UNC SCHOOL OF MEDICINE

COVID-19 NC Collaboratory Projects

Final Narrative
February 12, 2021

Therapeutics I: Transitioning/Optimizing New Drugs For Pulmonary Delivery

PI: Ric Boucher, MD
Co-Is: Charles Esther, PhD, Ray Pickles, PhD, Brian Button, PhD, Alessandra Livraghi-Butrico, PhD, Claire Doerschuk, MD, Matt Wolfgang, PhD, Alexander Kabanov, PhD, Gauri Rao, PhD

EXECUTIVE SUMMARY

A large number of drugs that have promising antiviral activity for COVID-19 in the laboratory have proven ineffective clinically. For many of these agents, lack of clinical effectiveness reflects the challenges of getting sufficient drug into the lung to have antiviral activity using intravenous or oral dosing regimens. Our project explored the therapeutic potential of aerosolizing drugs directly to the airway cell surface using a pharmacokinetic/pharmacodynamic (PK/PD) system in cell culture that mimics what can be achieved using clinical nebulizers in patients. Our working hypothesis was that aerosolizing drugs directly into the airway will improve the therapeutic index by delivering high concentrations directly to airway epithelia (the site of SARS-CoV-2 infection) while minimizing systemic side effects.

Evaluations were performed on drugs that are active within the cell (remdesivir, hydroxychloroquine) as well as those that are only active on cell surfaces (surfactants/baby shampoo, reducing agents). For both remdesivir and hydroxychloroquine, we showed that a single aerosolized dose could generate intracellular concentrations of active drug similar to, and in some cases greater than, what can be achieved by standard intravenous or oral doses. For remdesivir, we demonstrated that aerosolized drug is metabolized to the active metabolite at concentrations predicted to be antiviral, though gene expression studies suggested that active SARS-CoV-2 infection could inhibit this conversion. For hydroxycholorquine, we showed that drug accumulates intracellularly, undergoes minimal metabolism, and stays in the cell for several days after dosing. For both drugs, we demonstrated that diffusion into the extracellular fluid is limited, suggesting that the inhaled route will avoid many of the side effects of systemic dosing.

Our studies on cell surface active agents demonstrated challenges for this class of therapeutics. We showed that while a gentle surfactant such as baby shampoo can readily kill SARS-CoV-2 and other viruses in a test tube and is safe to apply to airway epithelia, application of a baby shampoo/saline mix widely used for sinus disease did not have any impact on viral levels in infected cultured cells. These findings paralleled those from a clinical study by colleagues at Vanderbilt in which nasal rinses with baby shampoo/saline did not alter viral levels in the noses of COVID-19 patients. Mass spectrometric pharmacokinetics showed that baby shampoo contains a mix of short and longer chain surfactants, and that the longer chain surfactants that have antiviral activity are rapidly lost when delivered to airway epithelia, likely because they bind to and/or are incorporated into epithelial cell membranes. We observed similar overall findings with reducing agents, which had significant antiviral activity in test tube but limited duration of activity on cell surfaces.

In summary, we found that drugs that are active intracellularly, such as remdesivir and hydroxychloroquine, are excellent candidates for aerosolization. Even brief aerosolized treatments generated high intracellular concentrations similar or better that those achieved by systemic dosing to levels predicted to have antiviral activity. Although further work needs to be done, our studies demonstrate that an aerosolized approach not only improves the therapeutic index for these agents but also offers a non-invasive route of delivery that would permit treatment earlier in the course of disease when antivirals are likely to be more effective. Our studies also uncovered challenges to utilization of cell surface active agents. Though in each case the challenges were unique to the drug, the results highlight the value of our PK/PD system to identify such problems prior to clinical trials.

INTRODUCTION

The COVID-19 pandemic has led to an unprecedented search for antiviral agents. While many drugs have promising antiviral activity against SARS-CoV-2 *in vitro*, most have failed to demonstrate clinical efficacy when tested in patients. For many drugs, a significant limiting factor is the difficulty of achieving sufficient concentrations at the site of pulmonary infection (the airway epithelia, **Fig. 1**)[1] early enough in the disease course to be effective while limiting systemic side effects. A unique opportunity to enhance the efficacy of

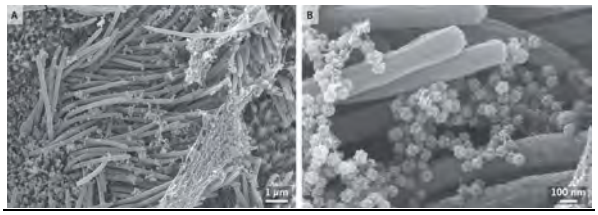

Fig. 1. SARS-CoV-2 Infection of Airway Epithelia. Scanning electron microscopy of cultured human bronchial epithelial cells 96 hours after SARS-CoV-2 infection [from Ehre et al, NEJM 2020].

pulmonary antiviral therapeutics exists because of the ability to deliver compounds via the aerosol route, which improves their therapeutic index by increasing concentrations in the target tissue while limiting systemic toxicities. Therefore, the goal of this project was to develop a world-class human respiratory epithelial testing facility to characterize the pharmacokinetic (PK) and pharmacodynamic (PD) properties of antiviral drugs delivered by aerosol vs systemic approaches in respiratory epithelia. The PK system employs novel aerosolized delivery of test agents to human bronchial or nasal epithelial cells (HBE, HNE) and utilized mass spectrometric-based analyses to assess test agent distribution between airway surface and intracellular compartments. The PD system utilizes model coronavirus test systems (*e.g.*, GFP – NL63) to provide a drug-screening platform to directly assess antiviral activity. These PK and PD systems were enhanced through utilization of confocal microscopy to assess impact of drugs on HBE and RNA sequencing technologies to assess expression of transporters and other proteins that can influence drug concentrations.

