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EFFECTS OF pH ALTERATION ON RESPIRATORY SYNCYTIAL VIRUS IN HUMAN AIRWAY EPITHELIAL CELLS

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Take Home Message:

Low airway surface pH impairs antimicrobial host defence and worsens airway inflammation. A novel, inhaled, alkaline buffer medication, Optate, raises airway pH and inhibits RSV infection in primary human airway epithelial cells in a dose dependent manner.

ABSTRACT

Respiratory syncytial virus (RSV) is a leading cause of respiratory distress and hospitalization in the paediatric population. Low airway surface pH impairs antimicrobial host defence and worsens airway inflammation. Inhaled Optate safely raises airway surface pH in humans and raises intracellular pH in primary human airway epithelial cells (HAECs) *in vitro*. We aimed to determine if raising intracellular pH with Optate would decrease infection and replication of RSV in primary HAECs.

We cultured HAECs from healthy subjects in both air-liquid interface (ALI) and submerged conditions. We infected HAECs with green fluorescent protein-labelled RSV (GFP-RSV; MOI 1) and treated them with Optate or phosphate buffered saline control. We collected supernatant after a four-hour incubation and then every 24 hours. We used fluorescence intensity, fluorescent particle counts, plaque assays, western blots, and enzyme-linked immunosorbent assays (ELISA) to quantitate infection.

In submerged culture, fluorescence intensity decreased in Optate-treated cells (48H $p=0.0174$, 72H $p=<0.0001$). Similarly, Optate treatment resulted in decreased fluorescent particle count (48H $p=0.0178$, 72H $p=0.0019$) and plaque forming units (PFUs) (48H $p=0.0011$, 72H $p=0.0148$) from cell culture supernatant. In differentiated HAECs cultured at ALI, Optate treatment decreased fluorescence intensity ($p \leq 0.01$), GFP via western blot and ELISA ($p<0.0001$), and RSV-fusion protein via ELISA ($p=0.001$). Additionally, RSV infection decreased as Optate concentration increased in a dose-dependent manner ($p < 0.001$).

In conclusion, Optate inhibits RSV infection in primary HAECs in a dose-dependent manner. These findings suggest Optate may have potential as an inhaled therapeutic for patients with RSV.

INTRODUCTION

Respiratory syncytial virus (RSV) is a member of the *Pneumoviridae* family that causes airway damage and is the leading cause of severe lower respiratory tract infections in children.[1, 2] In the United States, RSV accounts for over 2 million outpatient visits and nearly 60,000 hospitalizations in children under 5 years of age annually.[3, 4] Currently, no effective therapies or vaccines exist for RSV, and while preventive antibodies are on the market, they remain restricted to a small group of high-risk infants.[5] A safe treatment for RSV would be beneficial throughout the world.

Airway extracellular pH is acidic during viral respiratory infections and acidic extracellular pH in the airway leads to impaired mucociliary clearance, increased inflammation, and decreased host defense.[6] Many viruses, whether enveloped or not, use endocytic entry mechanisms to enter host cells, and an acidic pH serves as a trigger for penetration.[7, 8] Additionally, many viruses require acidic endosomal pH for viral surface protein activation.[9] While RSV was thought to enter the cell in a pH-independent manor via direct membrane fusion and release of RSV nucleocapsids into the cytoplasm, recent studies have shown that clathrin function, macropinocytosis and endocytosis play a key role in the virulence of RSV.[10, 11] Specifically, fusion protein cleavage via an endosomal protease that may require low pH for activation makes the virus infectious.[11-13]

Optate (IND #139144) is a safe, glycine-based, inhaled buffer that alkalinizes airway extracellular, intracellular, and endosomal pH. We have demonstrated that exposing human airway epithelial cells (HAECs) to Optate also alters endosomal trafficking and inhibits SARS-

CoV-2 infection in primary HAECs.[14, 15] Given the dependence of RSV on proteolytic cleavage of the fusion protein in the acidic endosome, we hypothesize that by increasing intracellular pH with Optate we will similarly inhibit RSV infection and replication.

MATERIAL AND METHODS

Study Design

The main objective of this study was to determine if raising intracellular pH with Optate would decrease infection and replication of RSV in primary HAECs.

Cell Culture and Infection Model

A compounding pharmacy prepared and assayed Optate (120 mM) for purity, potency, osmolality (target ~ 330 mOsm), pH (target 9.8), and sterility prior to all experiments (IND #139144; Arena District Pharmacy, Columbus, Ohio, USA).

