Suppression of influenza A virus replication in human lung epithelial cells by noncytotoxic concentrations bafilomycin A1

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First published October 31, 2014; doi:10.1152/ajplung.00011.2014.

INFLUENZA VIRUSES ARE COMMON pathogens of the upper respiratory tract and a substantial disease burden worldwide. It is estimated that seasonal epidemics affect 10–20% of the world’s population and the virus is associated with significant morbidity and mortality every year (22, 54). Although immunization is the single most effective way to protect against seasonal influenza infection, the vaccine’s effectiveness can be significantly compromised when it does not match the prevalent viral strain(s) in a given year (1, 46). That there are also reports of increasing numbers of drug-resistant flu strains further highlights a need to identify new anti-influenza strategies (19, 20, 53).

Influenza A viruses (IAV) are enveloped, single-stranded RNA viruses that belong to the family Orthomyxoviridae. The genome contains eight negative-sense segments of RNA that encode 10 or more different viral proteins depending on the virus strain (23, 55). The IAV envelope is made up of phospholipids with several embedded proteins including hemagglutinin (HA) and neuraminidase, which are commonly used to classify influenza virus strains. HA is the primary protein responsible for influenza virus binding to receptors on the host cell surface, facilitating cell entry by the virions (26).

Following virus attachment, host cells undergo de novo formation of clathrin-coated pits leading to primary uptake of the virus via endocytosis, although other clathrin- and caveolin-independent pathways also exist (26, 31). The endocytic pathway of IAV includes low-pH-mediated fusion followed by uncoating of the virus (6). Released viral nucleoproteins are subsequently imported into the nucleus for viral gene expression and replication (19). Progeny viral genomes leave the nucleus and assemble into infectious particles at the plasma membrane followed by budding from the apical plasma membrane (26, 29).

Targeting IAV trafficking at different stages of the infectious cycle could quell the ability of the influenza virus to spread and cause disease in its host. Bafilomycin A1 (Baf-A1) is a member of the plecomacrolide subclass of macrolide antibiotics isolated from Streptomyces griseus. Baf-A1 has been shown to be a highly specific vacuolar type H+-ATPase (V-ATPase) inhibitor in various cell types, at effective concentrations of ~0.1–1 μM (100–1,000 nM) (3, 11). Cellular V-ATPases transport protons across the plasma membrane as well as functioning to acidify intracellular compartments, including endosomes and lysosomes (7). Baf-A1 binds to the cellular V-ATPases and blocks proton translocation, resulting in the inhibition of lysosomal proteases that are activated at low pH (3, 60). These concentrations of Baf-A1 prevent V-ATPase-mediated degradation of seques-
tered material and block autophagy flux by interfering with late-stage autophagosome-lysosome fusion (12, 34, 58). When used for short duration in vitro at concentrations from 50 to 100 nM, Baf-A1 inhibits growth of influenza A and B viruses and rhinovirus by inhibiting V-ATPase and preventing endosomal acidification (40, 50). Although these findings are of interest conceptually, their impact is tempered by the fact that these Baf-A1 concentrations are also cytotoxic, thus precluding diarrhea virus by inhibiting V-ATPase and preventing endosomal rect in vivo translation (27). In the present study, we demonstrate that much lower Baf-A1 concentrations (below 1 nM) that are not sufficient to inhibit V-ATPase retain capacity to significantly impair the nuclear accumulation of IAV and dramatically attenuate virus replication and release in a human alveolar epithelial cell line.

**MATERIALS AND METHODS**

**Reagents.** Cell culture media, propidium iodide (PI), 3-(4,5-di-methyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), Baf-A1, and type-I agarose were obtained from Sigma (Sigma-Aldrich, Oakville, CA). Nitrocellulose membrane, prestained protein molecular weight markers, and BCA protein assay kit were obtained from Bio-Rad Laboratories (Mississauga, ON, Canada). pHRetro Red Dextran (pRRD) was from Molecular Probes. All other chemicals used were of the highest analytical grade and were purchased from Sigma (Sigma-Aldrich, Oakville, ON, Canada) or Fisher (Fisher Scientific, Ottawa, ON, Canada).

**Cells, media, and viruses.** Human alveolar lung A549 cell line was purchased from the American Type Culture Collection (ATCC, Manassas, VA) and grown in Dulbecco’s Modified Eagle’s Medium (DMEM, Invitrogen, Burlington, ON, Canada), supplemented with 10% fetal bovine serum (FBS, Invitrogen) and 1% penicillin/streptomycin (Invitrogen). Cells were maintained as monolayers in 10% CO2 at 37°C and passaged by trypsinization at 80–90% confluence. Human influenza virus strain A/PR/8/34 virus (H1N1) was grown in 10-day-old embryonated hens’ eggs from laboratory stocks. The chorioallantoic fluid was harvested, aliquotted, and tailed in Madin-Darby canine kidney (MDCK) cells by standard procedures (5).