The Therapeutics I Team. The team was led by Drs. Boucher and Esther. Dr. Boucher is an adult pulmonologist and director of the Marsico Lung Institute (MLI) with extensive experience developing therapeutics for respiratory disorders. Dr. Esther is a pediatric pulmonologist and director of the pharmacokinetics/pharmacodynamics core for drug measurement within MLI. Other members of the team included Drs. Brain Button, Raymond Pickles, and Guari Roa. Dr. Button has extensive experience in cultured human bronchial epithelial and designed an aerosolized drug delivery device for cultured cells that mimics the properties of clinical nebulizers. Dr. Pickles is an experienced virologist who tested the antiviral impacts of drugs, and Dr. Rao provided pharmacological expertise to help guide the experiments. Drs. Esther and Pickles received direct support from this award, as did two research specialists who performed the cell culture studies directed by the Button laboratory. All personnel supported on this award were employees of the University of North Carolina at Chapel Hill.

The Therapeutics I team met (virtually) at regular intervals to plan experiments and discuss results. Given the fairly short time frame of support available (6 months), the team elected to focus primary efforts on four agents: the intracellularly active drugs hydroxychloroquine and remdesivir, a cell surface active surfactant (baby shampoo), and novel cell surface active reducing agents. The team also completed pilot studies of other potential antiviral agents such as ivermectin and camostat.

RESULTS

Remdesivir. The nucleoside inhibitor remdesivir is one of the few FDA approved drugs for COVID-19, and while it demonstrated significant clinical benefit in SARS-CoV-2 infected patients in U.S. trials, subsequent international trials failed to demonstrate any efficacy for this agent[2]. These findings suggest that improving therapeutic index by aerosolized dosing may translate to better clinical outcomes. Furthermore, since remdesivir can currently only be given intravenously, its current use is effectively limited to hospitalized patients. Like other antiviral agents, it is likely that remdesivir would be most effective if given early in the course of disease. A non-invasive, inhaled route of administration could facilitate drug delivery earlier after initial infection or even prophylactically in exposed individuals.

To explore the aerosolized potential of remdesivir, we compared HBE treated with remdesivir given by aerosolization (Aero) to cells treated with drug in basolateral media to mimic systemic dosing (Baso). For these experiments, the basolateral treatment was 0.5 μM remdesivir in media, representing typical serum concentrations of the drug when given intravenously. For aerosolization, we utilized 0.5 mM remdesivir (maximum solubility in saline) given by aerosolization at doses mimicking a single nebulizer treatment for various time intervals: a typical 10 min treatment as well as longer 20 min (2x) and 40 min (4x) inhaled treatments. Concentrations of remdesivir were measured by mass spectrometry in the airway surface liquid (ASL), the cell culture media (basolateral side), and in intracellular lysates 24 hours after dosing. Because remdesivir must be converted to a triphosphate metabolite for antiviral activity, we also measured intracellular concentrations of the triphosphate metabolite. Since ASL and intracellular contents are diluted during cell lysis and processing, in parallel experiments we assessed the extent of this dilution by adding urea into the basolateral media and allowed it to diffuse throughout all extra-and intracellular compartments prior to analysis, then measuring urea concentrations by mass spectrometry [ref]. This allowed us to determine that ASL was diluted 10-fold and intracellular contents were diluted 50-fold during processing, and measured values were corrected for this dilution factor.

In these experiments, remdesivir was not detected in ASL when given by basolateral dosing, but ASL remdesivir increased in a dose dependent manner when drug was given by aerosolization (**Fig. 2A**). Contrasting results were observed in media. In the basolateral treatment group, substantial drug remained in media (~25% of original concentrations), whereas drug concentrations in media were substantially lower in HBE treated by aerosolization (**Fig. 2B**). Intracellular remdesivir concentrations were very low in basolaterally treated cells, suggesting extensive metabolism, but were significantly higher in cells treated by aerosolization (**Fig. 2C**). Interestingly, the intracellular remdesivir concentrations were not dose dependent, suggesting possible saturable compartmentalization of drug. Most importantly, the concentrations of the active triphosphorylated remdesivir metabolite exhibited dose dependent increases with aerosolization, with concentrations at the highest aerosolized dose exceeding that of drug given basolaterally (**Fig. 2D**).

