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ARTICLE

Aurintricarboxylic acid increases yield of HSV-1 vectors

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Production of large quantities of viral vectors is crucial for the success of gene therapy in the clinic. There is a need for higher titers of herpes simplex virus-1 (HSV-1) vectors both for therapeutic use as well as in the manufacturing of clinical grade adeno-associated virus (AAV) vectors. HSV-1 yield increased when primary human fibroblasts were treated with anti-inflammatory drugs like dexamethasone or valproic acid. In our search for compounds that would increase HSV-1 yield, we investigated another anti-inflammatory compound, aurintricarboxylic acid (ATA). Although ATA has been previously shown to have antiviral effects, we find that low (micromolar) concentrations of ATA increased HSV-1 vector production yields. Our results showing the use of ATA to increase HSV-1 titers have important implications for the production of certain HSV-1 vectors as well as recombinant AAV vectors.

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INTRODUCTION

Recombinant adenovirus-associated virus (rAAV) vectors have been successfully introduced in several human gene therapy clinical trials because of their nonpathogenic nature, low toxicity, minimal immunogenicity, and long-term persistence. Production of large quantities of clinical grade rAAV vectors for gene therapy has been challenging due to limitations in scalability of the commonly used co-transfection protocol.[1] AAVs are not able to replicate by themselves and were first found to propagate only when adenoviruses or herpes viruses coinfected the same cells.[2,3] The first scalable rAAV protocol was based on adenovirus infection of rAAV/Rep-Cap cell lines.[4] Besides adenoviruses, also herpesviruses have been shown to provide complete helper virus functions for the production of AAV virions.[5,6] The minimal set of herpes simplex virus type-1 (HSV-1) genes required for AAV replication and packaging has been identified as the HSV-1 early genes *UL5*, *UL8*, *UL52*, and *UL29*.[7] These genes encode components of the HSV-1 core replication machinery—the helicase, primase, and primase accessory proteins (U_L5, U_L8, and U_L52) and the single-stranded DNA–binding protein (U_L29). A protocol for production of rAAV serotype 2 (rAAV2) vectors using HSV-1 amplicons expressing AAV2 Rep and Cap in combination with rHSV-1 helper vectors has been described,[8] and this protocol was modified and further optimized by several groups using coinfection of two rHSV-1 vectors, both replication-deficient infected-cell protein ICP27-mutants, one carrying rAAV provirus and a second bearing AAV2 Rep-cap genes,[9–11] The use of rHSV-1 vectors has been historically limited by their relatively low titers and the rAAV vector yields in the rHSV-based manufacturing, thus would be affected by the titers of rHSV-1 helper.[12,13] Therefore, several methods to improve rHSV-1 yield have been studied, i.e., changing rHSV-1 propagation conditions[12–14] or using anti-inflammatory compounds known to inhibit the host defense mechanism, like dexamethasone or valproic acid.[15,16] In our search for compounds that would

increase HSV-1 yield, we investigated another anti-inflammatory compound, aurintricarboxylic acid (ATA). ATA is a heterogeneous mixture of polymers accredited with a continuously growing number of biological activities.[17–20] ATA is mostly known as an antiviral agent to several viruses like HIV, herpesvirus HHV-7, SARS-CoV, and others.[20–23] However, ATA did not block the replication of adenovirus type 5[24] and has been reported to increase adenovirus type 5 titer in human embryonic kidney (HEK)-293 cells.[25]

Here, we investigate the effects of ATA on HSV-1 vector production yield in V27, Vero, and HEK-293 cells. We further tested the rHSV-1 stocks produced in the presence of ATA in the HSV-1–based rAAV manufacturing protocol.

