Comparative Therapeutic Potential of ALX-0171 and Palivizumab against Respiratory Syncytial Virus Clinical Isolate Infection of Well-Differentiated Primary Pediatric Bronchial Epithelial Cell Cultures

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ABSTRACT Respiratory syncytial virus (RSV) causes severe lower respiratory tract infections in young infants. There are no RSV-specific treatments available. Ablynx has been developing an anti-RSV F-specific nanobody, ALX-0171. To characterize the therapeutic potential of ALX-0171, we exploited our well-differentiated primary pediatric bronchial epithelial cell (WD-PBEC)/RSV infection model, which replicates several hallmarks of RSV disease *in vivo*. Using 2 clinical isolates (BT2a and Memphis 37), we compared the therapeutic potential of ALX-0171 with that of palivizumab, which is currently prescribed for RSV prophylaxis in high-risk infants. ALX-0171 treatment (900 nM) at 24 h postinfection reduced apically released RSV titers to near or below the limit of detection within 24 h for both strains. Progressively lower doses resulted in concomitantly diminished RSV neutralization. ALX-0171 was approximately 3-fold more potent in this therapeutic RSV/WD-PBEC model than palivizumab (mean 50% inhibitory concentration [IC₅₀] = 346.9 to 363.6 nM and 1,048 to 1,090 nM for ALX-0171 and palivizumab, respectively), irrespective of the clinical isolate. The number of viral genomic copies (GC) was determined by quantitative reverse transcription-PCR (RT-qPCR), and the therapeutic effect of ALX-0171 treatment at 300 and 900 nM was found to be considerably lower and the number of GCs reduced only moderately (0.62 to 1.28 log₁₀ copies/ml). Similar findings were evident for palivizumab. Therefore, ALX-0171 was very potent at neutralizing RSV released from apical surfaces but had only a limited impact on virus replication. The data indicate a clear disparity between viable virus neutralization and GC viral load, the latter of which does not discriminate between viable and neutralized RSV. This report validates the RSV/WD-PBEC model for the preclinical evaluation of RSV antivirals.

KEYWORDS antiviral agents, nanobody, primary cells, respiratory syncytial virus, respiratory viruses

Respiratory syncytial virus (RSV) is a member of the *Pneumoviridae* family, *Orthopneumovirus* genus (1). It is the leading cause of severe lower respiratory tract infections (LRTI) in infants worldwide (2, 3), with an estimated 33.8 million LRTI cases occurring yearly. RSV accounts for approximately 3.4 million hospitalizations and up to 199,000 deaths worldwide, predominantly in developing countries (4). Economic burden and childhood morbidity and mortality rates associated with RSV are, in many countries, comparable to those seen with influenza (5).

Severe RSV infection is associated with an increase in mucus production and a decrease in the number of ciliated cells in the airway epithelium. There is a large influx

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of immune cells to the airways consisting predominantly of neutrophils but also lymphocytes and macrophages (6). The cellular infiltrate, together with mucus and sloughed epithelial cells (ECs), causes lumen obstruction and inflammation of the airways. Associated with mucus plug formation and bronchiole occlusion, bronchiolitis is therefore more severe in smaller airways, such as those of young or preterm infants (7). Accordingly, 66% of RSV-related hospitalizations are in children <6 months old (8). Risk factors associated with the development of severe RSV-LRTI in infants include the following: prematurity, bronchopulmonary dysplasia, congenital lung or heart conditions, male gender, age of ≤ 6 months, neuromuscular disorders, and immunodeficiency (9). However, the majority of patients that require hospitalization due to severe RSV-related disease have no underlying health conditions that constitute a risk factor (3). There is mounting evidence to suggest that occurrence of severe RSV infection in early life is associated with the development of wheeze and subsequently of asthma (10).