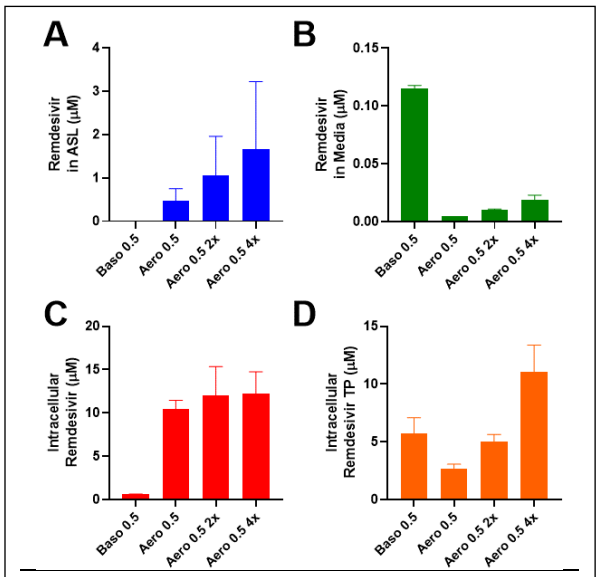

Fig. 2. **PK of remdesivir**. HBE were treated with remdesivir either basolaterally (Baso) or by aerosolization (Aero) as described in the text. Remdesivir concentrations (corrected for dilution during processing) are reported in A) ASL, B) media, and C) intracellularly. D) Intracellular concentrations of the active remdesivir triphosphate metabolite.

In summary, these experiments suggest that inhaled remdesivir has significant potential as a therapeutic for SARS-CoV-2. Reasonable aerosolization strategies can generate intracellular concentrations of the active metabolite that are higher than those expected from systemic dosing. Further studies are needed to identify the optimal dosing for antiviral efficacy and the duration of intracellular drug in HBE to guide dosing frequency.

Hydroxychloroquine. Another potential COVID-19 drug examined with the PK/PD system was hydroxychloroquine (HCQ). This drug was felt to be an ideal candidate for aerosolized drug delivery since it has 1) proven activity against SARS-CoV-2 and several other viruses *in vitro*, 2) a PK profile suggesting slow, suboptimal uptake into the lung with systemic dosing[3], and 3) known cardiac toxicity that can limit systemic dosing. To examine HCQ PK in airway epithelia, we treated HBE cells with 5 μM drug in media to mimic typical serum concentrations achieved with systemic dosing in comparison to HBE treated with aerosolization at rates mimicking a single nebulized dose at either low (10 mM) or high (40 mM) concentrations. Samples were collected 24 hours after dosing, and concentrations of HCQ and its metabolites were measured using mass spectrometric methods developed for this purpose and corrected for dilution. These experiments confirmed that HCQ accumulate intracellularly to high concentration. Intracellular HCQ concentrations from cells treated with low aerosolized dose were modestly lower than cells treated with systemic dosing, whereas a high aerosolized dose achieved levels higher than systemic dosing (**Fig. 3A**). We detected minimal metabolism of HCQ to its primary metabolites desethylchloroquine (DCQ) and didesethylchloroquine (DDCQ) with any treatment strategy (high dose aerosol shown in **Fig. 3B**). To assess durability of treatment, we maintained cells treated with low dose aerosolized HCQ for 96 hours after treatment, changing media every 24 hours. These experiments determined that

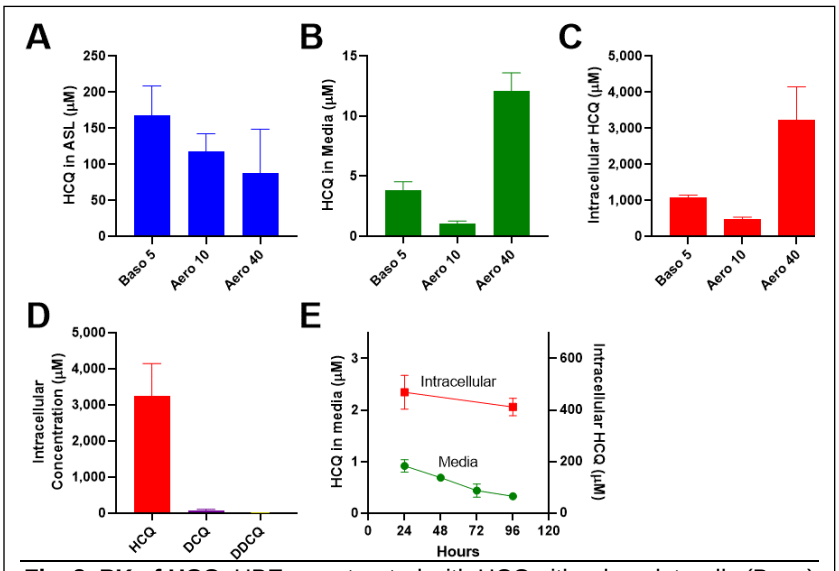

Fig. 3. **PK of HCQ**. HBE were treated with HCQ either basolaterally (Baso) or by aersoloization (Aero) as described in the text. HCQ concentrations are reported in A) ASL, B) media, and C) intracellularly. D) 24 hours after high dose (40 mM) aerosol treatment, minimal metabolism of HCQ to DCQ or DDCQ was observed. E) Cells treated with a single low dose (10 mM) aerosolized HCQ were maintained for 96 hours post treatment, with media changed every 24 hours. HCQ concentrations reported in both media and intracellular compartments.

intracellular HCQ slowly diffused out into the cell culture media over time. However, this loss was modest, with relatively low concentrations in media and preservation of intracellular drug concentrations even 96 hours post a single aerosolized dose (**Fig. 3C**).