Primary HAECs from three healthy, non-smoking donors were grown as previously described under submerged conditions and at air-liquid interface (ALI) at passages 3 or 4.[16-18] After optimizing techniques with one donor under submerged conditions and at ALI, two additional donors were used for all other ALI experiments. Graphs with data from multiple donors are color coded to allow for easy identification of each donor. Biological replicates are included in the datasets. HAECs were infected with RSV with green fluorescent protein (RSV-GFP, ViraTree, product #R121, RSV-A2) at a multiplicity of infection (MOI) of 1. Optate (at concentrations used in humans *in vivo*[14, 15]) or control (phosphate buffered saline, PBS, pH 7.2) were co-administered with RSV-GFP to HAECs. Negative control groups were cells that were not infected with RSV and were not treated with Optate or PBS. Supernatant was harvested and

stored at -80^{o}C after a four-hour incubation period and then every 24 hours for three days for submerged cells and up to ten days as needed to achieve optimal infection for cells cultured at ALI. Submerged cells were treated with Optate or PBS daily with media change, while ALI cells were treated apically for 20 minutes twice daily.

Quantifying Viral Infection by Fluorescence Intensity

Fluorescence intensity quantification was calculated using ImageJ (U. S. National Institutes of Health, Bethesda, Maryland, USA, https://imagej.nih.gov/ij/) on microscopic images obtained with the EVOS M5000 microscope (Thermo Fisher Scientific, Carlsbad, California, USA) as previously described.[14] Images were taken while microscopy was focused on the most apical layer of epithelial cells every 24 hours for either 3 days (submerged cells) or up to 10 days (cells cultured at ALI) using the Invitrogen GFP Light Cube (Thermo Fisher Scientific, Carlsbad, California, USA) prior to daily treatment with PBS or Optate. Fluorescence images were uploaded to ImageJ, image fields were selected, images were processed by subtracting the background using default settings, fluorescence intensity was analysed, and average fluorescence intensity was compared between all groups. This technique reflects the level of RSV-GFP infection by quantifying fluorescence from the GFP in infected cells.[19]

Fluorescent Particle Counting and Plaque Assays

Vero E6 (African green monkey kidney cell line, ATCC, Manassas, Virginia, USA) were plated on the day prior to the experiment. Plaque assays were performed as previously described using the stored supernatant from the previous experiments outlined above.[14] Fluorescent particles

were counted using fluorescence microscopy with Invitrogen GFP Light Cube on day 5 and results are reported as focus forming units per millilitre (FFU).

Detection of GFP and RSV Fusion Glycoprotein by Enzyme-Linked Immunosorbent Assay (ELISA)

After five days of infection with RSV and treatment with either Optate or PBS, growth medium was removed and cells were rinsed in PBS. Cells were then lysed by adding 50uL of 1X Cell Extraction Buffer PTR (GFP *in vitro* CatchPoint SimpleStep ELISA) and phosphatase inhibitor, which was directly applied to ALI filters. Cells were scraped off of filters using a flat pipette tip and lysate was transferred to a microfuge tube. Samples were centrifuged at 18,000 xg for 20 minutes at 4°C. The supernatants were transferred into clean tubes and the pellets were discarded. Samples were stored at -80°C until the assay was performed. The sample protein concentration in the extract was quantified using a protein assay (Pierce BCA Protein Assay, Thermo Fisher Scientific, Carlsbad, California, USA). Samples were diluted to desired concentration in 1X Cell Extraction Buffer PTR immediately prior to use. GFP *in vitro* CatchPoint SimpleStep ELISA (abcam, Cambridge, CB2 0AX, UK) was used to quantify GFP and RSV was quantified using RSV (A2) Fusion glycoprotein (RSV-F) ELISA Kit (SinoBiological, Pennsylvania, PA, USA). Technical replicates were averaged. Results were plotted on a 4-parameter logistic regression model per package instructions and concentrations were calculated.[20]

Immunoblots

Using the same protocol as that for ELISA above, cells were lysed in 1x cell extraction buffer PTR. Capillary electrophoresis was performed on the automated JESS system as previously described (ProteinSimple, San Jose, California).[14] Briefly, 0.25 ug/uL lysate was plated and run according to the manufacturer's recommendations. Antibodies to GFP (part number 2956T, Cell Signaling Technology, Boston, MA, USA) were used. Compass software (ProteinSimple) generated digitally rendered bands based on chemiluminescence electrophoretogram to quantify GFP.