**Antibodies.** Antibodies were used in this study for either Western blotting or immunofluorescence (IF), or both. Primary antibodies were mouse anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit-anti LC3 (Sigma-Aldrich, Oakville, CA), rabbit anti-early endosome marker (EEA1, ab2900) (Abcam, Cambridge, MA), and lysosomal-associated membrane protein 3 (LAMP3) (Proteintech Group, Chicago, IL, 12632-1-AP). Alexa Fluor 488 goat anti-rabbit, LysoTracker Red DND-99 (L7528, Molecular Probes) in the culture media for 10 min at 37°C. After incubation, cells were washed with PBS and immediately fixed for 15 min (4% paraformaldehyde/120 mM sucrose in PBS, pH 7.4), and permeabilized for 10 min with 0.3% Triton X-100 in PBS. After incubation with 3% BSA blocking solution for 60 min, cells were incubated overnight with the assigned primary antibodies at 4°C. Cells were then incubated with corresponding secondary antibodies diluted in 1% BSA in PBS for 1 h at room temperature. Cell nuclei were stained with DAPI dye or TO-PRO followed by mounting with ProLong Gold antifade reagent from Invitrogen Molecular Probes. The fluorescent signal was examined and analyzed with an Olympus Fluoview multilaser confocal microscope. Laser intensity and detector sensitivity settings remained constant for all image acquisitions within a respective experiment. The methods for the quantification of IAV nuclear transport have been described previously (62). In brief, following IF staining, the cells were analyzed by IF confocal microscopy and total number of infected cells as well as nuclear staining was counted. Data were then presented as average percentages of nuclear staining of IAV nuclear protein (vNP) in infected cells in Baf-A1-treated cells vs. nontreated control cells.

**Labeling of lysosomal compartments with LysoTracker.** Lysosomal compartments were labeled by incubating the live IAV-infected A549 cells (pretreated with different doses of Baf-A1 for 24 h) with 200 nM LysoTracker Red DND-99 (L7528, Molecular Probes) in the culture media for 10 min at 37°C. After incubation, cells were washed with PBS and immediately fixed for 15 min (4% paraformaldehyde/120 mM sucrose). Fluorescence images were captured by utilizing an Olympus Fluoview multilaser confocal microscope. Olympus Fluoview software, which measures the intensity of staining through threshold analysis, was used to quantify the amount of LysoTracker fluorescence detectable in the control and Baf-A1 cells (14).

**Measurement of lysosomal pH.** Lysosomal pH in was measured in A549 epithelial cells by using the pH-sensitive fluorescent indicator PRD (Molecular Probes). A549 cells were cultured (DMEM/10% FBS) on Nunc Lab-Tek four-well chambered coverglass slides. At confluence, the cultures were treated with Baf-A1 (0, 0.1, 1, and 10 ng/ml) for 24 h. Thereafter, cell nuclei were stained with 10 μg/ml Hoechst 33342 (Hank’s balanced salt solution-20 mM HEPES; pH 7.4) for 10 min (37°C). Cells were washed with HBSS then immediately incubated (40 min, 37°C) in HBSS containing pRRD (33 μg/ml). Cells were then washed with HBSS and the cells in each chamber were covered with HBSS containing the appropriate concentration of Baf-A1.

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Cellular lysosomal fluorescence resulting from pRRD uptake was quantitated by epifluorescence microscopy by using an Olympus IX70 inverted microscope coupled to a Retiga-SRV fast monochrome charge-coupled device camera and Nikon NIS-Elements imaging software. Each well of a chamber slide was imaged by capturing five images, each from distinct areas (image field dimensions were $2.7 \times 10^3 \, \mu m^2$). Integrated pRRD fluorescence over the entire area of each image field was obtained. Background fluorescence was sampled from three distinct areas and the mean was used to calculate the background fluorescence for each image field. The background-corrected integrated pRRD fluorescence of each image field was normalized for cell number by dividing by total nuclear counts to obtain the total integrated pRRD fluorescence per cell (TIFC). For each chamber well the TIFC values from the five separate image fields were averaged. Averaged TIFC values from triplicate experiments were combined to give a final mean TIFC value ($n=3$).

Analysis of cellular morphology. To assess viability of the cells based on gross cellular appearance (chromatin condensation and cell shrinkage), infected and mock-infected A549 cells treated with different doses of Baf-A1 (0–100 nM) in 12-well plates were examined by phase-contrast microscopy (Nikon TE-2000) and cells were photographed with a Canon-A700 digital camera.

MTT assay. Cytotoxic effects of Baf-A1 on the A549 cells was determined by MTT assays as previously described (14). Briefly, cells grown in 12-well plates were pretreated with Baf-A1 (0–100 nM, 24 h) and then infected with mock or A/PR/8/34 virus at a MOI of 1 PFU/cell for each experimental time point (10 and 24 h pi). The percentage of cell viability was calculated as (mean OD of treated cells/mean OD of control cells) × 100. For each time point, the treated cells were compared with control cells that had been treated only with medium. For each individual experiment, a vehicle control was performed at the appropriate time point.