These experiments confirmed that aerosolization has the potential to deliver high, long lasting concentrations of intracellular HCQ to airway epithelia with minimal systemic effects. Several clinical trials have shown that systemically administered HCQ does not have significant clinical efficacy in COVID-19[4], but lack of efficacy could reflect the known poor PK properties of systemically administered HCQ, with slow uptake into the lung coupled with systemic side effects that limit dosing[3]. Our studies suggest that aerosolized HCQ could overcome these limitations by delivering high, antiviral concentrations of drug directly to airway epithelia while minimizing drug metabolism and systemic toxicity. The persistence of intracellular HCQ suggests that even intermittent aerosolized dosing could provide persistent antiviral activity. The team is seeking support for further studies to determine the potential of aerosolized HCQ as a therapeutic for COVID-19 or other viral respiratory diseases.

Cell surface surfactant (baby shampoo). Detergents and surfactants are well-established antivirals properties since they readily disrupt the lipid membrane that surrounds SARS-CoV-, but most are too toxic to utilize directly on airway epithelia. Baby shampoos are much less toxic than other detergents, and otolaryngologists have long recommended dilute (0.5-1%) solutions of baby shampoo in sinus rinses to provide a gentle detergent effect[5]. Therefore, topical application of dilute baby shampoo solutions holds promise as an antiviral strategy, particularly in the nose as the initial portal of entry in SARS-CoV-2[6].

To explore the promise of topical surfactacts, we examined the safety and efficacy of dilute baby shampoo (Johnson's and Johnson's baby shampoo) at concentrations (0.5%-1.0% in normal saline) similar to those utilized in sinus rinses. Application of baby shampoo did not have any apparent toxic effects on human nasal epithelia (HNE) or impacts on cell composition as assessed using confocal microscopy (**Fig 4A**). Similarly, shampoo had only a mild and transient effect on transepithelial airway resistance as a measure of cellular integrity (**Fig. 4B**). To assess efficacy, SARS-CoV-2 was mixed 1:1 with 0.5% baby shampoo (250 µL shampoo in 24 mL normal saline) for 5 or 90 minutes, with titers of active virus assessed using standard methods[7]. Shampoo treatment decreased SARS-CoV-2 titers by ~2 orders of magnitude within 5 minutes of treatment, with further decreases after 90 minutes (**Fig. 4C**). Even greater potency was observed with other viruses, including the coronavirus NL63 (**Fig. 4D**) and respiratory syncytial virus (RSV) (**Fig. 4E**). For these experiments, we utilized genetically modified NL63 coronavirus and RSV that express Green Fluorescent Protein (GFP), allowing the Tissue Culture Infectious Dose (TCID50) to be easily tracked by the intensity of the fluorescent signal[8]. This approach allows us to rapidly test hypotheses without needing time in one of the few BSL-3 facilities that are required for experiments with SARS-CoV-2. An additional benefit is that data using NL63 is directly applicable to one of the viruses responsible for upper respiratory infections.

