Aurintricarboxylic acid increases yield of HSV-1 vectors

Peter Pechan1, Jeffery Ardinger1, Jyothi Ketavarapu1, Hillard Rubin1, Samuel C Wadsworth1 and Abraham Scaria1

INTRODUCTION

Recombinant adenovirus-associated virus (rAAV) vectors have been successfully introduced in several human gene therapy clinical trials because of their non-pathogenic 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 coinfect 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 (UL5, UL8, and UL52) and the single-stranded DNA–binding protein (UL29). A protocol for production of rAAV serotype 2 (rAAV2) vectors using HSV-1 ampiclons expressing AAV2 Rep and Cap in combination with rHSV-1 helper vectors has been described,3 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.8–11 The use of rHSV-1 vectors has been historically limited by their relatively low titers and the rAAV vector yields in the HSV-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 conditions12–14 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 524 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. Cytotoxic 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 (DPK/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...
ATA increases HSV-1 yield

P Pechan et al

Figure 1  Herpes simplex virus-1 (HSV-1) production protocol using aminooxycarbonyl 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 either in 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 DNA 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 × 107 or (b) 5 × 105 PFU/ml or DRP/ml.

ATA (Figure 1b,c). In this example, the supernatant titers were 2.7 ± 0.1 × 106 DRP/ml and 8.1 ± 0.7 × 107 PFU/ml in 50 µmol/l/ATA@step1 protocol, 2.0 ± 0.7 × 106 DRP/ml and 7.4 ± 1.4 × 107 PFU/ml in 20 µmol/l/ATA@step2 protocol, as compared with 7.3 ± 0.6 × 107 DRP/ml and 2.9 ± 0.6 × 107 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 ± 0.1 × 107 DRP/ml and 5.4 ± 0.3 × 107 PFU/ml (10% FBS) to 1.1 ± 0.2 × 107 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 rHSV-1/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 = 10) the McIntyre strain DRP/ml titers; from 1.7 ± 1.1 × 106 DRP/ml (−ATA) to 9.1 ± 2.1 × 106 DRP/ml (+ATA) (Figure 3a). The McIntyre virus ‘+’ ATA” titers were also elevated, from 2.6 ± 1.5 × 106 DRP/ml (−ATA) to 5.8 ± 1.1 × 107 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 ± 0.8 × 106 DRP/ml (−ATA) to 1.2 ± 0.5 × 107 DRP/ml (+ATA) (Figure 3a). The titers of KOS strain in HEK-293 cells, on the other hand, even dropped, from 1.1 ± 0.9 × 106 DRP/ml (−ATA) to 5.5 ± 4.9 × 105 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 ± 2.7 × 107 to 3.3 ± 1.2 × 108 DRP/ml and that DRP/ml titers of wtHSV-1 McIntyre strain in HEK-293 cells have significantly increased from 1.1 ± 0.5 × 108 to 1.0 ± 0.3 × 109 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 ± 1010 ± 2.4 × 109 DRP/ml (**P < 0.01) when
compared with the rAAV DRP/ml titer made by using naive rHSV-1 stocks, 2.8 × 10^10 ± 2.8 × 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 propagation conditions or by using reagents capable of decreasing host defense. VS–16 HSV-1 is known to both induce and partially evade host antiviral responses. VS 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. VS–16 ATA was reported to reduce inducible nitric oxide synthetase (iNOS) expression, to inhibit JAK-STAT signaling, or to prevent IFN-mediated transcriptional activation. VS–20 ATA has also previously been shown to increase adenovirus type 5 titer in HEK-293 cells. VS

HSV-1 infection activates innate immune system by inducing intracellular signaling pathways that lead to the expression of proteins with proinflammatory and microbiocidal activities, including cytokines and IFNs. VS–31 IFN signaling is one of the most important cellular defense mechanisms for viral clearance; VS–33 however, both Vero and HEK-293 cells have a dysfunctional intracellular antiviral signaling pathways. VS–34 Vero cells have inability to produce IFN-β, while HEK-293 cells do not produce IFN-α, which can explain their permissive nature to viral production. VS–38 ATA is known as an activator of Raf/MEK/MAPK pathway, insulin-like growth factor-1 receptor, and protein kinase C signaling. VS–40 It has been reported that ATA has a survival-promoting effect transduced 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). VS–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-MB-231 and MCF-7 cells, macrophage RAW 264.7 cells, and rat pheochromocytoma PC12 cells. VS–43

Interestingly, ATA in millimolar and higher concentrations is known as an antiviral agent. VS–32 In Vero, V27, and HEK-293 cells, we
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.20,24 To establish an exact mechanism of action of ATA in HSV-1 yield increase, therefore, would simply remain 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.

CONFLICT OF INTEREST

P.J., A.J., J.K., H.R., S.C.W., and A.S. are employees of Genzyme, a Sanofi Company.

ACKNOWLEDGMENTS

We thank Jennifer Tousignant for valuable comments on the manuscript.

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 co-infection 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).

MATERIALS AND METHODS

Cells and viruses

Vero-derived V27 cells65 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% 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% PenStrep/0.2% Gentamycin (Genzyme, Framingham, MA) was used to generate standard curves. The primer-probe set was specific for the vector genome UL36 sequence (HSV-UL36 F: 5′-AGCAATAGCATCACAAATTTCACAA-3′, HSV-UL36 R: 5′-GTTGGTTATGGGGGAGTGTGGA-3′, and HSV-UL36 Probe: 5′-6-FAM-CCAGGAGACTCCTGACGGCACCCTC-TAMRA). Amplification of the polymerase chain reaction (PCR) product was 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 MgCl2, 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 (Promega, Madison, WI) (50 U/ml final concentration) at 37 °C for 30 minutes was performed and then denaturation at 95 °C for 20 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 simian virus 40 (SV40) poly (A) sequence: rAAV-F: 5′-AGCAATAGCATCACAAATTTCACAA-3′, rAAV-R: 5′-GCAGATCGATAAGATACATTGATGAGTT-3′, and rAAV-Probe: 5′-6-FAMAGGATTTTTCACGTGCCGTTCGAGGGTGTGG-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 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 (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 and PFU/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′-GTTGGTTATGGGGGAGTGTGGAGTG, and HSV-UL36 Probe: 5′-6-FAM-CCAGGAGACTCCTGACGGCACCCTC-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.

rAAV production

HEK-293 cells (2.5 × 10⁶ 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, 1% PenStrep/0.2% Gentamycin, and then incubated in the presence of ATA at concentrations lower than 15 µmol/l. Results are representative of two independent experiments (n = 2) and are expressed as mean ± SD of rAAV (DRP/ml).

CONFLICT OF INTEREST

P.J., A.J., J.K., H.R., S.C.W., and A.S. are employees of Genzyme, a Sanofi Company.

ACKNOWLEDGMENTS

We thank Jennifer Tousignant for valuable comments on the manuscript.