Measurement of apoptosis by FACS analysis. Apoptosis was measured by the Nicoletti method as previously described (37). Briefly, cells grown in 12-well plates were pretreated with Baf-A1 (0–100 nM, 24 h) and then infected with mock or A/PR/8/34 virus at a MOI of 1 PFU/cell for each experimental time point (10 and 24 h pi). After scraping, the cells were harvested by centrifugation at 1,500 g for 5 min, washed once with PBS, and resuspended in hypotonic PI lysis buffer (1% sodium citrate, 0.1% Triton X-100, 0.5 mg/ml RNase A, 40 μg/ml PI). Cell nuclei were incubated for 30 min at 37°C and subsequently analyzed by FACS. Nuclei to the left of the G1 peak containing hypodiploid DNA were considered apoptotic.

Immunoblotting. Western blotting was used to detect pNP, NS1, LC3β, and GAPDH. Briefly, cell pellets were lysed on ice for 30 min in lysis buffer [20 mM Tris·HCl (pH 7.5), 0.5% Nonidet P-40, 0.5 mM MOPS, 100 μM β-glycerol 3-phosphate, and 0.5% protease inhibitor cocktail]. After a high-speed spin (13,000 g for 10 min), supernatant protein content was determined by Lowry protein assay. Equal amounts of proteins were size fractionated by SDS-PAGE and transferred to nitrocellulose membranes. After membranes were blocked with 5% nonfat dried milk and 0.1% Tween 20, blots were incubated overnight with the primary antibodies at 4°C. Blots were then probed with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature and visualized using an enhanced chemiluminescence detection (Amersham-Pharmacia Biotech).

Transmission electron microscopic analyses. For transmission electron microscopy (TEM), cells were fixed in 2.5% glutaraldehyde in PBS (pH 7.4) for 1 h at 4°C and postfixed with 1% osmium tetroxide before being embedded in Epon. TEM images were captured via a CM-10 transmission electron microscope (Philips, Netherlands) at 80 kV, on sectioned cells (100 nm on 200 mesh grids) stained with uranyl acetate and counterstained with lead citrate (15).

Statistical analysis. Data are expressed as means ± SD and statistical differences were evaluated by one-way or two-way ANOVA followed by Tukey’s or Bonferroni’s post hoc test, with GraphPad Prism 5.0 to determine significant differences at a P value <0.05. For all experiments data were collected in triplicate unless otherwise indicated.

RESULTS

Baf-A1 reduces IAV replication and release from A549 cells. Since 100 nM Baf-A1 is known to inhibit influenza virus replication in MDCK cells (40), we assessed the anti-IAV activity of Baf-A1 at different concentrations in A549 human alveolar epithelial cells. Cells were treated with 0–100 nM Baf-A1 for 24 h and then infected with A/PR/8/34 virus at an MOI of 1 PFU/cell. The highest concentrations of Baf-A1 (10 and 100 nM), which are known to effectively suppress V-ATPases, completely blocked viral replication, confirming previous reports. However, at lower concentrations (0.1, 0.5, and 1 nM), which are well below the Baf-A1 IC50 (13), we also observed significantly diminished viral replication, as evidenced by a reduction in expression of the IAV nonstructural protein 1 (NS1) ($P < 0.001$, Fig. 1A) and vNP ($P < 0.01$, Fig. 1B). To examine the effects of Baf-A1 on the production of infectious virus, supernatants from infected A549 cells pretreated with Baf-A1 were collected and assessed using plaque assays (Fig. 1C). Baf-A1 suppressed generation of replicating IAV in a dose-dependent manner, with 0.1 nM Baf-A1 reducing the release of virus by a factor of 1 log10 ($P = 0.003$). Consistent with prior reports, IAV infectivity was also inhibited by ~3 log10 ($P < 0.001$) with 10 and 100 nM Baf-A1. In another experiment we tested whether Baf-A1 treatment was responsible in A/PR/8/34 virus uncoating and affected viral replication. A549 were pretreated with Baf-A1 (0.1 nM, 24 h) and infected with A/PR/8/34 virus (MOI of 1 PFU/cell) or Baf-A1 were added to the infected cells (0.1 nM) 2, 3, and 5 h after A/PR/8/34 virus infection (MOI of 1 PFU/cell). After 24 h of infection cell lysates were prepared and viral proteins (NS1 and NP) protein were investigated by immunoblotting. Baf-A1 suppressed IAV when added to the A549 cells 24 h before infection and did not significantly affect IAV in postinfection treatment ($P < 0.001$) (Fig. 1, D–F).

IAV binding to A549 cells is not affected by Baf-A1. Because influenza virus entry begins with attachment to the surface of the host cell (8, 31, 49), and to gain insight into the mechanism...
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A

B

C

D

E

F

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for Baf-A1 inhibition of IAV replication, we next determined the impact of Baf-A1 on virus binding. Influenza virus was allowed to bind for 60 min on ice to A549 cells that had been pretreated with various Baf-A1 concentrations for 24 h. Cells were then washed and fixed without permeabilization. Cell-associated virus particles were determined by IF confocal microscopy and FACS with use of anti-vNP antibodies. IF confocal microscopy did not reveal any clear qualitative dif-