RESULTS

ATA effect on HSV-1 yield in V27 cells

To test whether ATA could increase the yield of rHSV-1 d27-1 (d27-1) vector in V27 cells, ATA was applied to the media during the infection (ATA@step1) or dilution steps (ATA@step2) (Figure 1a). Interestingly, ATA treatment delayed HSV-1 plaque formation or cell lysis in V27 cell monolayers. Cytopathic effect at the time of harvest, 72 hours postinfection was between 20 and 60% as compared with 100% cytopathic effect in the absence of ATA (data not shown). To determine which concentrations and conditions for ATA addition have impact on HSV DNase resistant particles per milliliter (DRP/ml) and plaque-forming units per milliliter (PFU/ml) titers, ATA was added at varying concentrations (0–60 μmol/l ATA) either in six-well plates in the first step (ATA@step1) (Figure 1b) or in T150 flasks in both steps (ATA@step1 and ATA@step2) (Figure 1c). Both protocols show HSV yield increase, and the optimal conditions would be either with 50 μmol/l ATA during the infection step (50 μmol/l ATA@step1) that is further diluted to the final concentration of 20 μmol/l ATA or by adding ATA at dilution step (20 μmol/l ATA@step2) to achieve the final concentration of 20 μmol/l
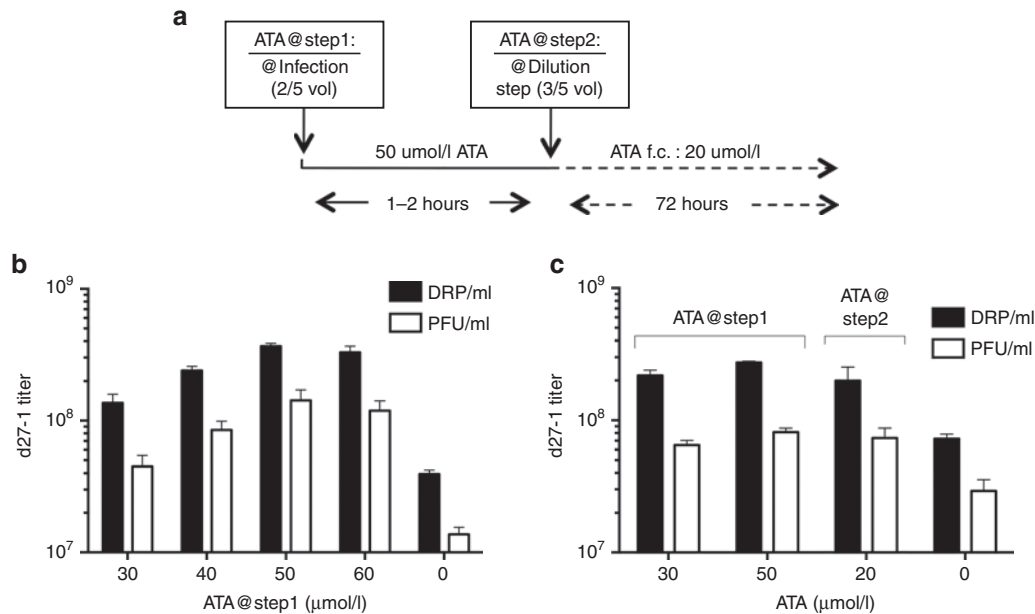
Figure 1 Herpes simplex virus-1 (HSV-1) production protocol using aurintricarboxylic acid (ATA) rationale and protocol optimization. (**a**) HSV-1 production protocol using ATA (ATA-HSV protocol) rationale showing steps, timeline, and when ATA was added to the media: whether it is during the infection (step 1) or dilution (step 2). (**b**) Optimization of ATA-HSV protocol of HSV-1 d27-1 vector production in V27 cells in six-well plates where the ATA concentrations 0–60 µmol/l added during the infection step (ATA@step1) were further reduced to a range between 0 and 24 µmol/l by the addition of 3/5 of final media volume. The optimal concentration in the condition + ATA@step1 was 50 µmol/l ATA (50 µmol/l ATA@step1). Results are representative of two independent experiments ($n = 2$) and are expressed as mean + SD. (**c**) Optimization of ATA-HSV protocol in T150 flasks cultures, where ATA and 10% fetal bovine serum were added ATA in either of step 1 (ATA@step1; 30 or 50 µmol/l) or in step 2 (20 µmol/l ATA@step2). The highest HSV-1 d27-1 titers were achieved in both conditions, ATA@step1 (50 µmol/l ATA) or ATA@step2 (20 µmol/l ATA). The HSV titers in both **b** and **c** are expressed as mean values + SD of DNase resistant particles per milliliter (DRP/ml), shown as black bars, and as mean values + SD of plaque-forming units per milliliter (PFU/ml), shown as white bars. Results are representative of two independent experiments ($n = 2$). The y-axis is in linear scale starting with values (**a**) 1×10^7 or (**b**) 5×10^7 PFU/ml or DRP/ml.

ATA (Figure 1b,c). In this example, the supernatant titers were $2.7 \pm 0.1 \times 10^8$ DRP/ml and $8.1 \pm 0.7 \times 10^7$ PFU/ml in 50 µmol/l ATA@step1 protocol, $2.0 \pm 0.7 \times 10^8$ DRP/ml and $7.4 \pm 1.4 \times 10^7$ PFU/ml in 20 µmol/l ATA@step2 protocol, as compared with $7.3 \pm 0.6 \times 10^7$ DRP/ml and $2.9 \pm 0.6 \times 10^7$ PFU/ml of untreated control (0 µmol/l ATA) (Figure 1c). Results are expressed as means ± SD from two independent experiments ($n = 2$).

Importance of serum presence in ATA-HSV protocol
We tested the effect of serum-free media on DRP/ml and PFU/ml HSV-1 titers in the above-described ATA-HSV protocol, which used 10% fetal bovine serum (FBS) (Figure 2). In this example of ATA@step2 protocol, serum-free media resulted in d27-1 titer reduction from $3.2 \pm 0.1 \times 10^8$ DRP/ml and $5.4 \pm 0.3 \times 10^7$ PFU/ml (10% FBS) to $1.1 \pm 0.2 \times 10^7$ DRP/ml (0% FBS), where PFU/ml titer value was below the detection limit (Figure 2). Even more dramatic effect of serum-free media was seen in the ATA@step1 protocol when both DRP/ml and PFU/ml titer values were below the detection limit (data not shown).