RSV infection remains a major unmet medical treatment need. Other than the antiviral ribavirin, there is no licensed RSV vaccine or therapeutic, despite the considerable medical importance of this virus. Palivizumab, a neutralizing monoclonal antibody that recognizes a conserved epitope in the viral fusion surface glycoprotein (RSV F site II) (11), is administered prophylactically to high-risk infants, e.g., those diagnosed with chronic lung disease of prematurity, congenital heart disease, or premature birth (typically limited to those with gestational age of less than 29 weeks for cost/benefit reasons). This is an expensive approach, costing \$6,000 to \$20,000 per patient for 1 RSV season (12). In addition to cost, as indicated above, a major limitation of this approach is that the majority of infants hospitalized with RSV do not fall into these high-risk categories. Palivizumab was assessed as a therapeutic treatment in patients who were hospitalized with RSV but who failed to demonstrate a reduction in viral titers from nasal aspirates or in disease severity (13). Therefore, understanding how RSV causes disease in humans and development of therapeutics remain important medical objectives.

One potential limitation to RSV antivirals being effective is that the viral load might have peaked by the time that infants are hospitalized. However, a study of RSV clearance in hospitalized children demonstrated that higher viral titers at day 3 of hospitalization were not associated with risk factors such as weight, gestational age, sex, or age at time of admission but were associated with the requirement for intensive care and respiratory failure, indicating a potential therapeutic window even in hospitalized infants (14). Results seen with oseltamivir (Tamiflu), an antiviral against influenza virus, demonstrate the importance of the time of administration following infection for effective treatment; administered within 48 h of symptom onset in clinically confirmed cases of influenza, it is effective at reducing the length of illness in patients hospitalized with influenza (15). Administered after that time, however, oseltamivir failed to have any effect on virus titers, disease severity, or illness duration (16).

The majority of RSV pathogenesis, antiviral, and prophylaxis studies have been performed in animal models or continuous cell lines, neither of which represents an optimal setting. Animal models, especially mouse models, are semipermissive for RSV replication and do not exhibit high viral titers or pulmonary pathology associated with RSV in infants unless very high inocula are employed (17–19). Continuous cell lines, e.g., HEp-2 and A549 cells, are poorly representative of the complexities of cell interactions in the human lung. The development of the well-differentiated primary pediatric bronchial epithelial cell (WD-PBEC) culture model has provided an authentic surrogate facilitating the elucidation of mechanisms of RSV pathogenesis in pediatric airways (20, 21) and, thereby, the study of RSV-specific antivirals. WD-PBECs reproduce hallmarks of RSV infection *in vivo* and can be infected for prolonged experiments without extensive damage to the cultures (22). Several studies have demonstrated RSV neutralization in human airway epithelial cells (HAECs) that was not evident in experiments using continuous cell lines such as HeLa or HEp-2 (23, 24).

There are several RSV therapeutics currently in development, including entry inhib-

FIG 1 Duplicate WD-PBEC cultures ($n = 3$ donors) were infected apically with either RSV BT2a (A) or RSV Memphis 37 (B) (multiplicity of infection [MOI] = 0.1) for 2 h at 37°C and then washed 5 times. The fifth wash was retained as the 2 hpi time point for virus titrations. At 24 hpi (and every 24 h thereafter), apical washes were undertaken and harvested for virus titrations. Following the apical washes at 24 hpi (and every 24 h thereafter), the cultures were apically treated with 100 μ l ALX-0171 at the indicated concentrations. After 1 h, the treatment was removed and replaced with 10 μ l ALX-0171 at the same concentration, which remained on the apical surfaces for the duration of the interval between washes. All apical washes were titrated on HEp-2 cells to determine viral titers, which were reported as log₁₀ 50% tissue culture infective doses per milliliter $\pm$ standard errors of the means (SEM). LOD, limit of detection.

itors and nucleoside analogues (25). One potential RSV therapeutic developed by Ablynx, ALX-0171, is a trivalent non-extended-half-life nanobody that specifically binds to RSV F site II. ALX-0171 is composed of 3 heavy chain variable region (VHH) domains providing strong binding to RSV, and it potently neutralizes infectious virions (26). The neutralizing and therapeutic activity of ALX-0171 against RSV was previously demonstrated in the neonatal lamb RSV infection model using RSV strain Memphis 37 (27). In this current study, we exploited our well-differentiated pediatric primary bronchial epithelial cell (WD-PBEC) culture model of RSV infection to assess the relative ALX-0171 50% inhibitory concentration (IC_{50}) values for the two RSV clinical strains, BT2a and Memphis 37, in comparison to palivizumab. We also aimed to establish whether any adjustment in the targeted lung lining fluid concentration, as determined in the RSV lamb model, is needed based on strain sensitivity differences.

