with the exception that reactions were performed at 1 mM pNPP and were preincubated with serial dilutions of ATA from 0 to 53 μg/ml. A concentration-dependent relationship between the quantity of ATA used and H1L activity was observed (Fig. 4). Enzyme activity was completely abolished at 53 μg/ml. Enzyme inhibition was still seen at the lowest concentration of ATA used (~0.83 μg/ml). The IC$_{50}$ value was found to be approximately 2.2 μg/ml.

The potential threat of bioterrorism using smallpox and the increasing emergence of monkeypox infection have highlighted the need for the development of therapeutic strategies for poxvirus infection. In this study, we demonstrate that the replication of vaccinia virus, which is closely related to smallpox and monkeypox viruses, is highly susceptible to ATA. Two possible mechanisms through which ATA may exert this effect are the down-regulation of ERK signaling and the inhibition of vaccinia virus H1L phosphatase activity. The activity of both of these factors is required during the early phase of vaccinia virus replication (1, 19).

ATa inhibited effect of the vaccinia virus replication, as titers reached the level of untreated controls (Fig. 3A, lanes 6 to 8).
Treatment of cells with ATA strongly inhibited virus replication. At 400 µg/ml of ATA, replication of vaccinia virus was almost completely inhibited. Several lines of evidence point to a specific mechanism of inhibition of vaccinia virus replication by ATA that is not due to general cytotoxicity. First, the CC50 values of ATA for Huh7, HeLa, and AD293 cells were found to be 1,250, 1,905, and 1,025 µg/ml, respectively. The highest concentration of ATA used (400 µg/ml) is well below the CC50 value. The 50% lethal dose of ATA has also been reported to be 0.34 g/kg in mice (2). The selectivity index of ATA has been reported to be 187 in Vero cells, meaning that the concentration that causes cell toxicity is much higher than the concentration required to inhibit viral replication (15). Second, GAPDH gene transcription proceeds normally during vaccinia virus infection at high MOIs without ATA. In contrast, host gene transcription is blocked at late times during vaccinia virus infection at high MOIs without ATA. Third, Ad replication is not inhibited in the presence of the chemical. These observations suggest that the antiviral action of ATA against vaccinia virus is not due to associated cytotoxicity.

The RT-PCR results indicate that ATA inhibits vaccinia virus early gene transcription. To further address this issue, we added ATA at different times postinfection. It was observed that the presence of ATA was required during the first hour of infection to have any significant inhibitory effect (Fig. 3A). The absence of ATA in the first hour of infection resulted in viral titers approaching those of untreated controls. This demonstrates that the presence of ATA is required at the early stage of replication for effective inhibition of viral replication. Moreover, treatment of cells or virus with ATA, followed by the removal of the ATA prior to infection, had no effect on viral replication. Collectively, these results indicate that ATA inhibits its early viral gene transcription but not virus entry into the cell.

Upon treatment of HeLa cells with ATA, the activation of a key component of the mitogen-activated protein kinase (MAPK) pathway, ERK, was significantly inhibited, as evidenced by a drastic decrease in its phosphorylated state. ATA can block ERK activation as early as 10 min posttreatment. This inhibition was sustained throughout the time course, while the levels of total ERK and β-actin remained relatively constant. An ATA-induced decrease in ERK activation in RAW 246.7 macrophages but only in addition to treatment with lipopolysaccharide has previously been reported (28). Previous studies have shown the activation of ERK signaling by ATA in NB2 cells (4). We found that ATA had no obvious effect on ERK phosphorylation in this cell line. These cells are also relatively nonpermissive to vaccinia virus infection (data not shown). Nonetheless, this should be taken into consideration in any future animal experiments.