Effect of ATA on wild-type HSV-1 titers in culture
The effects of ATA on DRP/ml viral titers of wild-type (wt) HSV-1 strains (KOS and McIntyre) were tested when propagated in HEK-293 or Vero cells using the 50 µmol/l ATA@step1 protocol (Figure 3a). In Vero cells, one-way analysis of variance and Tukey's multiple comparison test have shown that ATA significantly increased (***$P < 0.001$; $n = 4$) only the KOS strain DRP/ml titers; from $1.7 \pm 1.1 \times 10^8$ DRP/ml (−ATA) to $9.1 \pm 2.1 \times 10^8$ DRP/ml (+ATA) (Figure 3a). The McIntyre virus

"+ ATA" titers were also elevated, from $2.6 \pm 1.5 \times 10^8$ DRP/ml (−ATA) to $5.8 \pm 1.1 \times 10^8$ DRP/ml (+ATA), but according analysis of variance, the difference was not significant ($P > 0.05$; $n = 4$); (Figure 3a). On the contrary, in HEK-293 cells, only McIntyre strain titers were significantly increased (***$P < 0.001$; $n = 4$) by ATA, from $1.4 \pm 0.8 \times 10^8$ DRP/ml (−ATA) to $1.2 \pm 0.5 \times 10^9$ DRP/ml (+ATA) (Figure 3a). The titers of KOS strain in HEK-293 cells, on the other hand, even dropped, from $1.1 \pm 0.9 \times 10^8$ DRP/ml (−ATA) to $5.5 \pm 4.9 \times 10^7$ DRP/ml (+ATA), but according analysis of variance, the difference was not significant ($P > 0.05$; $n = 4$) (Figure 3a). In a larger study ($n = 10$) conducted in six-well plates, two-way analysis of variance and Sidak's multiple comparison test have shown that ATA significantly increased (**$P < 0.01$; $n = 10$) ICP27-deficient vector rHSV-enhanced green fluorescent protein (EGFP) (d27-GFP) DRP/ml titers in V27 cells, from $5.4 \pm 2.7 \times 10^7$ to $3.3 \pm 1.2 \times 10^8$ DRP/ml and that DRP/ml titers of wtHSV-1 McIntyre strain in HEK-293 cells have significantly increased from $1.1 \pm 0.5 \times 10^8$ to $1.0 \pm 0.3 \times 10^9$ DRP/ml (***$P < 0.001$) (Figure 3b).

Effect of residual ATA in HSV stocks on the production of rAAV virions
Because our ultimate goal is rAAV production, we investigated whether the presence of ATA in rHSV-1 stocks would influence rAAV yields. The rAAV-GFP vector was produced by coinfection of rHSV-rep2/cap2 and rHSV-EGFP vectors in HEK-293 cells using rHSV-1 stocks not containing ATA, or rHSV-1 stocks prepared under ATA@step1 protocol (see Materials and Methods). The effect of residual ATA in rHSV-rep2/cap2 stock (~3 µmol/l) resulted in a small 1.3-fold (statistically significant) increase in rAAV titer to $3.8 \times 10^{10} \pm 2.4 \times 10^9$ DRP/ml (**$P < 0.01$) when
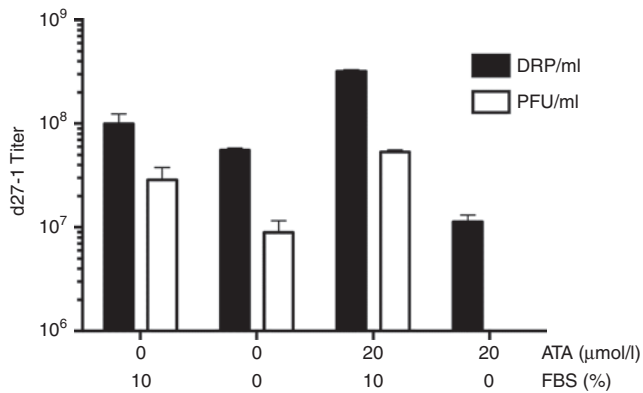
Figure 2 The importance of fetal bovine serum (FBS) in ATA-HSV protocol. Following experiments were conducted to determine optimal conditions, and the effect of the presence or absence of 10% FBS on HSV titer in the ATA@step2 protocol. In this example, 20 µmol/l ATA and 10% FBS were either added or omitted in step 2 (ATA@step2). When ATA was added without presence of 10% FBS, the DNase resistant particles per milliliter (DRP/ml) titer of HSV-1 d27-1 vector was reduced, and the plaque-forming units per milliliter (PFU/ml) titer was below detection limit. The HSV-1 d27-1 titers (d27-1) are expressed as mean values + SD of DRP/ml, shown as black bars, and as mean values + SD of PFU/ml, shown as white bars. Results are representative of two independent experiments ($n = 2$) and are expressed as mean + SD. ATA, aurintricarboxylic acid; HSV, Herpes simplex virus.

compared with the rAAV DRP/ml titer made by using naive rHSV-1 stocks, $2.8 \times 10^{10} \pm 2.8 \times 10^{9}$ DRP/ml ($n = 4$). As shown in Figure 4, ATA also slightly increased rAAV yield (DRP/cell) when 10 µmol/l ATA was spiked directly into HEK-293 cell media during the rHSV-1 coinfection step. This effect was not observed when the ATA concentrations were higher than 15 µmol/l.