RESULTS

There was a clear dose effect of ALX-0171 treatment on RSV BT2a and Memphis 37 growth kinetics. RSV BT2a titers diminished dramatically by 24 h of treatment with 900 nM ALX-0171, with a 4.74 log₁₀ reduction in mean virus titers (Fig. 1A). The virus was almost completely neutralized by this time under these conditions, and virus neutralization was maintained for the duration of the experiment. Treatment using a 300 nM concentration also resulted in reductions in the levels of apically released RSV BT2a, with a >2 log₁₀ reduction in mean virus titers evident at 144 h postinfection (hpi) relative to buffer-treated RSV BT2a-infected control cultures. A less marked reduction in mean RSV BT2a titers was observed following treatment with a concentration of 100 nM relative to buffer-treated controls.

Substantial reductions were also evident in RSV Memphis 37 titers by 24 h of treatment with 900 nM ALX-0171, with a 4.2 log₁₀ reduction in mean virus titers, although low virus titers were continually detectable in these cultures until 144 hpi (Fig. 1B). Lower, but nonetheless substantial, reductions in RSV Memphis 37 titers were also evident following treatment with 300 nM ALX-0171, whereas treatment with 100 nM did not result in substantially reduced viral titers. In contrast, treatment with <100 nM ALX-0171 concentrations did not influence growth kinetics of either RSV strain relative to buffer-treated control cultures under these experimental conditions.

Unlike ALX-0171, infectious RSV BT2a was detected following treatment with all palivizumab concentrations. However, there was a clear dose effect on virus titers following palivizumab treatment of RSV BT2a-infected WD-PBEC cultures. Treatment with 900 nM palivizumab resulted in >2 log₁₀ reductions in viral titers. Treatment with

FIG 2 Duplicate WD-PBEC cultures ($n = 3$ donors) were infected apically with RSV BT2a (A) or RSV Memphis 37 (B) (MOI = 0.1) for 2 h at 37°C and then washed 5 times. The fifth wash was retained as the 2 hpi time point for virus titrations. At 24 hpi (and every 24 h thereafter), the cultures were apically washed and harvested for virus titrations. Following the apical washes at 24 hpi (and every 24 h thereafter), the cultures were apically treated with 100 μ l palivizumab at the indicated concentrations. After 1 h, the treatment was removed and replaced with 10 μ l palivizumab at the same concentration, which remained on the apical surfaces for the duration of the intervals between washes. All apical washes were titrated on HEp-2 cells to determine viral titers, which were reported as log₁₀ 50% tissue culture infective doses per milliliter $\pm$ SEM. LOD, limit of detection.

300 or 100 nM palivizumab also resulted in reductions in viral titers that were substantial but lower than those seen with the buffer-treated controls (Fig. 2A). However, treatment with 33, 11, or 4 nM palivizumab had no effect on the growth kinetics of RSV BT2a.

Similar results in RSV Memphis 37 titers were also observed following treatment with palivizumab. Treatment with either 900 or 300 nM palivizumab resulted in substantial reductions in viral titers (Fig. 2B). However, only slight reductions in mean Memphis 37 titers were evident following treatment with 100 nM palivizumab. Treatment with <100 nM palivizumab did not alter the growth kinetics of RSV Memphis 37 relative to the buffer control.

Treatment with a 900 nM, 300 nM, or 100 nM concentration of either ALX-0171 or palivizumab resulted in substantial reductions in RSV BT2a titers. At the highest concentration tested (900 nM), ALX-0171 appeared to be considerably more potent than palivizumab, reducing the viral titers to the limit of detection (Fig. 3A). The two highest concentrations of ALX-0171 (900 nM and 300 nM) and only the highest con-

FIG 3 WD-PBEC cultures ($n = 3$ donors and duplicated cultures for each conditions) were infected apically with RSV BT2a (A) or RSV Memphis 37 (B) (MOI = 0.1) for 2 h at 37°C and then washed 5 times. The fifth wash was retained as the 2 hpi time point for virus titrations. At 24 hpi (and every 24 h thereafter), apical washes were undertaken and harvested for virus titrations. Following the apical washes at 24 hpi (and every 24 h thereafter), the cultures were apically treated with 100 μ l of either ALX-0171 or palivizumab at the indicated concentrations. After 1 h, the treatment was removed and replaced with 10 μ l of either ALX-0171 or palivizumab at the same concentration, which remained on the apical surfaces for the duration of the intervals between washes. All apical washes were titrated on HEp-2 cells to determine viral titers, which were reported as log₁₀ 50% tissue culture infective doses per milliliter $\pm$ SEM. LOD, limit of detection.