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**Fig. 2.** Baf-A1 treatment has no effect on influenza A virus (IAV) binding. A549 cells were treated with different concentrations of Baf-A1 for 24 h and then incubated with A/PR/8/34 virus at a MOI of 10 plaque-forming units (PFU)/cell. Cells were exposed to influenza virus on ice for 90 min to allow viral binding, then washed, fixed, and analyzed by immunofluorescence (IF) microscopy and FACS by using anti-vNP antibody. **A:** typical confocal images of A549 cells stained for IAV particles with a mouse anti-vNP monoclonal antibody. Initial attachment and binding of IAV was not affected by Baf-A1 treatment since both Baf-A1-treated (c, d, e, and f) and Baf-A1-untreated (b) A549 cells showed comparable high levels of virus binding. vNP, IAV nuclear protein. **B:** representative FACS histograms of Baf-A1 pretreated A549 cells infected with influenza virus, showing number of attached virus particles in Baf-A1-treated cells (in red) compared with nontreated cells (in black). **C:** quantified data of the FACS histograms displayed in **B.** Treatment of A549 cells with different concentrations of Baf-A1 did not affect the numbers of viruses attached to the cells. Error bars represent the standard deviation for 3 independent experiments. Ø, No Baf-A1 treatment; Nuc, nucleus; NS, not significant.
Fig. 3. Entry of influenza virus ribonucleoproteins (RNPs) into the nucleus is blocked in a dose-dependent manner by Baf-A1. A549 cells grown on glass coverslips and pretreated with different concentrations of Baf-A1 (0–100 nM) for 24 h were infected with A/PR/8/34 virus at MOI of 1 PFU/cell for 24 h. Cells were analyzed by IF confocal microscopy using anti-vNP, anti-EEA1, and anti-LAMP3 antibodies. TO-PRO-3 was used for nuclear staining. A–C: examination of the effect of different concentrations of Baf-A1 (0–100 nM) on subcellular localization of IAV particles by confocal fluorescence microscopy. In the absence of Baf-A1, expression of virus protein NP (red) was observed in the nuclei (blue) of infected cells (Aa and B). The number of vNP within nucleoli was lower in cells treated with 0.1 nM Baf-A1 (Ad). No vNP positive cells (red) were seen in cells treated with high concentrations of Baf-A1 (10 and 100 nM) (A, g and j). The majority of virus particles (red) are trapped in the early endosomes (Ai and li) or late endosomes (Bi and li) of cells treated with 10 and 100 nM Baf-A1.

D: quantitative determination of IAV nuclear transportation. Bar graph shows quantitative analysis for ratios of vNP nuclear imported cells/total infected cells (Fig. 3D). About 42% of vNPs were translocated to the cell nuclei in the control cells but this number was reduced to ~7% in cells treated with 0.1 nM Baf-A1. Translocation of vNP to the cell nuclei was significantly blocked in the cells treated with high concentrations of Baf-A1 (10 and 100 nM). Data show the mean values and SE scored from counting total and infected cells from 5 nonadjacent ×40 high-power fields of view (average 180 cells/field) and presented as average percentages of vNP nuclear staining in infected cells treated with Baf-A1 vs. nontreated control cells. Values are expressed as means ± SD from 3 independent experiments (n = 3), and statistical differences were evaluated by 1-way or 2-way ANOVA followed by Tukey’s or Bonferroni’s post hoc test, using GraphPad Prism 5.0. P < 0.05 was considered significant. ***P < 0.001.
ferences in virus binding to A549 cells treated with any Baf-A1 concentration (Fig. 2A). Quantitative determination of IAV particles bound to A549 cells by FACS analysis confirmed this observation (Fig. 2, B and C). These data indicate that Baf-A1 does not directly affect IAV binding to A549 cells.

**Baf-A1 attenuates IAV intracellular trafficking to nuclei and nuclear import.** Since viral particles are taken up into coated pits and undergo endocytosis (26, 31), we next determined the effect of different concentrations of Baf-A1 on intracellular virus trafficking, with vNP evident in the nucleus as well as in the cytoplasm, where it appeared to colocalize with LAMP3 and had many areas of overlap with small EE1A-positive punctate EE1A (Fig. 3, A and B, a–c). In cells treated with 0.1 nM Baf-A1, reduced labeling of nuclear vNP was evident, but overlapping staining with EE1A and LAMP3 in the cytoplasm was retained (Fig. 3, A and B, d–f). This effect was enhanced in cells treated with higher, 10 and 100 nM Baf-A1 concentrations (Fig. 3, A and B, g–i and j–l). Consistent with data from prior studies (9), our observations suggest that IAVs are retained in early and late endosomes in cells treated with relatively high concentrations of Baf-A1.

We also quantified abundance of the vNP during IAV infection of A549 cells (24 h pi) pretreated with different concentrations of Baf-A1. Quantitative analysis of vNP abundance by IF microscopy showed that at least 42% of vNP in infected cells accumulated in nuclei of Baf-A1-naive control cells (Fig. 3, Ca and D). Nuclear vNP was significantly decreased to ~7% in cells pretreated with 0.1 nM Baf-A1 (Fig. 3, Cb and D). Nuclear vNP was virtually absent in cells treated with 10 or 100 nM Baf-A1 (Fig. 3, Cc, Cd, and Dc). Collectively, these data show that, even at low concentration, Baf-A1 attenuates nuclear accumulation of IAV NP protein, suggesting that endosomal and vesicular trafficking are significantly impacted.