DISCUSSION

In this study, we have shown for the first time that micromolar concentrations of ATA increase titers of certain HSV-1 strains in various cell lines. In rHSV-1–based rAAV manufacturing protocol, the yield of rAAV is limited by the maximal titer of helper rHSV-1 vectors, and in addition, the presence of residual ATA in rHSV-1 stocks did not negatively influence rAAV yield. Thus, these findings are important both for large-scale rHSV-1 vector production and rAAV vector production. Previously, several groups were investigating possibilities to increase HSV-1 titers by changing the propagation conditions or by using reagents capable of decreasing host defense.[13–16] HSV-1 is known to both induce and partially evade host antiviral responses.[26] Both dexamethasone and valproic acid have been shown to increase HSV-1 yield by either inhibition of cellular defense against viral propagation or induction of interferon (IFN)-responsive antiviral genes.[15,16] ATA was reported to reduce inducible nitric oxide synthase (iNOS) expression, to inhibit JAK-STAT signaling, or to prevent IFN-mediated transcriptional activation.[27,28] ATA has also previously been shown to increase adenovirus type 5 titer in HEK-293 cells.[25]

HSV-1 infection activates innate immune system by inducing intracellular signaling pathways that lead to the expression of proteins with proinflammatory and microbicidal activities, including cytokines and IFNs.[29–31] IFN signaling is one of the most important cellular defense mechanism for viral clearance;[32,33] however, both Vero and HEK-293 cells have a dysfunctional intracellular antiviral signaling pathways.[34–38] Vero cells have inability to produce IFN-β, while HEK-293 cells do not produce IFN-α,
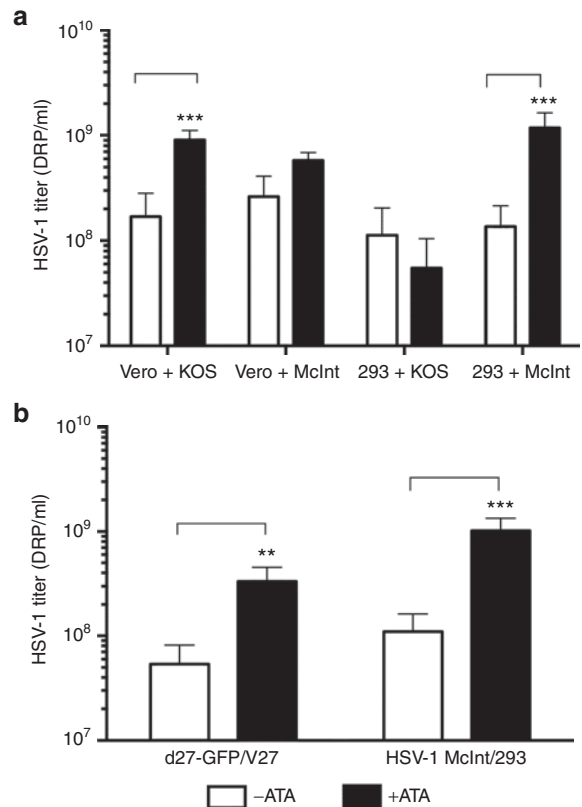

Figure 3 Aurintricarboxylic acid (ATA) effect on wild-type herpes simplex virus-1 (wtHSV-1) KOS and McIntyre strains titers in culture. (**a**) wtHSV-1 strains KOS and McIntyre were propagated in human embryonic kidney (HEK)-293 and Vero cells using the ATA@step1 protocol (50 µmol/l ATA@step1) in six-well plates, and HSV-1 titers were assessed in supernatant harvested 72 hours postinfection. The DNase resistant particles per milliliter (DRP/ml) HSV-1 titers are representative of four independent experiments ($n = 4$) and are expressed as mean + SD, where the black bars represent ATA-treated samples (+ATA) and white bars represent untreated controls (−ATA). (**b**) DRP/ml titers of HSV-1 d27-GFP in V27 cells (d27-GFP/V27) and wtHSV-1 McIntyre strain in HEK-293 cells (HSV-1 McInt/293) using the 50 µmol/l ATA@step1. The black bars represent ATA-treated samples (+ATA), and white bars represent untreated controls (−ATA). The HSV-1 titers are expressed as mean values + SD of DRP/ml ($n = 10$). The y-axis is in log10 scale starting with a value of 1×10^{7} DRP/ml. Statistics for **a** were analyzed by one-way analysis of variance (ANOVA) and Tukey's multiple comparison test and for **b** by two-way ANOVA and Sidak's multiple comparison test. −ATA versus +ATA: ***$P < 0.001$, **$P < 0.01$; nonsignificant: $P > 0.05$. GFP, green fluorescent protein; KOS, wtHSV-1 KOS strain, McInt, wtHSV-1 McIntyre strain.