TABLE 1 IC_{50} of ALX-0171 and palivizumab for RSV BT2a or RSV Memphis 37

Strain and antiviral	IC_{50} (95% confidence interval) in nM
RSV Memphis 37 ^a	
ALX-0171	363.6 (276.3–472.9)
Palivizumab	1,090 (727.2–2,126)
RSV BT2a ^b	
ALX-0171	346.9 (262.2–468.3)
Palivizumab	1,048 (869–1,337)

^a P value for palivizumab versus ALX-0171, 0.0038.^b P value for palivizumab versus ALX-0171, 0.0001.

centration of palivizumab substantially reduced the viral titers of RSV Memphis 37 compared to buffer-treated controls. However, treatment with ALX-0171 resulted in reductions in viral growth kinetics that were greater than those seen with treatment with palivizumab at equimolar concentrations, except for treatment with 100 nM ALX-0171 and 100 nM palivizumab, which showed similar viral titers at each time point (Fig. 3B).

The IC_{50} for each treatment against each virus over the time course of the experiment was calculated (Table 1). The palivizumab IC_{50} (1,090 nM) was significantly higher than the IC_{50} of ALX-0171 (363.6 nM) against Memphis 37. Similarly, the IC_{50} of palivizumab (1,048 nM) against BT2a was also significantly higher than that of ALX-0171 (346.9 nM). The IC_{50} values calculated from the area under the curve (AUC) data were not significantly different for either ALX-0171 (P value = 0.751) or palivizumab (P value = 0.858) against the two RSV strains, indicating that the two viruses had comparable sensitivities to neutralization in the WD-PBEC culture model.

A trend toward a reduction in RSV BT2a viral loads, as determined by quantitative reverse transcription-PCR (RT-qPCR), was evident following treatment with both ALX-0171 and palivizumab. At 144 hpi, ALX-0171 900 and 300 nM doses reduced mean viral loads by 0.8 $\log_{10}$ genome copies (GC)/ml and 1.3 $\log_{10}$ GC/ml, respectively, versus the buffer-treated cultures (Fig. 4A). At the same time point and for the same doses, palivizumab reduced mean viral loads by 0.3 and 0.8 $\log_{10}$ GC/ml versus the buffer-treated cultures, respectively. Similarly, reduced viral loads were observed with both

FIG 4 WD-PBEC cultures ($n = 3$ donors and duplicated cultures for each conditions) were infected apically with RSV BT2a (A) or RSV Memphis 37 (B) (MOI = 0.1) for 2 h at 37°C and then washed 5 times. The fifth wash was retained as the 2 hpi time point. At 24 hpi (and every 24 h thereafter), apical washes were harvested. Following the apical washes at 24 hpi (and every 24 h thereafter), the cultures were apically treated with 100 μ l of ALX-0171 or palivizumab at the indicated concentrations. After 1 h, the treatment was removed and replaced with 10 μ l of either ALX-0171 or palivizumab at the same concentration, which remained on the apical surfaces for the duration of the intervals between washes. RNA was extracted from apical washes and RT-qPCR performed. Data were plotted as $\log_{10}$ genome copies per milliliter $\pm$ SEM.

ALX-0171 treatment and palivizumab treatment of Memphis 37-infected WD-PBEC cultures. At 144 hpi, 900 and 300 nM ALX-0171 reduced mean viral loads by 0.62 log₁₀ and 0.76 log₁₀ GC/ml, respectively, versus the buffer-treated cultures (Fig. 4B). At the same time point and for the same doses, palivizumab reduced mean viral loads by 0.27 and 0.57 log₁₀ GC/ml, respectively, versus the buffer-treated control cultures.