Intracellular pH Assays

Intracellular pH was evaluated as previously described using two fluorescent dyes: 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxymethyl ester (BCECF-AM, Invitrogen, Carlsbad, California) and pHrodo Red (Invitrogen).[14] After washing with PBS, cells were treated with Optate (clinical solution, 1:1 dilution in medium) or PBS.

Analysis

All statistical analysis were calculated using GraphPad Prism (GraphPad Software, San Diego, California, USA) and R (R Core Team, Vienna, Austria). To evaluate differences in fluorescence intensity, fluorescent particle counts, viral plaques, and RSV-F and GFP protein quantities between negative control and treatment groups, two-sample two-tailed Student's *t*-test was used for Gaussian distributed data and Wilcoxon rank-sum test was used for non-Gaussian distributed data (Supplement Table 1). For cases with more than two groups, robust ANOVA models were considered with pairwise Tukey's test. With multiple times points, the tests were performed for each time point separately. To study the association with pH, robust linear regression models

were fitted. For ELISA data, a four-parameter logistic regression model was used.[20] A *p*-value of less than 0.05 was considered statistically significant.

Biosafety and Ethical Statement

All experiments were conducted at Indiana University in a biosafety level 2 laboratory (IBC# IN-1127). Primary cells were obtained under Indiana University Institutional Review Board Protocol #1408855616. All subjects provided informed consent.

RESULTS

Optate treatment inhibits RSV infection in submerged cells

In submerged HAECs, RSV infection was lower in cells treated with Optate compared to control at 48 and 72 hours. Figure 1A shows fluorescence microscopy images taken 72 hours post infection and demonstrates the difference in RSV infection between Optate treated cells and PBS control. Figure 1B demonstrates a significant decrease in fluorescence intensity quantified using ImageJ during primary infection (48H n=4, p=0.0174, 72H n=4, p<0.0001). These results were confirmed by fluorescent particle count and plaque assays from supernatant (48H n=9, p=0.0178, 72H n=9, p=0.0019 and 48H n=7, p=0.0011, 72H n=7, p=0.0148, respectively, Figure 2A and 2B). Immediately following the incubation period, supernatant collected from Optate-treated cells had a significantly higher viral load indicated by increased fluorescent particle counts (n=7, p=0.0017) and PFUs (n=8, p=0.0011) compared to PBS control (Figure 2C and 2D).

Inhibition of RSV by Optate is dose-dependent

RSV infection in submerged HAECs decreased in a dose-dependent manner as shown in Figure 3. Figure 3A shows the decrease in viral fluorescence intensity quantified using ImageJ. Optate significantly decreased RSV infection to the level of the negative control group at doses ≥ 50% ($n=4$, $p<0.001$). Furthermore, pH decreased as the dose of Optate decreased (Figure 3B). Finally, Optate pH strongly correlated with intracellular fluorescence intensity in cells treated with pH-sensitive intracellular dye pHrodo Red ($R^2=0.8406$, Figure 3C).

Optate treatment inhibits RSV infection in organotypic, differentiated primary HAECs

In primary HAECs cultured at ALI, fluorescence intensity decreased in cells treated with Optate compared to control during primary infection ($p \leq 0.05$, Figure 4A, B, and C). Cells were treated twice daily with Optate or PBS control and fluorescent images were obtained every 24 hours post infection. There was also a significant decrease in the amount of GFP quantified using JESS (5A and B) and ELISA (Figure 5C), along with a significant reduction in RSV-F protein quantified using ELISA (Figure 6).

DISCUSSION

These experiments demonstrate that Optate inhibits RSV infection and replication in primary HAECs grown under both submerged conditions and at ALI. Furthermore, RSV infection decreased with increasingly concentrated Optate administration. Our group has previously shown that treatment with Optate raises both intracellular and extracellular pH, is not cytotoxic, is safe and well tolerated in humans, and prevents SARS-CoV-2 replication in primary HAECs (Supplemental Figure 1).[14, 15]

Of note, fluorescent particle count and PFUs in the plaque assays of supernatant from Optate-treated cells increased immediately after RSV inoculation (Figure 2C and 2D), despite the fact that Optate reduced intracellular RSV infection and replication at later timepoints (Figure 2A and 2B). This suggests decreased uptake of the virus into the cells. Altered endosomal trafficking caused by Optate may explain the decrease in viral entry into the cell.[14] At later time points, a decrease in fluorescent particle count and PFUs in plaques assays of Optate treated cells suggests that Optate also inhibits RSV replication within the cell. Figure 7 illustrates our proposed mechanisms for RSV inhibition by Optate.