which can explain their permissive nature to viral production.[34–38] ATA is known as an activator of Raf/MEK/MAPK pathway, insulin-like growth factor-1 receptor, and protein kinase C signaling.[39,40] It has been reported that ATA has a survival-promoting effect transducted via activation of the insulin-like growth factor-1 receptor signaling pathway and Akt, MAP, and p42/p44 mitogen-activated protein kinases (Erk-1 and -2).[39,41] We have observed that ATA treatment delayed HSV-1 plaque formation, cell lysis in V27 cell monolayers, and CPE, which may suggest its antiapoptotic properties. ATA has also been shown to prevent apoptotic cell death in a variety of cell types including breast cancer MDA-231 and MCF-7 cells, macrophage RAW 264.7 cells, and rat pheochromocytoma PC12 cells.[28,41–43]

Interestingly, ATA in millimolar and higher concentrations is known as an antiviral agent.[20–23] In Vero, V27, and HEK-293 cells, we
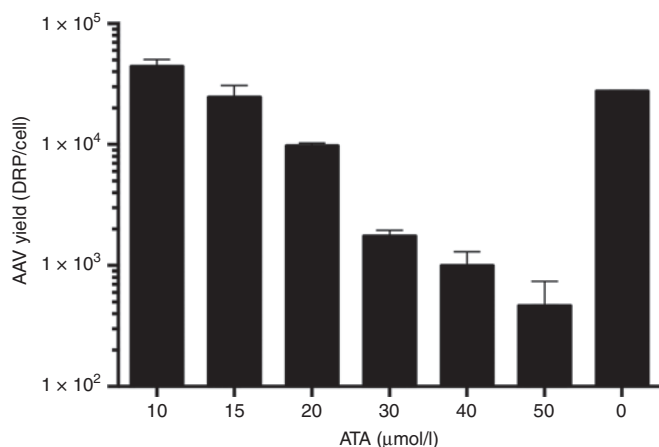
Figure 4 Effect of aurintricarboxylic acid (ATA) residues in herpes simplex virus-1 (HSV-1) stocks on the production or recombinant adeno-associated virus (rAAV) virions. The rAAV-GFP vector was produced by coinfection of rHSV-rep2/cap2 and rHSV-EGFP vectors in human embryonic kidney (HEK)-293 cells 60-mm plates. ATA was shown to slightly increase rAAV yields (DNase resistant particles (DRP) per cell) when 10 μmol/l ATA was spiked directly into HEK-293 cell media during 2 hours of HSV coinfection step. This effect was not observed when the ATA concentrations in HEK-293 cells during 2 hours of HSV coinfection were higher than 15 μmol/l. Results are representative of two independent experiments ($n = 2$) and are expressed as mean + SD of rAAV (DRP/cell. eGFP, enhanced GFP, GFP, green fluorescent protein.

find that HSV-1 titer actually increases when ATA was at micromolar concentrations; however, a possible antiviral effect was observed when ATA was added into serum-free media. Because ATA, being a polycarboxylate, would bind by electrostatic interactions to any protein that contain positively charged residues given the myriad of possible interaction sites, it was considered as a nonspecific enzyme inhibitor.[20,44] To establish an exact mechanism of action of ATA in HSV-1 yield increase, therefore, would simply remain unknown at this point. Our findings that ATA increases HSV-1 yield has important implications both for large-scale rHSV-1 production and rAAV vector production.

MATERIALS AND METHODS
Cells and viruses
Vero-derived V27 cells[45] and HEK-derived 293 cells (HEK-293 cells)[46] were obtained from the Applied Genetic Technologies Corporation (Alachua, FL). Vero cells were purchased from American Type Culture Collection (Manassas, VA). All cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% FBS (HyClone, South Logan, UT) and either 50 mg/ml G418, Geneticin (Invitrogen Life Technologies, Grand Island, NY) for V27 cells or 1% penicillin/streptomycin (Corning Cellgro Mediatech, Manassas, VA) for the other cells. The wtHSV-1 KOS strain and the ICP27-deficient derivatives of the wtHSV-1 KOS strain: vectors d27-1,[47] rHSV-rep2/cap2, and rHSV-EGFP,[11] and their producer ICP27-complementing V27 cell line were obtained from the Applied Genetic Technologies Corporation. The wtHSV-1 McIntyre strain was purchased from Advanced Biotechnologies (Columbia MD).

ATA experiments
A 0.5 mmol/l stock of ATA (Sigma-Aldrich, St Louis, MO), generated in 100 mmol/l sodium bicarbonate water solution, was further diluted in DMEM ± 10% FBS into concentration ranges of 12–60 μmol/l ATA (8.5–21 μg ATA/ml).

HSV-1 production
The ICP27-deficient vectors d27-1, rHSV-rep2/cap2, and rHSV-EGFP strains were propagated in ICP27-complementing V27 cell line. The wtHSV-1 KOS strain and wtHSV-1 McIntyre strain were propagated in Vero or HEK-293 cell