**Low concentrations of Baf-A1 have no effect on lysosome number.** In addition to the suspected requirement of acidic endosomes for uncoating and fusion of viral and endosomal membranes (6), the formation of vacuoles with low intracellular pH is required for autophagosome-lysosome fusion during autophagy, a process that has been shown to be induced in association with IAV replication (33, 61). Because our data indicate that high concentrations of Baf-A1 greatly diminish nuclear staining for vNP (Fig. 3), we next investigated whether this observation is associated with incomplete virus uncoating and reduced formation of acid lysosomes.

In cells treated with LysoTracker Red DND-99, the dye normally accumulates in acidic vacuoles, in particular lysosomes. Thus this compound can reveal abnormalities in formation of vacuoles with low pH that are capable of fusion with autophagosomes (16, 21, 57). We pretreated A549 cells with different concentrations of Baf-A1 for 24 h, infected the cells with IAV at MOI = 1 for another 24 h, and then labeled the cells with LysoTracker Red DND-99 for 10 min. Thereafter cells were fixed and then analyzed by fluorescence microscopy. Treatment with 0.1 nM Baf-A1 did not appear to alter the pattern of lysosome staining compared with IAV-infected cells that were not treated with Baf-A1 (Fig. 4A, top). This suggests that this very low Baf-A1 concentration had no effect on V-ATPase activity, formation of vacuoles with low pH, or the number of lysosomes. Consistent with prior reports (60), higher concentrations of Baf-A1 diminished LysoTracker Red DND-99 staining (Fig. 4A, bottom). Indeed, no detectable labeling appeared in cells treated with 100 nM Baf-A1 (Fig. 4A, bottom right). To quantify these responses we used Olympus Fluoview software to determine the average pixel intensity in confocal images. This analysis revealed a significant reduction in the number of stained lysosomes in cells treated with 10 and 100 nM Baf-A1, compared with IAV-infected A549 cells treated with either no or with low-concentration (0.1 nM) Baf-A1 (P < 0.001). Collectively, the altered pattern and reduction in the number of stained lysosomes suggests that high, but not low, concentrations of Baf-A1 block vATPase activity to diminish the abundance of acidic lysosomes. We also confirmed the effect of different concentrations of Baf-A1 on lysosomal pH using a newly developed pH-sensitive dye, pRRD. We used 0.1, 1, and 10 nM concentration of Baf-A1. Our result showed that low concentration of Baf-A1 (0.1, 1 nM) did not significantly affect lysosomal pH whereas higher concentration of Baf-A1 (10 nM) significantly diminished lysosomal pH (P < 0.001) (Fig. 4, C and D).
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A

Control 0.1 nM
10 nM 100 nM

B

Baf-A1 Concentration (nM) 0 0.1 10 100
LysoTracker Red fluorescent intensity

C

Control (No Baf-A1) 0.1 nM Baf-A1 1 nM Baf-A1 10 nM Baf-A1
Red Channel: pHrodo Red Dextran Blue Channel: Hoechst 33342 Nuclei

D

Integrated Fluorescence per Cell (Arbitrary Unit)
Control Baf-A1 0.1 nM Baf-A1 1 nM Baf-A1 Baf-A1 10 nM

E

Baf-A1 (nM) 0 0 0.1 1 2.5 10 100
Virus – + + + + + +

F

LC3b II expression (Ratio of intensity)
C 0 0.1 1 2.5 10 100

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Baf-A1 effects on autophagy and autophagosome formation in A549 cells. Recent studies indicate that autophagy pathways are induced during various pathogenic infections (10, 52), although the precise role of autophagy during influenza virus infection is not yet elucidated (61). Autophagy is a multistep homeostatic lysosome-dependent degradation pathway that can be greatly increased by stress stimuli, such as viral infection. Autophagy involves formation of a double-membrane phagophore that elongates to form autophagosomes vesicles. These structures undergo maturation and ultimately form autolysosomes, either through direct fusion with a lysosome or by first forming an amphisome (fusion with a late endosome) that associates with a lysosome (10). Baf-A1 treatment blocks autophagy flux as inhibition of V-ATPase interferes with fusion of autophagosomes and lysosomes, preventing autophagosome-linked degradation (24, 58).

Western blot analyses of microtubule-associated protein 1 light chain, LC3, is considered a marker for autophagosome formation because during autophagosome maturation it is cleaved and becomes conjugated with phosphatidylethanolamine to form the LC3-II variant (33). We used immunoblotting and densitometry to measure cellular levels of LC3-II in A549 cells pretreated with different concentrations of Baf-A1 for 24 h then subjected to IAV infection. Infection alone was sufficient to significantly induce an increase in LC3-II, but pretreatment with 0.1 and 1 nM Baf-A1 suppressed infection-induced LC3-II accumulation (Fig. 4, E and F). This effect of Baf-A1 could be due to either a decrease in formation or an increase in degradation of autophagic vacuoles. As expected, increasing Baf-A1 concentrations that are known to inhibit autophagy flux augmented infection-induced LC3-II accumulation (Fig. 4, E and F).