Our study has several limitations. All the data presented from our cell culture models *in vitro* may not translate to *in vivo* systems and disease processes. Also, our use of fluorescence microscopy to quantify infection may be confounded by the presence of multiple layers of cells in ALI. Additionally, our current results support the hypothesis that Optate inhibits RSV through alterations in endosomal trafficking, but further studies are needed to confirm this mechanism. One of the challenges of studying airway pH is exact measurements of pH are difficult to obtain and require direct sampling during bronchoscopy. Even then, the act of sampling airway pH may affect the results. Exhaled breath condensate pH and changes in exhaled nitric oxide are therefore often used as surrogate markers of airway pH. Although a normal range of pH has been established a using exhaled breath condensate, exact airway pH measurements are unknown. Using exhaled breath condensate pH and changes in exhaled nitric oxide, we know Optate transiently raises airway pH in humans *in vivo*, but to what extent is unknown.[15]

In conclusion, Optate reduces RSV infection in primary HAECs. Our results suggest this is due to increased intracellular pH.[14] These findings suggest that Optate might be the focus of additional studies aimed at developing a treatment for patients with RSV. These studies could include further mechanistic experiments in addition to clinical studies.

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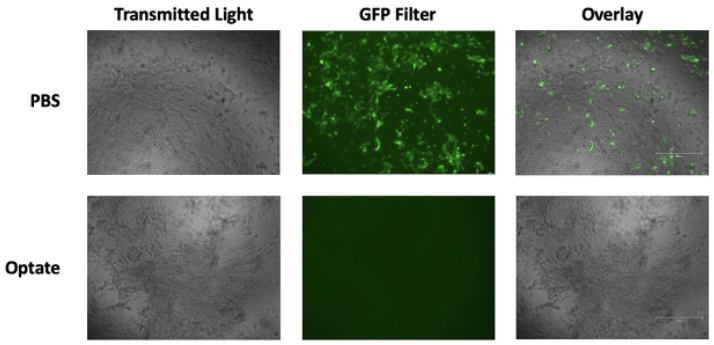
1A



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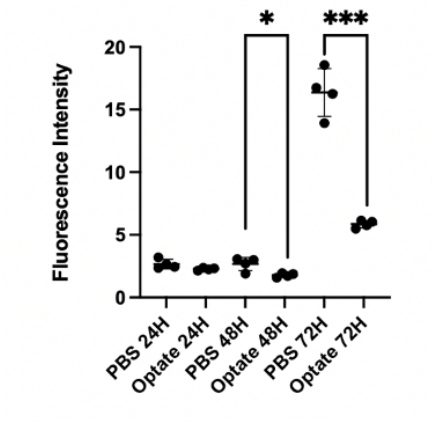

Figure 1: RSV infection is significantly decreased in submerged HAECs treated with Optate compared to PBS control

A. Fluorescence microscopy images taken using transillumination and GFP-filter at 72 hours to demonstrate the difference in infection between Optate-treated cells compared to PBS control. No difference was noted in cell viability between the two groups.

B. ImageJ software was used to quantify fluorescence intensity which is significantly decreased in the Optate-treated groups at the 48-hour (n=4, p=0.0174) and 72-hour (n=4, p <0.001) timepoints compared to control.