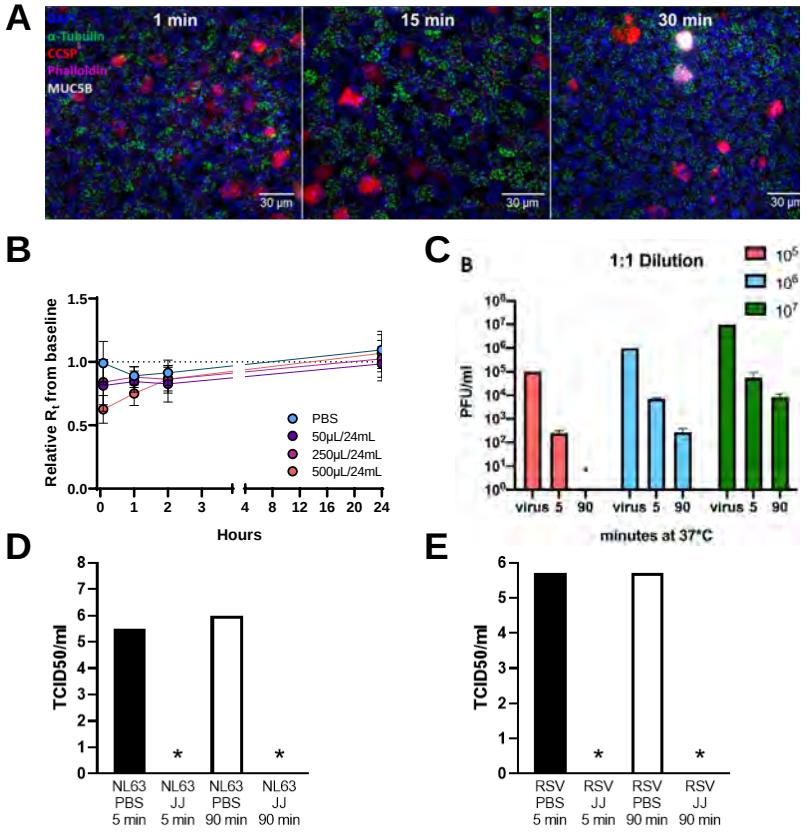

Fig. 4. Baby Shampoo Safety and in vitro Efficacy. A) Confocal microscopy showing that application of baby shampoo to HNE did not result in obvious evidence of cellular toxicity. B) Application of shampoo to HNE resulted in short term decreases in transepithelial airway resistance (Rt) only at higher concentrations, suggesting minimal toxicity. C) Treating SARS-CoV-2 1:1 with shampoo reduced titers by two or more orders of magnitude. D) Treating GFP-labeled NL-63 coronovirus 1:1 with shampoo reduced tissue culture infectious dose (TCID) measured using fluorescence.

Having established safety and antiviral activity, we then explored efficacy of baby shampoo in an HNE model system of active viral infection. HNE were infected with SARS-CoV-2 at an MOI of 0.1, and titers were obtained at 48 hours to verify active infection. At 72 hours, cells were treated with 200 µl of 0.5% shampoo or PBS as a control, with the treatment removed by aspiration after 10 minutes to mimic typical mucociliary clearance time in the nose. Viral titers were then measured four hours later (76 hours) and again at 96 hours. As expected, viral titers were high at all measured time points in the PBS (control) treated cells (**Fig. 5A**). However, somewhat surprisingly shampoo treatment had no significant impact on viral titers at any timepoint. Lack of efficacy in our HNE system parallels conclusions from a clinical trial by colleagues at Vanderbilt, who found that nasal lavage of 0.5% baby shampoo had no significant impact on nasal viral load in patients infected with SARS-CoV-2 (**Fig. 5B**). To better understand why baby shampoo did not exhibit antiviral activity on nasal epithelia, we utilized MS to assess the PK properties of shampoo when applied to HNE. Baby shampoo is a proprietary mixture of detergents and surfactants, but the dominant signal from full scan MS was found in six peaks with run times between 7.6 and 10.1 that had masses and fragmentation patterns consistent with various chain lengths of cocamidopropyl betaine (CAPB) (**Fig. 5C**). CAPB is the first listed surfactant in the baby shampoo ingredient list, and the relative abundances of the different chain lengths, as assessed by peak area on chromatograms, was similar to the known pattern of chain lengths in CAPB: mostly C12 with lesser quantities of lower and higher chain lengths (**Fig. 5D**). We then evaluated samples in which shampoo was applied to HNE then washed after 1 or 30 minutes. We found that shorter chain lengths of CABP (C8 and C10) were readily recovered by HNE wash, but that the concentrations of C12 were reduced significantly and the longest chain lengths (C14, C16, C18) were below detection limits of the assay. These differences were observed in samples lavaged very quickly (one minute) after application of baby shampoo to HNE and persisted in samples lavaged later (**Fig. 5D**).

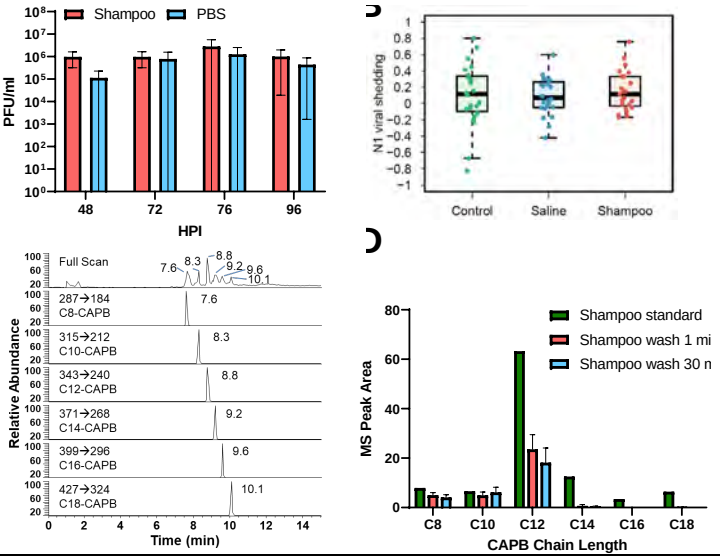

Fig. 5. **Baby Shampoo PK/PD**. A) No differences in SARS-CoV-2 titers were observed in HNE infected at day zero and treated with shampoo or PBS at 72 hours B) Baby shampoo nasal washes did not affect SARS-CoV-2 viral shedding relative to control and saline alone, measured as log10(change values from Ct value at first day to the max value of Ct)/days between two values) of the N1 nucleocapsid expression. C). Full scan MS of baby shampoo dominated by six peaks that had masses and fragmentation patterns of various chain lengths of CAPB. D). Longer chain CAPB were depleted after baby shampoo was applied to then washed off HNE.