Baf-A1 effects on ultrastructural features of IAV-infected A549 cells. To fully characterize cellular responses to treatment with different Baf-A1 concentrations, we examined A549 cells 24 h after infection by TEM. In infected A549 cells without Baf-A1 exposure, or exposed to low (0.1 nM) Baf-A1 concentration, we observed organelles, nuclei, and chromatin with typical morphology. Numerous electron-dense lysosomes that appear as dark vesicles, indicating lysosomal activation, were evident scattered throughout the cytoplasm (Fig. 5A, a and b). In contrast, but consistent with our observations using LysoTracker labeling (Fig. 4), cells treated with 10 or 100 nM Baf-A1 possessed markedly fewer dark lysosomes but accumulated both double-membrane vacuole structures resembling autophagosomes and vesicles that contained what appeared to be undegraded material (Fig. 5A, c and d). To more fully appreciate these TEM-detected features, we performed semi-quantitative analysis for the number of lysosomes, identifying dark-stained lysosomes in the cytoplasm. This confirmed there was a significant reduction in the number of active electron-dense lysosomes in infected cells treated with 10 or 100 nM Baf-A1 compared with counterparts that either were unexposed to Baf-A1 or were treated with 0.1 nM Baf-A1 (Fig. 5B) (P < 0.001 vs. control group).

Using high magnification TEM, we also examined the presence and contents of the double-membrane autophagosomes. These structures, containing virus particles, were readily seen in IAV-infected cells (Fig. 5C, a and e). In cells treated with 0.1 nM Baf-A1 prior to IAV infection, autophagic vacuoles with intact membrane containing virus particles were visible (Fig. 5C, b and f). However, in cells pretreated with 10 or 100 nM Baf-A1 we observed accumulation of incomplete autophagic vacuoles that appeared to envelop both viral particles and undigested material (Fig. 5C, c, d, g, and h). Collectively, these data suggest that, although both low and high concentrations of Baf-A1 reduced IAV replication in A549 cells, the mechanisms of action, with respect to autophagy flux, are different. Cells treated with Baf-A1 at higher concentrations (10 and 100 nM) displayed lysosomal acidification inhibition, attenuated fusion between autophagosomes and lysosomes, blockage of the autophagy pathway, and accumulation of IAV within vesicles that lead to an impaired virus life cycle. However, lysosomal pH and fusion between autophagosomes and lysosomes were unaffected in cells treated with 0.1 nM Baf-A1.

Baf-A1 induces cell death in a concentration-dependent manner. Previous studies have shown that higher Baf-A1 concentrations have cytotoxic effects in vitro (27, 56). To study cytotoxicity of Baf-A1 in our system, we examined cellular morphology of mock- and virus-infected A549 cells pretreated with different doses of Baf-A1, focusing on chromatin condensation and cell shrinkage. Virus-infected cells exhibit characteristic morphological features of cytopathic effects and cell death, such as cell shrinkage, cell disassembly, and rounding and membrane blebbing (Fig. 6A, arrows). Cells treated with 0.1 nM Baf-A1 show relatively fewer signs of death and appear more normal in both infected and noninfected cells compared with virus-infected cells without Baf-A1 pretreatment (Fig. 6A). In contrast, treatment of cells with 10 or 100 nM Baf-A1 resulted in cells with morphological changes in shape and appearance as well as reduced the number of adherent cells, revealing cytotoxic effects of Baf-A1 at these concentrations (Fig. 6A).

The concentration-dependent effects of Baf-A1 exposure on mock- and virus-infected A549 cell viability was also examined using the MTT assay (38). Infection of A549 cells with IAV significantly reduced cell viability compared with mock-infected cells (P < 0.001). In IAV-infected as well as uninfected A549 cells, we observed reduced cell viability, with
higher doses of 10 and 100 nM Baf-A1 inducing significant levels of cell death after 48 h \( (P < 0.001) \) (Fig. 6B). These data confirm that the low concentration of Baf-A1 used in our study has limited, if any, cytotoxic effect on uninfected and IAV-infected human lung epithelial cells.

To better understand the mechanism underpinning reduced cell viability in cells treated with high concentrations of Baf-A1, we performed FACS analysis to identify cells with sub-G1 DNA content (i.e., apoptotic) by labeling with PI (45). Treatment of uninfected cells with 10 and 100 nM Baf-A1 for 24 h
also significantly increased the number of cells in sub-G1 phase \( (P < 0.001) \) (Fig. 7, A and B). Conversely, 0.1 nM Baf-A1 alone had no statistically significant effect on the number of cells with sub-G1 DNA content \( (P > 0.05) \) (Fig. 7, A and B). Infection without Baf-A1 pretreatment was sufficient to increase the fraction of sub-G1-phase A549 cells (Fig. 7, A and B), and this effect was increased when cells were pre-treated with 10 or 100 nM Baf-A1 \( (P < 0.001) \) (Fig. 7, A and B). \( \text{sup} \)