lines. ATA was applied to the media during the infection (ATA@step1) or dilution step (ATA@step2) (Figure 1a). The HSV-1 infection at multiplicity of infection of 0.15 (typically 6×10^5 cells in a six-well plate) was performed in 40% (2/5 vol) of the total final media volume for 1–2 hours, and the remaining media (60% or 3/5 of the total final volume) was added during the dilution step. The cells were then incubated for 72 hours and supernatant harvested to perform assays to obtain titers in DRP/ml and PFU/ml. Infectious vector particles were harvested 72 hours postinfection by collecting the culture supernatant. The titers of HSV-1 stocks in DRP/ml were determined by Taqman assay. Viral genomes within crude culture medium were quantified via treatment in the presence of DNase I (Promega, Madison, WI) (50 U/ml final concentration) at 37 °C for 60 minutes, followed by digestion with proteinase K (Invitrogen Life Technologies) (1 U/ml final concentration) at 50 °C for 60 minutes, and then denatured at 95 °C for 30 minutes. Linearized plasmid pZero 195 UL36, obtained from Applied Genetic Technologies Corporation, was used to generate standard curves. The primer–probe set was specific for the vector genome UL36 sequence (HSV-UL36 F: 5′-GTTGGTTATGGGGGAGTGTGG, HSV-UL36 R: 5′-TCCTTGTCTGGGGTGTCTTCG, and HSV-UL36 Probe: 5′- 6FAM – CGACGAAGACTCCGACGCCACCTC-TAMRA). Amplification of the polymerase chain reaction (PCR) product was achieved with the following cycling parameters: 1 cycle at 50 °C for 2 minutes, 1 cycle at 95 °C for 10 minutes; 40 cycles at 95 °C for 15 seconds, and 40 cycles at 60 °C for 60 seconds. The results were expressed as a mean of rHSV DRP/ml titers ± SD and were statistically analyzed by Prism 5.0d GraphPad Software (GraphPad Software, La Jolla, CA). The titers of HSV-1 stocks in PFU/ml were also determined within crude culture medium. PFU/ml were quantified by serial dilutions in DMEM 10% FBS, 1% penicillin/streptomycin, and treatment to either V27 or HEK-293 cells. At 6 hours post-treatment, infectious media content was adjusted to DMEM/10% FBS/1% PenStrep/0.2% γ-globulins by spiking an appropriate 4% (w/v) γ-globulin in phosphate-buffered saline solution into DMEM/10% FBS/1% PenStrep. At 72 hours postinfection, media is removed, and plates dried and then fixed. Cell monolayers are treated with horse radish peroxidase–conjugated α-HSV antibody (Dako, Carpinteria, CA) followed by Vector VIP Peroxidase Substrate Kit (Vector Labs, Burlingame, CA) for enumeration and PFU titer calculation. The results were expressed as a mean of rHSV PFU/ml titers ± SD and were statistically analyzed by Prism 5.0d GraphPad Software.

rAAV production
HEK-293 cells (2.5×10^6 cells) were simultaneously coinfected with both rHSV-rep2/cap2 and rHSV-EGFP vectors as described by Kang et al.[11] At 2–4 hour postinfection, infectious medium was exchanged with DMEM + 10% FBS equivalent to double the preinfection culture volume. At the time of harvest, the cell pellet was frozen at −80 °C. DRP titers were quantified by real-time PCR in a 96-well block Applied Biosystems 7500 Fast Real-Time PCR System (Applied Biosystems Life Technologies, Grand Island, NY). Crude samples were subjected to three cycles of freezing and thawing, then incubated in the presence of 250 U/ml of Benzonase Endonuclease (EMD Millipore, Billerica, MA) in 2 mmol/l $MgCl_2$, 1% final concentration protein grade Tween 80, and incubated at 37 °C for 60 minutes, followed by 0.25% Gibco Trypsin (Invitrogen Life Technologies) digestion at 50 °C for 60 minutes. Finally, treatment with DNase I (50 U/ml final concentration) at 37 °C for 30 minutes was performed and then denaturation at 95 °C for 20 minutes. Linearized plasmid pDC67/+SV40 (Genzyme, Framingham, MA) was used to generate standard curves. The primer–probe set was specific for the simian virus 40 (SV40) poly (A) sequence: rAAV-F: 5′- AGCAATAGCATCACAAATTTCACAA-3′, rAAV-R: 5′-GCAGACATGATAAGATACATTGATGAGTT-3′, and rAAV-Probe: 5′ 6-FAM-AGCATTTTTTTCACTGCATTCTAGTTGTGGTTTGTC-TAMRA-3′. Amplification of the PCR product was achieved with the following cycling parameters: 1 cycle at 50 °C for 2 minutes, 1 cycle at 95 °C for 10 minutes; 40 cycles at 95 °C for 15 seconds, and 40 cycles at 60 °C for 60 seconds. The results were expressed as a mean of rAAV DRP/ml titers ± SD and were statistically analyzed by Prism 5.0d GraphPad Software.

REFERENCES

1 Xiao, W, Chirmule, N, Berta, SC, McCullough, B, Gao, G and Wilson, JM (1999). Gene therapy vectors based on adeno-associated virus type 1. *J Virol* **73**: 3994–4003.

2 Casto, BC, Atchison, RW and Hammon, WM (1967). Studies on the relationship between adeno-associated virus type I (AAV-1) and adenoviruses. I. Replication of AAV-1 in certain cell cultures and its effect on helper adenovirus. *Virology* **32**: 52–59.

3 Atchison, RW (1970). The role of herpesviruses in adenovirus-associated virus replication *in vitro*. *Virology* **42**: 155–162.