Taken together, these findings indicated that while baby shampoo has antiviral properties and can be safely applied to nasal epithelia, it does not have efficacy as a therapeutic. The most likely explanation is that the antiviral activity of baby shampoo arises mainly from the longer chain surfactants, which are rapidly depleted from baby shampoo applied to HNE by binding to or intercalating into epithelial membranes. These findings are consistent with previous studies suggest that longer chain length surfactants are the most potent as antivirals but bind readily to cells. Their rapid depletion on cell surfaces limits the clinical efficacy of baby shampoo and likely other surfactant based therapies.

Reducing agents. Infection with SARS-CoV-2 depends upon an interaction between the viral spike protein and a cellular receptor (ACE2). The viral spike protein has to have a specific configuration to bind well to the receptor, which is maintained in part by the presence of disulfide bonds within the protein. Therefore reducing agents, which break disulfide bonds, might change the configuration of the viral spike protein and reduce infectivity. We tested this hypothesis through evaluation of two novel reducing agents from Parion Sciences, 2119 and 2165, that are being developed as inhaled therapeutics for chronic lung diseases. Consistent with our hypothesis, both 2119 and 2165 were able to reduce the infectivity of SARS-CoV-2 treated prior to infection (**Fig 6A**). Similar findings were seen with NL63 coronavirus and parainfluenza virus (PIV) (**Fig 6B**), both of which also rely on viral protein-receptor interactions for infectivity. However, our previous studies have demonstrated that the inhaled reducing agents are quickly oxidized on the cell surface (**Fig 6C**), suggesting a lack of durable antiviral action.

Thus, we predict that the antiviral activity of reducing agents will be lost quickly after aerosolization. Further development of newer agents with prolonged cell surface activity would be needed for reducing agents to be effective as clinical antiviral therapies.

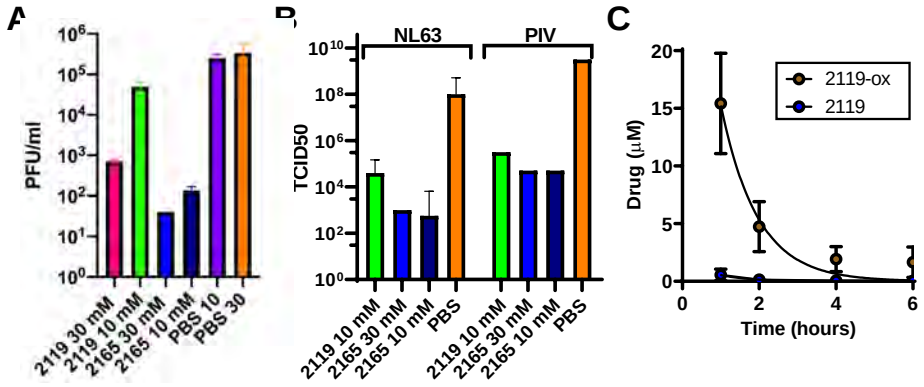

Fig. 6. Reducing Agents. A) SARS-CoV-2 infectivity was significantly reduced by pre-treatment with the reducing agents 2119 or 2165. B) Similar impact of reducing agents was observed in NL63 and PIV. C) In bronchoalveolar lavage obtained from sheep at various time intervals after treatment with nebulized 2119, very little native drug was detected. Oxidized 2119 (2119-ox) was present and cleared over time.

Other agents. While the limited time of the award precluded extensive investigation of other agents, we did lay the groundwork to examine other potential therapies for COVID-19. Camostat has potential antiviral activity at the cell surface by blocking cleavage of the viral spike protein needed for cell entry. Because the drug is rapidly metabolized, we developed methods to measure its primary (and active) metabolite FOY-251 (**Fig. 7A**). We also developed methods to measure ivermectin (**Fig. 7B**), an anti-parasitic drug that inhibits SARS-CoV-replication *in vitro* but likely has poor distribution to the lungs with standard oral dosing[9]. Development of these methods puts us in strong position for future studies of these and other drugs that have antiviral activity.

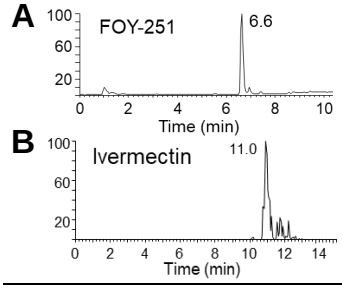

Fig. 7. Other Agents. Mass spectrometric chromatograms of A) FOY-251, the active metabolite of camostat, and B) ivermectin

Viral effects on gene expression. Viral infection is known to affect gene expression in airway epithelia, which could alter expression of genes involved in transport and metabolism of antiviral drugs. To assess potential impacts of viral infection on expression of relevant genes, we performed RNA sequencing studies on cultured HBE infected with SARS-CoV-2. HBE were obtained from donors of a variety of ages from infants to elderly, allowing us to also assess age related changes in expression. In these experiments, gene expression was measured at day 1, 3, 7, and 14 post infection, with viral titers noted to be highest on day 3 of infection. We initially focused on genes involved in transport and metabolism of remdesivir, since this drug relies on multiple transporters and enzymes to be converted to its active, triphosphate form. Remdesivir is transported intracellularly via the SLC29A3 nucleoside transporter and is then cleaved from its pro-drug form by carboxylesterase 1 (CES1) or cathepsin A (CTSA). The drug is then converted into the nucleoside monophosphate by a phosphoramidase (HINT1) and further phosphorylated to the active triphosphate by adenylate kinase (AK2). Active drug can be desphosphorylated and inactivated by nucleotidases such as NTSC3B and NT5C2. In our gene expression