Significant differences denoted on graphs with *p=<0.05, **p=<0.01 ***p=<0.001

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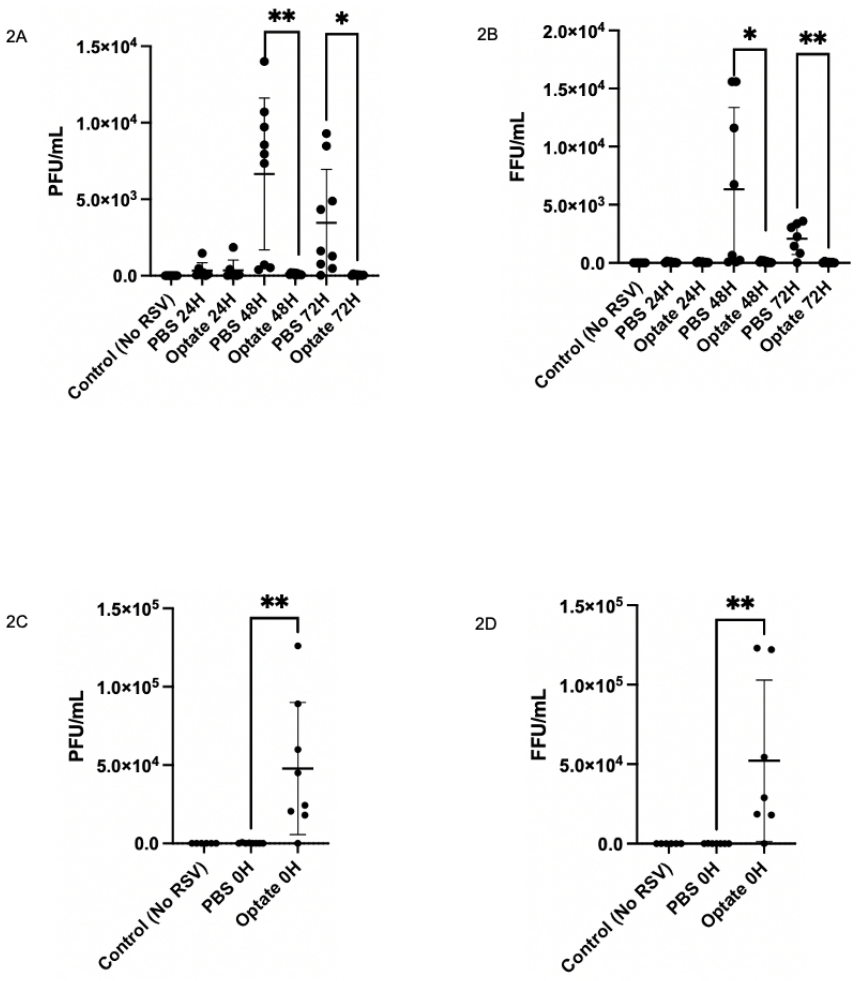

Figure 2: RSV is significantly decreased in the supernatant of submerged HAECs treated with Optate compared to control

A. PFUs are significantly reduced in Optate-treated cell supernatant at 48-hour (n=9, p=0.0011) and 72-hour (n=9, p=0.0148) timepoints compared to control.

B. Fluorescent particle count by fluorescence microscopy performed on day 5 of plaque assays prior to fixing the plates was significantly reduced in cell supernatant from Optate-treated cells at the 48-hour (n=7, p=0.0178) and 72-hour (n=7, p=0.0019) timepoints compared to control. Fluorescent particles were counted and are reported as focus forming units per mililitre (FFU/mL)

C. PFUs are significantly increased (n=8, p=0.0011) in the supernatant of Optate-treated cells immediately following incubation period.

D. FFUs are significantly increased (n=7, p=0.0017) in the supernatant of Optate-treated cells immediately following the incubation period.

Significant differences denoted on graphs with *p=<0.05, **p=<0.01 ***p=<0.001

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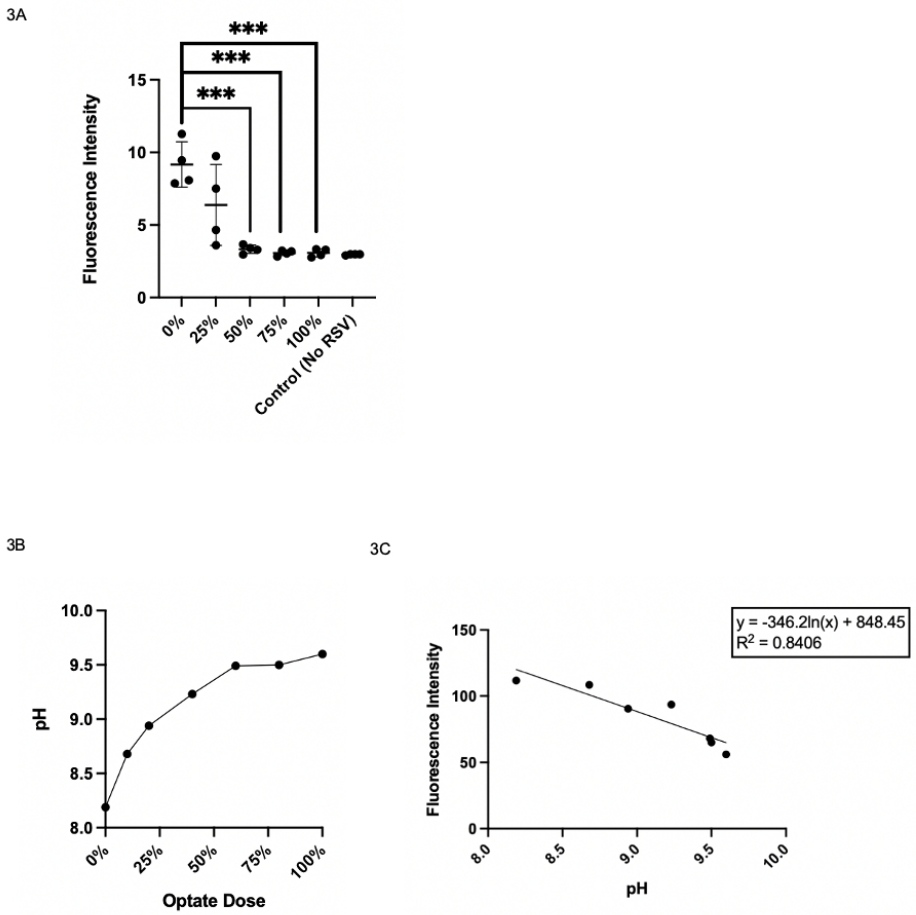