4 Clark, KR, Voulgaropoulou, F, Fraley, DM and Johnson, PR (1995). Cell lines for the production of recombinant adeno-associated virus. *Hum Gene Ther* **6**: 1329–1341.

5 Buller, RM, Janik, JE, Sebring, ED and Rose, JA (1981). Herpes simplex virus types 1 and 2 completely help adenovirus-associated virus replication. *J Virol* **40**: 241–247.

6 Mishra, L and Rose, JA (1990). Adeno-associated virus DNA replication is induced by genes that are essential for HSV-1 DNA synthesis. *Virology* **179**: 632–639.

7 Weindler, FW and Heilbronn, R (1991). A subset of herpes simplex virus replication genes provides helper functions for productive adeno-associated virus replication. *J Virol* **65**: 2476–2483.

8 Conway, JE, Zolotukhin, S, Muzyczka, N, Hayward, GS and Byrne, BJ (1997). Recombinant adeno-associated virus type 2 replication and packaging is entirely supported by a herpes simplex virus type 1 amplicon expressing Rep and Cap. *J Virol* **71**: 8780–8789.

9 Conway, JE, Rhys, CM, Zolotukhin, I, Zolotukhin, S, Muzyczka, N, Hayward, GS *et al*. (1999). High-titer recombinant adeno-associated virus production utilizing a recombinant herpes simplex virus type I vector expressing AAV-2 Rep and Cap. *Gene Ther* **6**: 986–993.

10 Booth, MJ, Mistry, A, Li, X, Thrasher, A and Coffin, RS (2004). Transfection-free and scalable recombinant AAV vector production using HSV/AAV hybrids. *Gene Ther* **11**: 829–837.

11 Kang, W, Wang, L, Harrell, H, Liu, J, Thomas, DL, Mayfield, TL *et al*. (2009). An efficient rHSV-based complementation system for the production of multiple rAAV vector serotypes. *Gene Ther* **16**: 229–239.

12 Lim, F and Neve, RL (2001). Generation of high-titer defective HSV-1 vectors. *Curr Protoc Neurosci* **6**: 4.13.1–4.13.17.

13 Ozuer, A, Wechuck, JB, Russell, B, Wolfe, D, Goins, WF, Glorioso, JC *et al*. (2002). Evaluation of infection parameters in the production of replication-defective HSV-1 viral vectors. *Biotechnol Prog* **18**: 476–482.

14 Wechuck, JB, Goins, WF, Glorioso, JC and Ataai, MM (2000). Effect of protease inhibitors on yield of HSV-1-based viral vectors. *Biotechnol Prog* **16**: 493–496.

15 Erlandsson, AC, Bladh, LG, Stierna, P, Yucel-Lindberg, T, Hammarsten, O, Modéer, T *et al*. (2002). Herpes simplex virus type 1 infection and glucocorticoid treatment regulate viral yield, glucocorticoid receptor and NF-kappaB levels. *J Endocrinol* **175**: 165–176.

16 Otsuki, A, Patel, A, Kasai, K, Suzuki, M, Kurozumi, K, Chiocca, EA *et al*. (2008). Histone deacetylase inhibitors augment antitumor efficacy of herpes-based oncolytic viruses. *Mol Ther* **16**: 1546–1555.

17 Tscherne, JS and Pestka, S (1975). Inhibition of protein synthesis in intact HeLa cells. *Antimicrob Agents Chemother* **8**: 479–487.

18 Mikelens, PE, Woodson, BA and Levinson, WE (1976). Association of nucleic acids with complexes of N-methyl isatin-beta-thiosemicarbazone and copper. *Biochem Pharmacol* **25**: 821–827.

19 Vollgraf, U, Wegner, M and Richter-Landsberg, C (1999). Activation of AP-1 and nuclear factor-kappaB transcription factors is involved in hydrogen peroxide-induced apoptotic cell death of oligodendrocytes. *J Neurochem* **73**: 2501–2509.

20 De Clercq, E (2011). The next ten stories on antiviral drug discovery (part E): advents, advances, and adventures. *Med Res Rev* **31**: 118–160.

21 Cushman, M, Wang, PL, Chang, SH, Wild, C, De Clercq, E, Schols, D *et al*. (1991). Preparation and anti-HIV activities of aurintricarboxylic acid fractions and analogues: direct correlation of antiviral potency with molecular weight. *J Med Chem* **34**: 329–337.

22 Zhang, Y, Schols, D and De Clercq, E (1999). Selective activity of various antiviral compounds against HHV-7 infection. *Antiviral Res* **43**: 23–35.

23 Yap, Y, Zhang, X, Andonov, A and He, R (2005). Structural analysis of inhibition mechanisms of aurintricarboxylic acid on SARS-CoV polymerase and other proteins. *Comput Biol Chem* **29**: 212–219.

24 He, R, Adonov, A, Traykova-Adonova, M, Cao, J, Cutts, T, Grudesky, E *et al*. (2004). Potent and selective inhibition of SARS coronavirus replication by aurintricarboxylic acid. *Biochem Biophys Res Commun* **320**: 1199–1203.

25 Myskiw, C, Deschambault, Y, Jefferies, K, He, R and Cao, J (2007). Aurintricarboxylic acid inhibits the early stage of vaccinia virus replication by targeting both cellular and viral factors. *J Virol* **81**: 3027–3032.