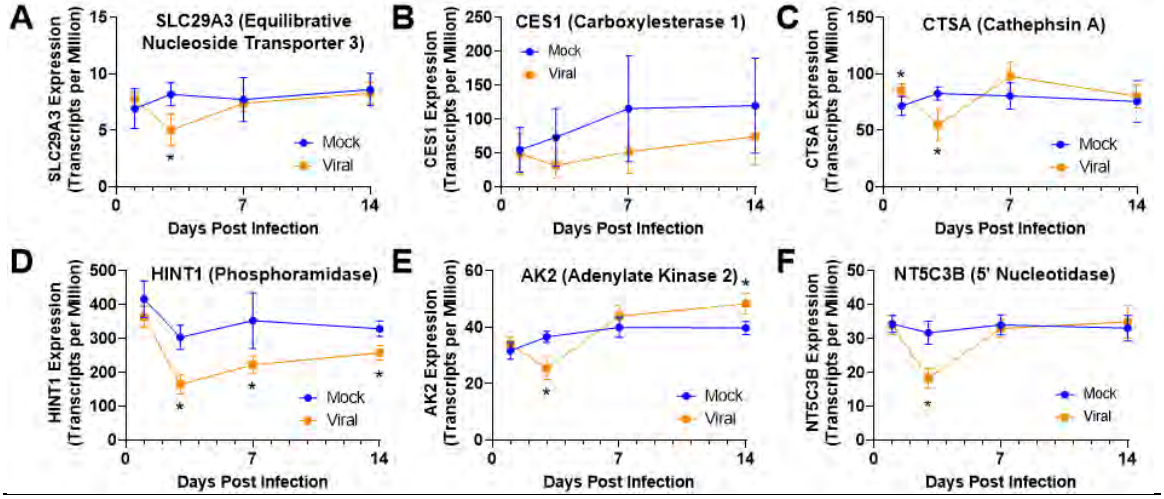

Fig. 8. **Gene Expression**. HBE were infected with SARS-CoV-2 viral infected (orange) and mock infected (blue), and expression over time was monitored for genes involved in remdesivir transport and metabolism including the nucleoside transporter SLC29A3 (A), the esterases CES1 (B) and CTSA (C), the phophoramidase HINT1 (D), the adenylate kinase AK2 (E), and the nucleotidase NT5C3B (F). Significant decreases in expression in virally infected cells were noted for SLC29A3, CTSA, HIT1, AK2, and NT5C3B,

studies, we observed that several of these genes were downregulated during active SARS-CoV-2 infection in HBE, with the most significant changes observed at day 3 of active infection for SLC29A3, CTSA, HINT1, AK2, and NTSC3B (**Fig. 8A-F**). These findings suggest that highly infected cells may have reduced ability to transport remdesivir and convert it to its antiviral metabolite. We did not observe any significant impact of the age of the donor on expression of these genes.

We performed a similar analysis for HCQ. Current literature suggests that movement of HCQ into the cell is not highly dependent on transporters, as the drug is sufficiently hydrophobic to readily cross lipid membranes. Furthermore, intracellular accumulation is not transporter driven but results from protonation of HCQ in acidic compartments such as lysosomes that reduces its lipid permeability, effectively trapping it within the cell. However, some studies have suggested that the efflux pump P-glycoprotein (ABCB1) may influence intracellular HCQ concentrations, with evidence that HCQ inhibits P-glycoprotein activity at high concentrations, and overexpression of P-glycoprotein reduces HCQ toxicity[10]. We do not believe this mechanism would meaningfully impact HCQ concentrations in viral infection, since in our RNA sequencing studies expression on ABCB1 in HBE was low and not significantly influenced by viral infection (**Fig. 9**). We also do not believe viral infection would influence HCQ metabolism, since the cytochrome P450 system likely involved in HCQ metabolism (CYP2C8, CYP3A4, CYP2D6) are primarily expressed in the liver. Furthermore, we did not observe significant metabolism of HCQ in HBE (see **Fig 3**). Therefore, unlike with remdesivir, we do not believe viral infection is likely to influence intracellular HCQ concentrations in treated epithelia.

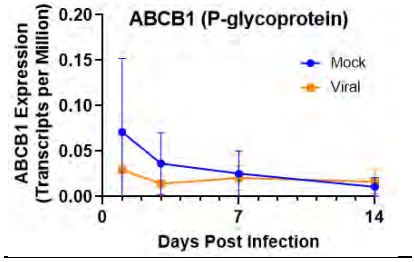

Fig. 9. **Gene Expression**. Gene expression of P-glycoprotein (ABCB1) was low and not influenced by SARS-CoV-2 viral infection in HBE.