We show that ATA can block ERK phosphorylation within 1 h of treatment. We also demonstrate that ATA is required during the first hour of infection to have an inhibitory effect. This suggests that ATA targets a factor(s) required at the early stage of replication. When infected cells were treated with ATA (for 1, 3, or 6 h) and then washed and grown in fresh medium overnight, viral replication again approached the level of untreated controls. The level of ERK phosphorylation in these cells also returned to control levels. Collectively, these results correlate the inhibition of viral replication with the inhibition of ERK phosphorylation. The ERK signaling cascade mediates cellular responses to growth and survival signals (26). Vaccinia virus infection has been shown to induce sustained activation of the ERK cascade as early as 1 hpi (1). Activation is mediated by the MAPK ERK kinase and induces the phosphorylation of the Elk-1 transcription factor, leading to the expression of the early growth response gene (egr-1). Inhibition of the ERK pathway by selective kinase inhibitors greatly reduces vaccinia virus replication, demonstrating the essential role of this pathway during vaccinia virus infection (1, 20). Therefore, it seems plausible that the inhibition of ERK activation remains unknown. Since ATA has been shown to inhibit the activity of kinases (22), it is possible that ATA inhibits a kinase upstream of ERK, such as MAPK ERK kinase, blocking its activation.

Previous studies have shown that ATA also inhibits protein tyrosine phosphatase activity (9, 18). To further elucidate the mechanism of action of ATA, we performed enzyme activity assays on vaccinia virus H1L. In vitro, ATA was found to be a potent inhibitor of H1L tyrosine phosphatase activity. A dose-dependent relationship between ATA and H1L activity was observed, with the IC50 value of ATA against H1L being approximately 2.2 µg/ml (∼5.3 µM). The IC50 value of ATA against the human homologue of H1L, termed vaccinia H1-related (VHR), has been reported to be 1.2 µM (18). The gene encoding H1L is well conserved among the poxviruses, with homologues identified in all chondroviroses studied (29). H1L is necessary for initiating viral early gene transcription, and its immediate role in infection is highlighted by the finding that it is packaged in mature virions (19). Therefore, the inhibi-

![FIG. 4. ATA inhibits H1L tyrosine phosphatase activity. Reactions were performed at a substrate concentration equal to the \(K_m\) value (1 mM), at 37°C, with 2.5 µg of H1L in the presence of serial dilutions of ATA (0 to 53 µg/ml). Reactions were performed in triplicate. Error bars represent the standard errors of the means.](http://jvi.asm.org/)

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bition of H1L by ATA might contribute to the observed down-regulation of viral early gene transcription and replication. Although it has been reported that H1L dephosphorylates Stat1 in vaccinia virus-infected, gamma interferon-treated cells (24), this is unlikely to be related to the transcriptional down-regulation of vaccinia virus early genes observed. We attempted to determine whether ATA treatment during infection could reverse Stat1 dephosphorylation. However, this effect could not be observed, as ATA treatment alone was able to prevent Stat1 phosphorylation (data not shown). The vaccinia virus F18 protein has also been reported to be a substrate for H1L (19). Due to a lack of reagents, we were unable to test the effect of ATA on F18 phosphorylation.

It should also be noted that the IC₅₀ value for H1L is much lower than the concentration required to inhibit 50% virus replication in tissue culture. The negatively charged nature of ATA may hinder its ability to cross the lipid bilayer of the cell (18), and therefore, the bioavailability of ATA in the cytoplasm may be compromised.

This study provides evidence that ATA has the potential to be of therapeutic use against poxviruses by blocking the early stage of replication. We have shown that ATA blocks ERK activation, an important cellular event for vaccinia virus replication. ATA also inhibits the activity of H1L, a viral enzyme essential for the initiation of viral gene transcription. The activity of both of these factors is required at the early stage of vaccinia virus replication. To our knowledge, this is the first example of an antiviral chemical that targets both cellular and viral factors required for virus replication. The low toxicity of ATA, as demonstrated by CC₅₀ analysis and the easy reversibility of its effect on cells, combined with its potent and selective inhibition of vaccinia virus replication make it an ideal drug candidate for further study.

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