Fig. 6. Analysis of cellular morphology. Phase-contrast microscopy images to capture gross cellular appearance (chromatin condensation and cell shrinkage) at different doses of Baf-A1 in the presence and absence of virus in A549 cells. A: reduced density and poor morphological appearance of cells treated with high concentrations of Baf-A1 (10 and 100 nM), indicating cytopathicity at high concentrations of Baf-A1. At low concentration of Baf-A1 (0.1 nM), both infected and noninfected cells appeared healthy compared with virus-infected cells without pretreatment with Baf-A1. B: quantification of cytopathic effects of Baf-A1 on A549 cells. Cells grown in 12-well plates were pretreated with Baf-A1 for 24 h, and either mock-infected or infected with virus and examined by MTT assay. Low-dose Baf-A1 (0.1 nM) was not toxic to A549 cells. Treatment with Baf-A1 at 10 and 100 nM decreases cell viability. Baf-A1 (0.1 nM) also protected virus-infected cells against cell death. Results represent means ± SD from 6 independent experiments. *** \( P < 0.001 \), * \( P < 0.05 \) vs. matched control; \( SP < 0.001 \), virus only vs. Mock; \( @P < 0.001 \), virus + 0.1 nM Baf-A1 vs. virus + 10 nM Baf-A1; \# \( P < 0.001 \), 0.1 nM Baf-A1 vs. 10 and 100 nM Baf-A1.

Fig. 7. Apoptotic cell death induced by different concentrations of Baf-A1 (Baf). Representative FACS histograms for A549 cells treated with different concentrations of Baf-A1 (24 h) (indicated by text in relevant panels), and infected with influenza A/PR/8/34 virus (MOI 1 FFU/cell). For controls, cultures either underwent Mock infection or were not treated at all (labeled as Control). Cells were lifted, and nuclei were stained with propidium iodide, then subjected to FACS analysis to identify apoptotic cells with a reduced DNA content, called the sub-G1 subgroup (delineated as M1). A: FACS histograms for A549 cells treated with different concentrations of Baf-A1 (Baf-A1 pretreatment) and infected with influenza A/PR/8/34 virus (MOI 1 FFU/cell). B: bar graph showing mean data for fraction of cells with sub-G1 DNA content. Results represent means ± SD from 6 independent experiments. *** \( P < 0.001 \) compared with Control. * \( P < 0.01 \) compared with Mock; ** \( P < 0.001 \) compared with Mock.
**Suppression of Influenza Replication with Low-Dose Baf-A1**

**A**

- **Control**
- **Mock**
- **A/PR/8/34**

Cell Count vs. Fluorescence Intensity for different Baf-A1 concentrations and influenza virus conditions.

**B**

- **% Apoptosis (Sub G1 Population)**
  - **24 hpi, n=6**
  - Bar chart showing the percentage of apoptosis over different Baf-A1 concentrations and virus conditions.

*References and citations may be included here.*
B). Notably, pretreatment of cells with 0.1 nM Baf-A1 did not increase infection-induced apoptosis, confirming that at this low-concentration Baf-A1 has very limited, if any, cytotoxic effect and, compared with the nontreated cells, is protective.

**DISCUSSION**

The antagonistic effect of Baf-A1 on the replication of viruses, including influenza A and B, has been reported by other groups (30, 32, 36, 40, 50). The antiviral activity of Baf-A1 at concentrations ≥10 nM has been attributed to its ability to inhibit V-ATPase, an enzyme that acidifies intracellular compartments, including lysosomes (60). Although this demonstrates that preventing the genesis and/or maintenance of acid lysosomes impacts viral uncoating to prevent replication, the toxicity of such high concentrations of Baf-A1 limits its antiviral therapeutic potential in vivo (27).

Lysosomes are cellular acidic organelles that rely on V-ATPases for the maintenance of their intraluminal acidity. Thus, to explore the effect of different concentrations of Baf-A1 on acid lysosomes, LysoTracker Red was employed. This fluorescent acidotropic probe consists of a fluorophore linked to a weak base that is only partially protonated at neutral pH. LysoTracker Red is freely permeant to cell membranes and typically concentrates in acidic organelles; thus staining with the dye has been used to track and measure formation of acidic vacuoles such as endosomes, trans-Golgi vesicles, and lysosomes. Consistent with our electron microscopy data, LysoTracker Red staining confirmed that very low Baf-A1 concentration had no effect on the number of lysosomes. Similarly, using a lysosome-selective, pH-sensitive dye, we observed that low concentrations of Baf-A1 were without effect on vacuolar pH. In contrast, with higher concentrations of Baf-A1, the number of lysosomes as well as vacuolar pH was significantly reduced. Here we also demonstrate that Baf-A1 used at concentrations up to 1,000 times less than previously reported, and insufficient to fully inhibit V-ATPases, is sufficient to markedly limit influenza A replication in human lung epithelial host cells in vitro. This effect appears to occur without impact on lysosomal pH and is without cytotoxic effect. This suggests a novel antiviral mechanism for low-dose Baf-A1 and suggests this pleomacrolide antibiotic may offer future therapeutic potential in vivo.