DISCUSSION

The aims of this study were 2-fold: to assess the efficacy of ALX-0171 as an anti-RSV therapeutic and to evaluate the WD-PBEC model for use in preclinical studies. Despite extensive research into RSV pathogenesis and mechanisms of disease, vaccines and treatments have remained elusive. Ablynx NV has developed ALX-0171 to address the need for a RSV treatment option. In this study, we used our RSV/WD-PBEC model to assess the therapeutic potential of ALX-0171 or of palivizumab to neutralize two different clinical strains of RSV. Palivizumab is the only licensed neutralizing anti-RSV monoclonal antibody. It is administered prophylactically, and its use is reserved for infants deemed at high risk of severe RSV infection, in large part because of its high cost. However, the majority of children hospitalized due to severe RSV infection are not classified as high risk and, as such, do not receive palivizumab prophylaxis. A therapeutic intervention that can be administered after the onset of symptoms would reduce the huge economic burden of RSV and potentially reduce the number and/or duration of hospitalizations.

Growth kinetics for RSV Memphis 37 and BT2a followed similar patterns in the WD-PBECs. The two strains reached similar peak viral titers between 72 and 96 hpi. RSV BT2a and Memphis 37 demonstrated similar susceptibilities to neutralization by ALX-0171 or palivizumab. The highest concentration of ALX-0171 (900 nM) reduced viral titers to near or below the limit of detection by 24 h posttreatment (~ 5 log₁₀ reduction), whereas palivizumab treatment at the same concentration was less effective (~ 3 log₁₀ reduction). These differences were reflected in the respective IC₅₀ values for the two molecules.

The RT-qPCR data demonstrated that ALX-0171 treatment or palivizumab treatment resulted in a trend toward reduced viral replication for both RSV BT2a and Memphis 37, but the results did not reach statistical significance. However, the differences were much less marked than those seen with the 50% tissue culture infective dose (TCID₅₀) data. When the TCID₅₀ and RT-qPCR data were considered together, they suggested that both ALX-0171 treatment and palivizumab treatment resulted in efficient neutralization of RSV released from the WD-PBEC cultures (TCID₅₀ results) but had a limited effect on intracellular virus replication. Interestingly, a similar effect was seen following administration of motavizumab to infants hospitalized with RSV; a significant reduction in infectious viral titers was reported coincident with a much lower reduction in virus copy numbers (28). However, the RT-qPCR assay does not distinguish between released virus that was neutralized and virus that remained infectious, thereby masking the effect of treatment. Similarly, it was shown that the natural rate of viral load decline was less steep using an RT-qPCR method than using quantitative infectivity culture and that this can confound determinations of the antiviral efficacy of test compounds targeting RSV replication (29).

The respiratory systems of human infants and young lambs have similarities, suggesting that lambs represent interesting models for studies of asthma, drug delivery, lung development, and vaccine efficacy (30). A neonatal lamb-RSV Memphis 37 model was also used to assess the efficacy of ALX-0171. There are several similarities in the results from the neonatal lamb model and the WD-PBEC model. Both models showed peak viral titers between 72 to 96 hpi. ALX-0171-treated lambs also demonstrated reduced clinical signs of disease and diminished lung pathology. Importantly, similar IC₅₀ values for inhibition of viral growth kinetics following ALX-0171 treatment were obtained from WD-PBECs and neonatal lambs.

The highest Memphis 37 titers reached in the lamb model and the WD-PBEC model for the buffer-treated cultures in the current study were 4.83 log₁₀ FFU/ml (27) and 7.05

$\log_{10}$ TCID₅₀/ml, respectively. As such, RSV evidently reached much higher peak viral titers in the WD-PBEC model than in the lamb model under these experimental conditions. However, RSV infectivity titers in nasal and/or tracheal aspirates from hospitalized infants were reported previously to range from $\sim 10^1$ to $\sim 10^7$ PFU/ml (14), suggesting that virus replication may reflect virus growth kinetics in infants in both models. In the lamb model, viral titers in the lungs were markedly reduced by day 8. However, persistent RSV infection was reported previously in a WD-PBEC model over a 3-month period, with limited damage to the culture (31). Despite these differences, the WD-PBEC culture model and the neonatal lamb model provided similar IC₅₀ values. As the reductions in viral titers were similar in the neonatal lamb model and the WD-PBEC model and as the lambs treated with ALX-0171 had lower clinical severity scores, it is possible that the titer reductions observed in WD-PBECs following ALX-0171 treatment might be predictive of lower clinical severity scores in infants. However, this evidently remains to be confirmed. Nonetheless, our data suggest that our RSV/WD-PBEC model may be of interest in helping to bridge the gap between poorly predictive preclinical animal models and clinical trials to further support the rationale for developing promising RSV therapeutics.