Confocal Microscopy. Funding from this proposal supported purchase of a new confocal microscope that offers improved resolution over our existing instruments. Our primary purpose was to utilize this instrument to assess for drug toxicities in our PK/PD system, as we did for baby shampoo (see **Fig. 4A**). This instrument also proved useful in other studies of COVID-19, including evaluation of autopsied lungs from patients who died of COVID-19. We were able to demonstrate increases in inflammasome activation (**Fig. 10A**) as well as increased levels of neutrophil elastase and its naturally occurring inhibitor alpha-1 antitrypsin (**Fig. 10B**). These experiments highlight the role of inflammatory responses in COVID-19, which are known to underlie much of the morbidity and mortality of this disease. They also identify potential targets for anti-inflammatory treatments that are key aspects of COVID-19 treatment.

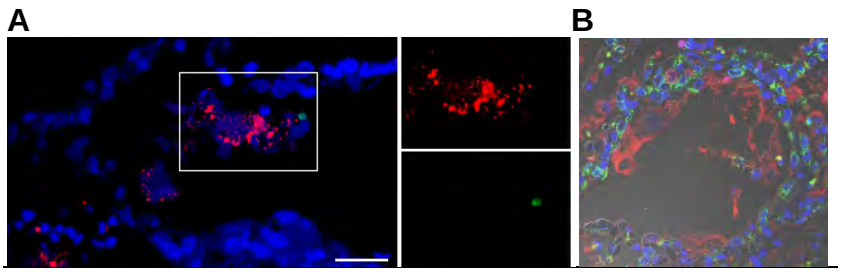

Fig. 10. **Confocal Microscopy**. A) Confocal image of showing inflammasome activation (PYCARD staining, green) in proximity to SARS-CoV-2 (red) in a lung autopsy from a patient who died from COVID-19. Cell nuclei are stained blue with DAPI. B) Airway from a COVID-19 patient showing prominent staining of human neutrophil elastase (ELANE, green) and naturally occurring protease inhibitor (SERPINA1, red).

CONCLUSIONS

The goal of this project was to develop a respiratory epithelial PK/PD system to assess the therapeutic potential of drugs for COVID-19, focusing on therapeutic agents suitable for aerosolization that were either active on the cell surface and or required intracellular accumulation for efficacy. Our studies successfully demonstrated the aerosolization potential of two intracellularly active drugs, remdesivir and HCQ. For both drugs, antiviral intracellular concentrations could be achieved quickly using aerosolization strategies that equivalent to those achievable with existing nebulizers. Although more work remains to be done, we anticipate that our findings can be readily translated into clinical studies of aerosolized treatment early in disease when infection burden is less or even prophylactically in exposed individuals. Our PK/PD system was also able to uncover challenges with cell surface therapies that likely limit clinical utility. Although we have hypothesized that removal of drug by mucociliary clearance would limit the potential of cell surface drugs, in fact lack of efficacy could be traced to rapid inactivation of drug on cell surfaces. While disappointing, our system allows further testing of such agents to identify strategies to overcome these challenges and increase the likelihood of success in future clinical trials.

Indeed, funding from this proposal has left us well-positioned for future studies of aerosolized drug therapies for COVID-19 and other viral infections.

References

1. Ehre C. SARS-CoV-2 Infection of Airway Cells. N Engl J Med 2020;383:969.
2. Cohen J, Kupferschmidt K. 'A very, very bad look' for remdesivir. Science 2020;370:642-3.
3. McChesney EW. Animal toxicity and pharmacokinetics of hydroxychloroquine sulfate. Am J Med 1983;75:11-8.
4. Maisonnasse P, Guedj J, Contreras V, et al. Hydroxychloroquine use against SARS-CoV-2 infection in non-human primates. Nature 2020;585:584-7.
5. Chiu AG, Palmer JN, Woodworth BA, et al. Baby shampoo nasal irrigations for the symptomatic post-functional endoscopic sinus surgery patient. Am J Rhinol 2008;22:34-7.
6. Hou YJ, Okuda K, Edwards CE, et al. SARS-CoV-2 Reverse Genetics Reveals a Variable Infection Gradient in the Respiratory Tract. Cell 2020;182:429-46 e14.
7. Sheahan TP, Sims AC, Zhou S, et al. An orally bioavailable broad-spectrum antiviral inhibits SARS-CoV-2 in human airway epithelial cell cultures and multiple coronaviruses in mice. Sci Transl Med 2020.
8. Donaldson EF, Yount B, Sims AC, Burkett S, Pickles RJ, Baric RS. Systematic assembly of a full-length infectious clone of human coronavirus NL63. J Virol 2008;82:11948-57.
9. Chaccour C, Abizanda G, Irigoyen-Barrio A, et al. Nebulized ivermectin for COVID-19 and other respiratory diseases, a proof of concept, dose-ranging study in rats. Scientific reports 2020;10:17073.
10. Weiss J, Bajraktari-Sylejmani G, Haefeli WE. Interaction of Hydroxychloroquine with Pharmacokinetically Important Drug Transporters. Pharmaceutics 2020;12.