26 Mossman, KL, Macgregor, PF, Rozmus, JJ, Goryachev, AB, Edwards, AM and Smiley, JR (2001). Herpes simplex virus triggers and then disarms a host antiviral response. *J Virol* **75**: 750–758.

27 Chen, CW, Chao, Y, Chang, YH, Hsu, MJ and Lin, WW (2002). Inhibition of cytokine-induced JAK-STAT signalling pathways by an endonuclease inhibitor aurintricarboxylic acid. *Br J Pharmacol* **137**: 1011–1020.

28 Tsi, CJ, Chao, Y, Chen, CW and Lin, WW (2002). Aurintricarboxylic acid protects against cell death caused by lipopolysaccharide in macrophages by decreasing inducible nitric-oxide synthase induction via IkappaB kinase, extracellular signal-regulated kinase, and p38 mitogen-activated protein kinase inhibition. *Mol Pharmacol* **62**: 90–101.

29 Sainz, B Jr and Halford, WP (2002). Alpha/Beta interferon and gamma interferon synergize to inhibit the replication of herpes simplex virus type 1. *J Virol* **76**: 11541–11550.

30 Haller, O, Kochs, G and Weber, F (2006). The interferon response circuit: induction and suppression by pathogenic viruses. *Virology* **344**: 119–130.

31 Paludan, SR, Bowie, AG, Horan, KA and Fitzgerald, KA (2011). Recognition of herpesviruses by the innate immune system. *Nat Rev Immunol* **11**: 143–154.

32 Brandner, G and Mueller, N (1973). Wirkung von Interferon und poly(I):poly(C) auf Genomfunktionen von Herpes-simplex-Virus Typ 1. *Hoppe Seylers Z Physiol Chem* **354**: 1176.

33 de Vries, W, Haasnoot, J, van der Velden, J, van Montfort, T, Zorgdrager, F, Paxton, W *et al*. (2008). Increased virus replication in mammalian cells by blocking intracellular innate defense responses. *Gene Ther* **15**: 545–552.

34 Desmyter, J, Melnick, JL and Rawls, WE (1968). Defectiveness of interferon production and of rubella virus interference in a line of African green monkey kidney cells (Vero). *J Virol* **2**: 955–961.

35 Mosca, JD and Pitha, PM (1986). Transcriptional and posttranscriptional regulation of exogenous human beta interferon gene in simian cells defective in interferon synthesis. *Mol Cell Biol* **6**: 2279–2283.

36 Xanthoudakis, S, Alper, D and Hiscott, J (1987). Transient expression of the beta interferon promoter in human cells. *Mol Cell Biol* **7**: 3830–3835.

37 Alexopoulou, L, Holt, AC, Medzhitov, R and Flavell, RA (2001). Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. *Nature* **413**: 732–738.

38 Ali, S and Kukolj, G (2005). Interferon regulatory factor 3-independent double-stranded RNA-induced inhibition of hepatitis C virus replicons in human embryonic kidney 293 cells. *J Virol* **79**: 3174–3178.

39 Beery, R, Haimsohn, M, Wertheim, N, Hemi, R, Nir, U, Karasik, A *et al*. (2001). Activation of the insulin-like growth factor 1 signaling pathway by the antiapoptotic agents aurintricarboxylic acid and evans blue. *Endocrinology* **142**: 3098–3107.

40 Chen, N, Ma, WY, She, QB, Wu, E, Liu, G, Bode, AM *et al*. (2001). Transactivation of the epidermal growth factor receptor is involved in 12-O-tetradecanoylphorbol-13-acetate-induced signal transduction. *J Biol Chem* **276**: 46722–46728.

41 Haimsohn, M, Beery, R, Karasik, A, Kanety, H and Geier, A (2002). Aurintricarboxylic acid induces a distinct activation of the IGF-I receptor signaling within MDA-231 cells. *Endocrinology* **143**: 837–845.

42 Mesner, PW, Winters, TR and Green, SH (1992). Nerve growth factor withdrawal-induced cell death in neuronal PC12 cells resembles that in sympathetic neurons. *J Cell Biol* **119**: 1669–1680.

43 Batistatou, A and Greene, LA (1993). Internucleosomal DNA cleavage and neuronal cell survival/death. *J Cell Biol* **122**: 523–532.

44 Bina-Stein, M and Tritton, TR (1976). Aurintricarboxylic acid is a nonspecific enzyme inhibitor. *Mol Pharmacol* **12**: 191–193.

45 Rice, SA, Su, LS and Knipe, DM (1989). Herpes simplex virus alpha protein ICP27 possesses separable positive and negative regulatory activities. *J Virol* **63**: 3399–3407.

46 Graham, FL, Smiley, J, Russell, WC and Nairn, R (1977). Characteristics of a human cell line transformed by DNA from human adenovirus type 5. *J Gen Virol* **36**: 59–74.

47 Rice, SA and Knipe, DM (1990). Genetic evidence for two distinct transactivation functions of the herpes simplex virus alpha protein ICP27. *J Virol* **64**: 1704–1715.