Although palivizumab is licensed for use as a prophylactic, when tested therapeutically it resulted in modest but significant reductions in viral growth kinetics from tracheal aspirates. However, these viral reductions were insufficient to reduce clinical severity in patients hospitalized with RSV (13, 27). Motavizumab, an affinity-matured derivative of palivizumab which is not approved for prophylactic use, was assessed as a parenterally administered therapeutic following RSV infection. However, there are conflicting data on motavizumab efficacy, with one study indicating a reduction in viral load (28) and another showing no effect on viral load, clinical severity, or length of hospitalization (32). In preclinical studies, motavizumab and ALX-0171 were 16.8-fold (33) and 126-fold (26) more potent than palivizumab, respectively. Studies of G-specific antibodies administered postinfection showed a reduction in inflammation in a mouse model of RSV infection (34, 35). This indicates that there is potential for a monoclonal antibody to be used as an effective treatment for RSV infection, provided that the IC₅₀ is sufficient. The route of administration is an important consideration. In a study of an adenovirus-based RSV vaccine, intranasal administration but not intramuscular administration elicited strong IgA responses (36). Both palivizumab and motavizumab were administered intramuscularly, whereas ALX-0171 is inhaled. It is likely, therefore, that both the increased IC₅₀ values and the routes of administration might explain why ALX-0171 appears to have greater therapeutic efficacy *in vivo* than previously developed anti-RSV antibodies.

The determinants of RSV disease severity remain unclear and may involve multiple factors, including viral load, viral strain, and host susceptibility. It is also thought that the immune response to RSV infection plays a major role in the severity of disease. High viral titers are associated with higher levels of proinflammatory cytokines (14, 37). It has been theorized that a higher viral load may indirectly lead to more-severe disease due to an excessive immune response involving production of proinflammatory cytokines, leukocyte recruitment, and subsequent epithelial cell damage (38, 39). This would correlate with the data from the neonatal lamb model, which showed that reduced clinical severity scores were reflective of reduced viral titers. However, there are other studies that showed no correlation between viral load and disease severity (40). It is likely that a combination of host and viral factors contributes to the overall severity of disease.

Interestingly, the neonatal lamb model also resulted in a smaller reduction in virus copy numbers in the airways than in viral titers following ALX-0171 treatment, despite a significant reduction in clinical severity scores (27). This suggests that the viable virus titer is more indicative of disease severity than the viral load detected by RT-qPCR. Although viral load has been correlated with disease severity both in the human challenge model and in infants hospitalized with RSV (37, 41), it may be more appro-

priate to measure levels of replication-competent virus as an indicator of disease severity.

The development of RSV pharmaceuticals presents several challenges. These challenges relate primarily to the fact that much of the pathology associated with RSV infection is thought to be caused by the inflammatory immune responses to the virus infection rather than by direct viral cytopathogenesis. It is imperative, therefore, that RSV antivirals should result in both virus neutralization and modulation of the proinflammatory immune responses induced by infection. As has been demonstrated for influenza virus antivirals, such as oseltamivir, early treatment with potent RSV antivirals delivered at sufficiently high doses to the site of infection is likely to be required for effective disease therapy. Using the WD-PBEC model to perform preclinical and dose adjustment studies on antiviral treatments offers several benefits. The costs associated with large-animal *in vivo* studies are very high, and although WD-PBEC assays are more expensive than monolayer cell line assays, the cells can be cultured in a routine class II safety laboratory. Several parameters can be tested in parallel with WD-PBECs, and experiments on cultures from multiple donors can be carried out in duplicate. Furthermore, ethical considerations concerning the use of the neonatal lamb model must include a comprehensive rationale justifying the numbers of animals to be used.

The translation of preclinical model data to the clinic has so far proved elusive for RSV therapeutic drugs. The similarities between our RSV/WD-PBEC data and the neonatal lamb RSV infection model data suggest that use of our morphologically and physiologically authentic RSV/WD-PBEC therapeutic model may provide a basis for predicting drug efficacy in clinical trials at a level that is currently not possible. As such, ALX-0171 may have therapeutic potential against RSV in young infants and our data support its further clinical development.