Attachment of the host cell is the first crucial step in establishing a successful virus infection. Here we show that Baf-A1 treatment (at low and high concentrations) has no impact on IAV attachment to human lung epithelial cells. Although we show that both low and high concentrations of Baf-A1 disrupt nuclear import of IAV and attenuated virus replication and release, the mechanism of antiviral activity of a very low concentration of Baf-A1 at 0.1 nM did not involve pruning of acid lysosomes; indeed, this lower dose even appeared to limit infection-induced autophagosome formation. Conversely, markedly higher concentrations of Baf-A1 markedly attenuated lyosome number and autophagy flux, likely owing to inhibition of V-ATPase (4, 40, 58, 60), an effect that was concomitant with blocking IAV nuclear import and replication.

Reduced virus-induced LC3-II accumulation by low concentration of Baf-A1 could suggest an important role in lysosome-associated regulation of autophagy, a pathway that has been shown to be involved in IAV replication (61). Little is known about the molecular target(s) that low-dose Baf-A1 might affect. It is likely that low concentration of Baf-A1 may influence upstream factors that regulate autophagy such as mammalian TOR (mTOR) or hinder the Beclin 1/Bcl-2 interaction (42, 48). Additional research is warranted to further understand how low concentration of Baf-A1 limits autophagosome formation. In cerebellar granule neurons, Shacka and colleagues (47) showed that low concentration of Baf-A1 (≤1 nM) exhibits an inhibitory effect on chloroquine-induced caspase-3 activation and cell death in the absence of any effect on vacuolar acidification or autophagy. Similarly, this group has described a cytoprotective effect of ≤1 nM Baf-A1 in human neuroblastoma cells and Caenorhabditis elegans neurons cells treated with chloroquine, hydroxychloroquine amodiaquine, or staurosporine (43). Although this cytoprotective effect has been suggested to likely involve maintenance of the autophagy-lysosome pathway independent of V-ATPase inhibition (43), the exact mechanism of action remains unknown. Furthermore, similar to Pivitoraiko and colleagues’ work (43), but in contrast to Shacka et al.’s study (47), our data suggest that, although there was no effect of 0.1 nM Baf-A1 on lysosome number in A549 cells, the autophagy pathway was affected by low and high concentrations of Baf-A1. The reported differences may be due to different cell types used in these studies.

The antiviral activity of Baf-A1 makes it a potentially suitable drug candidate for the treatment of viral infection. However, the ionophoric properties of ≥100 nM Baf-A1 cause mitochondrial damage and apoptosis, precluding the in vivo pharmacological use of this macrolide (17, 51). Consistent with studies in a number of different cell types (4, 39, 56), we found that 10 and 100 nM Baf-A1 treatment reduced A549 cell viability and induced apoptotic cell death. This effect appears to be linked to Baf-A1-mediated inhibition of the acidification of intracellular compartments that are required for viral cellular processes, such as membrane turnover, autophagy, and endocytic uptake of nutrients, and severely limits the therapeutic use of Baf-A1 at a relatively high concentration (3, 18, 35). In a study conducted by Muller and colleagues (35), the investigators also found that low concentration of Baf-A1 (below 2 nM) could successfully inhibit viral replication. They also reported that three daily intraperitoneal injections of 350 ng/kg Baf-A1 in mice caused spleen and liver damage without impact on IAV infection. The effects of Baf-A1 at this dose could be attributed to the route of Baf-A1 administration and to processes of detoxification by the liver and kidney. Conceivably, other routes of drug administration such as nasal or inhalation would be more efficient to target the lung epithelium. Furthermore, it is not evident whether 350 ng/kg injection of Baf-A1 in mice is sufficient to inhibit V-ATPase activity. Other groups have used much higher concentrations of Baf-A1 to reach its inhibitory effect on V-ATPase. For instance, in a study conducted by Belibi and colleagues (2), mice were injected with 2 mg/kg Baf-A1, for 4 days, and in another study (41) 1 mg/kg of Baf-A1 was injected for 4 wk to reach inhibitory effects.

In an attempt to reduce its cytotoxic effects, others have examined the cellular effects of short-term (1 h) pretreatment with high-dose Baf-A1 (≥100 nM) (34, 58). It has been
suggested that, at high concentrations and short exposure times, Baf-A1 can reduce acid endosome and lysosome numbers, cause fusion block, inhibit autophagy (25), and lead to the inhibition of IAV replication (40). We opted for a nontoxic low-dose Baf-A1 concentration and an extended pretreatment (24 h), and this strategy succeeded in significantly reducing IAV replication. Although the complete molecular mechanism underlying the antiviral properties of low-dose Baf-A1 is not fully evident from our work, our data do indicate that this is unlikely to involve the ability of Baf-A1 to inhibit vacuolar acidification. Detailed investigation of this mechanism is currently underway by our group to understand how low-dose Baf-A1 has antiviral properties.

In conclusion, overall, our results reveal that low-concentration Baf-A1 is an effective inhibitor of IAV replication, without impacting host cell viability. Furthermore, these results suggest a unique mode of action of low concentration Baf-A1 that does not compromise host cell viability and thus has potential for in vivo therapeutic applications.

REFERENCES