Figure 3: Optate inhibits RSV infection and alters intracellular pH in a dose-dependent manner
A. Fluorescence intensity is decreased in a dose-dependent manner at 72 hours post-infection with GFP-RSV. Fluorescence intensity was quantified using ImageJ 72 hours post-infection. Optate significantly decreased RSV infection to the level of the negative control group at doses ≥ 50% (n=4, p=<0.001). Significant differences denoted on graphs with ***p=<0.001
B. pH decreases as the dose (buffer content) of Optate decreases. Per Figure A, Optate is most effective at doses above 50%, which are above pH 9.2. Dose is defined as % of glycine buffer in Optate, with 100% representing 120 mM, 75% representing 90 mM, 50% representing 60 mM, and 25% representing 30 mM.
C. Optate pH strongly correlates with intracellular fluorescence intensity in cells treated with pH-sensitive intracellular dye pHrodo Red.

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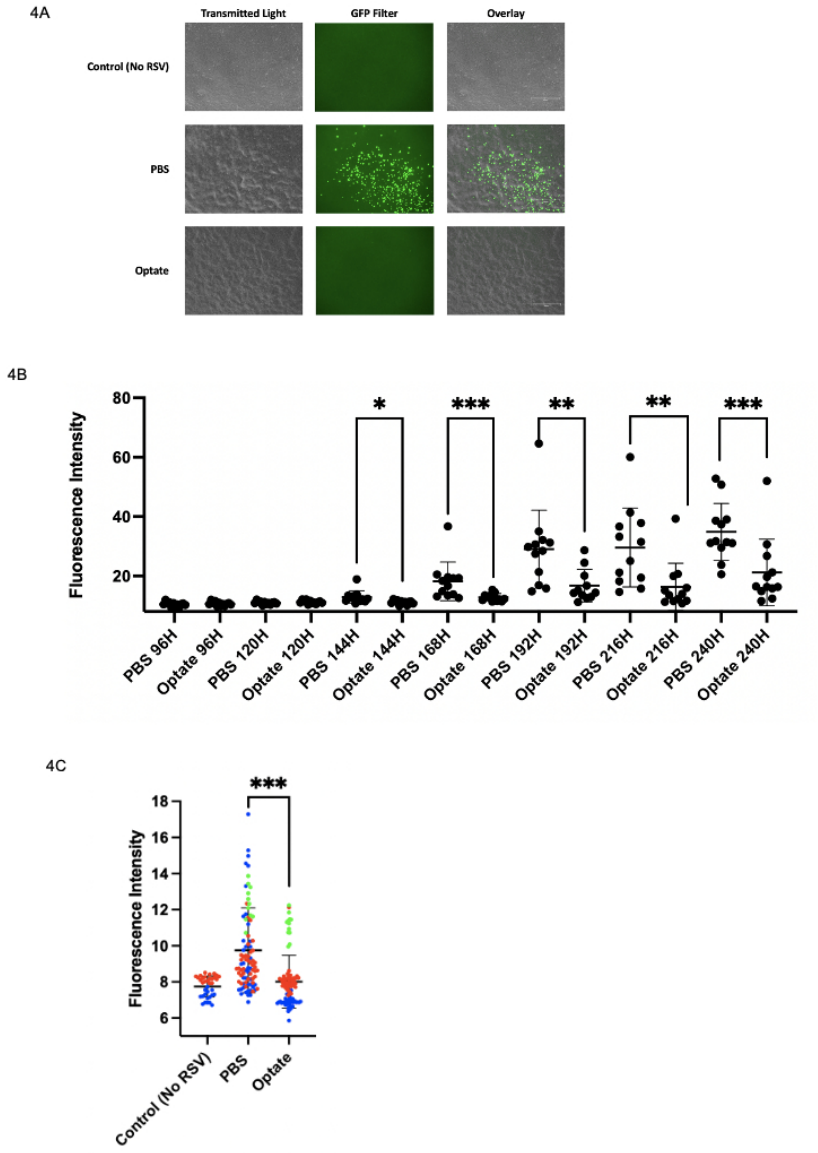

Figure 4: Optate inhibits RSV infection in HAECs cultured at ALI

A. Fluorescence microscopy images taken using transillumination and GFP-filter at 72 hours to demonstrate the difference in infection between Optate-treated cells compared to PBS control. No difference was noted in cell viability between the two groups.