MATERIALS AND METHODS

Well-differentiated primary pediatric bronchial cell culture. The generation of WD-PBEC cultures was described previously (20). Briefly, primary pediatric epithelial cells obtained from Lonza were expanded in collagen-coated flasks until almost confluent and transferred onto collagen-coated semi-permeable Transwells (Corning) (6-mm diameter, 0.4- μ m pore size). When confluence occurred, the apical medium was removed and an air-liquid interface (ALI) was established to promote differentiation. Cells were maintained in ALI for a minimum of 21 days. Cultures were used only when hallmarks of excellent differentiation were evident, including the absence of holes in the cultures, extensive coverage of beating cilia, and obvious mucus production.

Cell lines and viruses. RSV BT2a was originally isolated from a 4-month-old infant hospitalized with bronchiolitis in Belfast, United Kingdom. It was cultured in HEP-2 cells as previously described (42) and passaged a total of three times before use. RSV Memphis 37 was originally isolated from a 4-month-old male in Memphis, TN, USA, who presented with bronchiolitis. The virus was isolated and passaged in FDA-approved Vero cell cultures as previously described (43). RSV Memphis 37 was further passaged seven times on HEP-2 cells. RSV titers in biological samples were determined by a 50% tissue culture infectious dose (TCID₅₀) assay, as previously described (44), or by RT-qPCR (see below).

Infection and treatment. WD-PBECs were infected apically in duplicate (2 wells per condition per patient) for 2 h at 37°C. Apical rinses were carried out by adding low-glucose Dulbecco's modified Eagle's medium (DMEM) and performing gentle pipetting up and down several times. The recovered DMEM was added to cryovials and snap-frozen and stored in liquid nitrogen. Following a 24-hpi apical wash, the cultures were treated apically with 100 μ l of either ALX-0171 or palivizumab at the indicated concentrations, or with the buffer control, for 1 h at 37°C. To maintain the air-liquid interface, the treatment was removed and replaced with 10 μ l of the same concentration of ALX-0171 or palivizumab followed by incubation for 24 h, until the next apical rinse. After each subsequent apical rinse, 10 μ l of the indicated concentration of ALX-0171 or palivizumab was added to the apical surface. This was repeated every 24 h for 6 days.

Virus quantification. RSV titers in apical washes were determined on HEP-2 cells as previously described (44). To determine the viral load by RT-qPCR, RNA was extracted from apical washes (High Pure viral RNA kit; Roche). cDNA was prepared using 10 μ l RNA (High Capacity cDNA reverse transcription kit; ABI). A LightCycler 480 probe master kit (Roche) was used to amplify RSV cDNA. Primers and probes specific for the RSV L-gene were designed with Mega6 software based on alignment of multiple RSV L-gene sequences derived from GenBank representing both clinical and prototypic strains of RSV subgroups A and B (Table 2). Standard curves were generated using a plasmid containing the RSV-A2 genome in 10-fold dilutions.

Statistical analysis. The change in TCID₅₀ values over time per donor was summarized using the area under the curve (AUC) for each compound and concentration. AUC viral load data were computed using the trapezoid rule in GraphPad Prism, where the lower limit of detection of TCID₅₀ was used as a baseline for calculation. Subsequently, the AUC values corresponding to averages of the dose-response

TABLE 2 RT-qPCR primer and probe sequences^a

hRSV L-gene primer or probe	Sequence
Forward primer 3	5'-GATTGCAATGATCATAGTTTACC
Reverse primer 4	5'-TAAANTTTGCNGAACCTATNAG
Degenerate hydrolysis probe 12	5'-GACCATTCCTGCTACAGATG

^ahRSV, human respiratory syncytial virus.

data were fitted using a four-parameter logistic curve (4PL) with the bottom constrained to a value of 0 to calculate the IC₅₀ values. Since the variances were not assumed to be equal, a Welch's *t* test was performed and the degrees of freedom were approximated using the Welch-Satterthwaite equation. Two-sided *t* tests were performed at a 5% significance level based on the IC₅₀ data from the 4PL model.

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