B. HAECs from a single donor were used to determine time-course of infection at ALI. Fluorescence intensity is significantly decreased in the Optate-treated groups between the 144- and 240-hour timepoints compared to control. Significant differences denoted on graphs with *p=<0.05, **p=<0.01 (n=12)

C. Fluorescence intensity is significantly decreased in the Optate-treated groups during primary infection of HAECs from three donors. Negative control included for reference of autofluorescence. Error bars represent standard deviation around the mean. Significant differences denoted on graphs with ***p=<0.001 (n=96)

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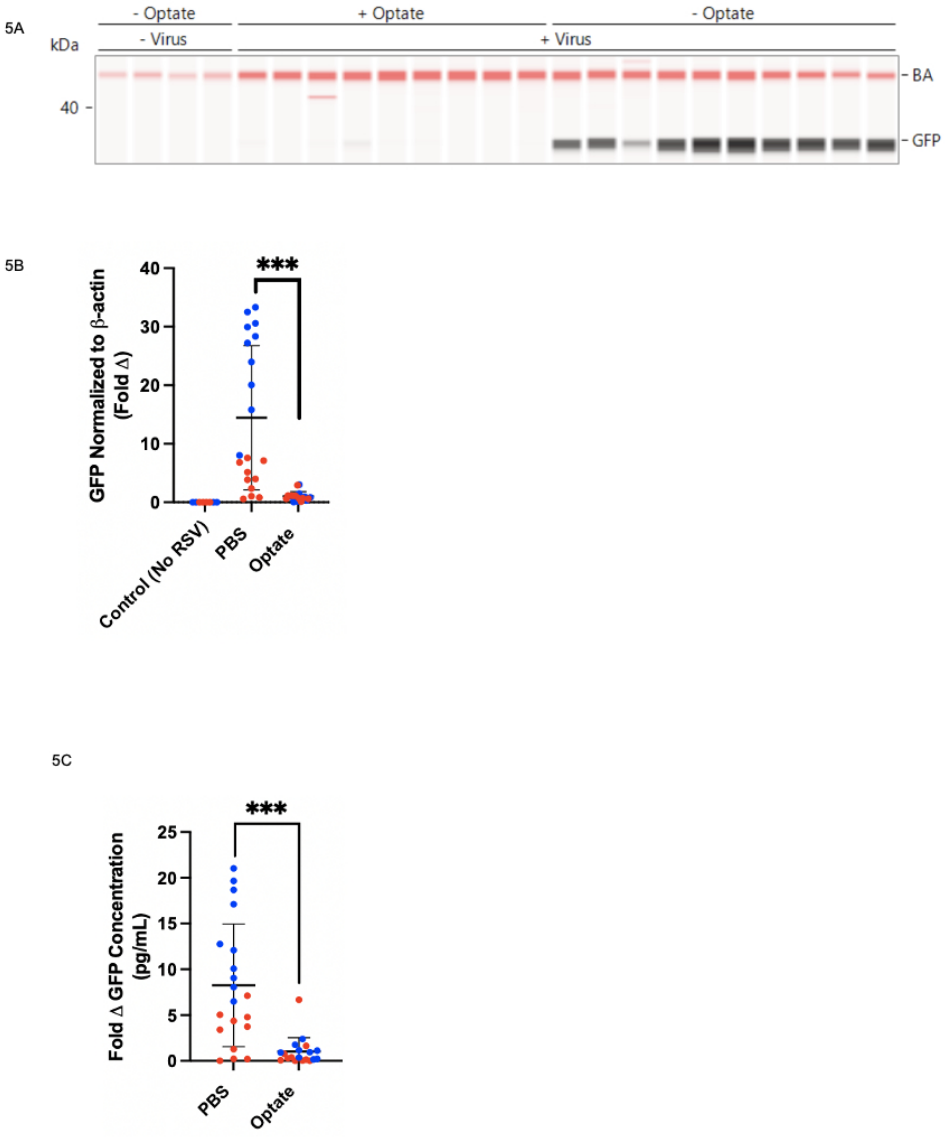

Figure 5: Optate inhibits RSV infection in HAECs cultured at ALI as evidence by a reduction in GFP via JESS and ELISA

A. Western blot demonstrating a reduction of GFP in samples treated with Optate compared to those treated with PBS

B. GFP is significantly reduced in cells treated with Optate compared to PBS control using JESS (n=20, $p<0.0001$). HAECs from two different donors were used to verify this observation. Samples were normalized to beta-actin for comparison and then data sets were normalized to reflect fold change.

C. GFP ELISA shows a significant reduction in GFP production in cells treated with Optate compared to PBS control (n=20, $p<0.0001$). HAECs from two different donors were used to verify this observation, and data were normalized to reflect fold change instead of reporting protein concentrations due to significant variation in relative concentrations between the donor cells.

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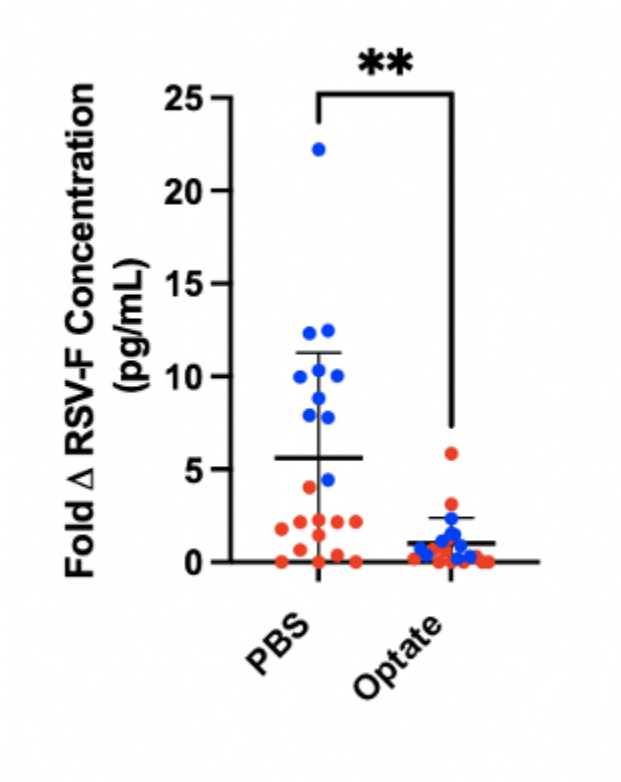

Figure 6: Optate inhibits RSV infection in HAECs cultured at ALI as evidence by reduction in RSV-fusion protein by ELISA (n=22, p=0.001). HAECs from two different donors were used to verify this observation, and data were normalized to reflect fold change instead of reporting protein concentrations due to significant variation in relative concentrations between the donor cells.

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7

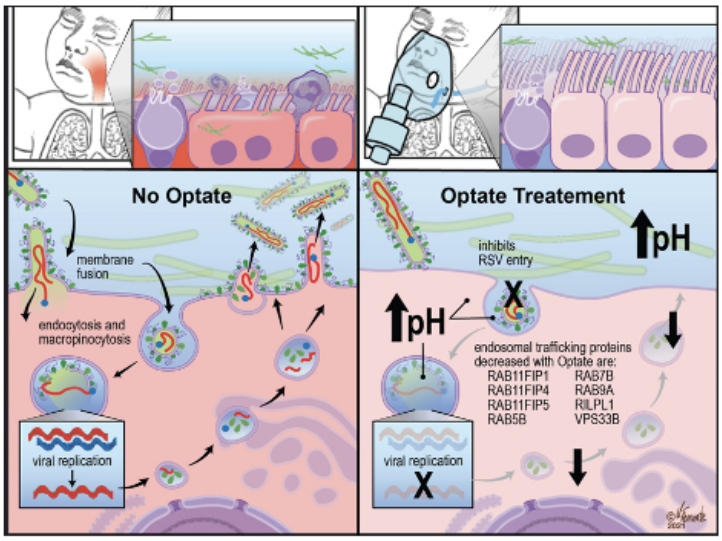

Figure 7: RSV inhibition by Optate
RSV is an enveloped virus that can enter the host cell via direct membrane fusion, endocytosis and macropinocytosis. Although it has several mechanisms to enter the host cell, replication relies on the endosome for cleavage of the fusion protein and thus its infectivity. Optate raises the extracellular, intracellular, and endosomal pH and alters endosomal trafficking. We speculate that Optate decreases RSV entry into the host cell via altered endosomal trafficking.

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