Comparative therapeutic potential of ALX-0171 and palivizumab against RSV clinical isolate 1 infection of well-differentiated primary pediatric bronchial epithelial cell cultures

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2 infection of well-differentiated primary pediatric bronchial epithelial cell cultures

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16 Running Head: Efficacy of ALX-0171 against two RSV strains

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19

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 characterise 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; Memphis 37), we compared the therapeutic potential of ALX-0171 with palivizumab, which is currently prescribed for RSV prophylaxis in high-risk infants. ALX-0171 treatment (900 nM) at 24 h post-infection 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 neutralisation. ALX-0171 was approximately 3 fold more potent in this therapeutic RSV/WD-PBEC model than palivizumab (mean IC_{50} = 346.9-363.6 nM and 1048-1090 nM for ALX-0171 and palivizumab, respectively), irrespective of the clinical isolate. When viral genomic copies (GC) were measured by RT-qPCR, the therapeutic effect was considerably less and GCs were only moderately reduced (0.62 – 1.28 Log_{10} copies/mL) by ALX-0171 treatment at 300 and 900 nM. Similar findings were evident for palivizumab. Therefore, ALX-0171 was very potent at neutralising RSV released from apical surfaces but only had a limited impact on virus replication. The data indicate a clear disparity between viable virus neutralisation and GC viral load, the latter of which does not discriminate between viable and neutralised RSV. This study validates the RSV/WD-PBEC model for the pre-clinical evaluation of RSV antivirals.

43

44 ## Introduction

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

51 Severe RSV infection is associated with an increase in mucus production and a decrease in the
52 number of ciliated cells in the airway epithelium. There is a large influx of immune cells to the
53 airways, predominantly neutrophils but also lymphocytes and macrophages (6). The cellular
54 infiltrate, together with mucus and sloughed epithelial cells, causes lumen obstruction and
55 inflammation of the airways. Associated with mucus plug formation and bronchiole occlusion,
56 bronchiolitis is therefore more severe in smaller airways, such as those of young or preterm
57 infants (7). Accordingly, 66% of RSV-related hospitalizations are in children <6 months old (8).
58 Risk factors associated with the development of severe RSV-LRTI in infants include:
59 prematurity, bronchopulmonary dysplasia, congenital lung or heart conditions, male gender, age
60 ≤6 months, neuromuscular disorders, and immunodeficiency (9). However, the majority of
61 patients that require hospitalization due to severe RSV-related disease have no underlying health
62 conditions that constitute a risk factor (3). There is mounting evidence to suggest that a severe
63 RSV infection in early life is associated with the development of wheeze and subsequently
64 asthma(10).

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

78 One potential limitation to RSV antivirals being effective is that the viral load may have peaked
79 by the time infants are hospitalized. However, a study of RSV clearance in hospitalized children
80 demonstrated that higher viral titers at day 3 of hospitalization were not associated with risk
81 factors such as weight, gestational age, sex, or age at time of admission but were associated with
82 the requirement for intensive care and respiratory failure, indicating a potential therapeutic
83 window even in hospitalized infants (14). Osteltamivir (Tamiflu), an antiviral against influenza
84 virus, demonstrates the importance of the time of administration following infection for effective
85 treatment; it is effective at reducing the length of illness in patients hospitalised with influenza
86 when administered within 48 h of symptom onset in clinically confirmed cases of influenza(15).
87 When administered after this time, however, osteltamivir failed to have any effect on virus titers,

88 disease severity or illness duration(16).

89 The majority of RSV pathogenesis, antiviral and prophylaxis studies have been performed in
90 animal models or continuous cell lines, neither of which are optimal. Animal models, especially
91 mice, are semi-permissive for RSV replication and do not exhibit high viral titers or pulmonary
92 pathology associated with RSV in infants unless very high innocula are employed (17–19).
93 Continuous cell lines, e.g. HEp-2 and A549 cells, are poorly representative of the complexities of
94 cell interactions in the human lung. The development of the well-differentiated primary pediatric
95 bronchial epithelial cell (WD-PBEC) culture model has provided an authentic surrogate
96 facilitating the elucidation of mechanisms of RSV pathogenesis in pediatric airways (20, 21), and
97 thereby, the study of RSV-specific antivirals. WD-PBECs reproduce hallmarks of RSV infection
98 in *vivo* and can be infected for prolonged experiments with extensive damage to the cultures(22).
99 Several studies have demonstrated RSV neutralisation in human airway epithelial cells (HAE)
100 that were not evident in experiments using continuous cell lines such as HeLa or HEp-2(23, 24).

101 There are several RSV therapeutics currently in development including entry inhibitors and
102 nucleoside analogues(25). One potential RSV therapeutic has been developed by Ablynx, , ALX-
103 0171, which is a trivalent non-half-life extended Nanobody® that specifically binds to RSV F
104 site II. ALX-0171 is composed of 3 heavy chain variable region (VHH) domains, providing
105 strong binding to RSV and it potently neutralises infectious virions (26). The neutralizing and
106 therapeutic ability of ALX-0171 against RSV was previously demonstrated in the neonatal lamb
107 RSV-infection model using the RSV strain Memphis 37 (27). In this current study, we exploited
108 our well-differentiated pediatric primary bronchial epithelial cell (WD-PBEC) culture model of
109 RSV infection to assess the relative ALX-0171 IC_{50} for the two RSV clinical strains, BT2a and
110 Memphis 37, in comparison to palivizumab. We also aimed to establish whether any adjustment

111 in the targeted lung lining fluid concentration, as determined in the RSV lamb model, is needed
112 based on strain sensitivity differences.

113

114 **Results**

115 There was a clear dose-effect of ALX-0171 treatment on RSV BT2a and Memphis 37 growth
116 kinetics. RSV BT2a titers diminished dramatically by 24 h of treatment with 900 nM ALX-0171
117 with a 4.74 log_{10} reduction in mean virus titers (Figure 1A). The virus was almost completely
118 neutralized by this time under these conditions and virus neutralization was maintained for the
119 duration of the experiment. Treatment with 300 nM also resulted in reductions in apically-
120 released RSV BT2a, with >2 log_{10} reduction in mean virus titers evident at 144 hpi relative to
121 buffer-treated RSV BT2a-infected control cultures. A less marked reduction in mean RSV BT2a
122 titers was observed following treatment with 100 nM relative to buffer-treated controls.

123 Substantial reductions were also evident in RSV Memphis 37 titers by 24 h of treatment with 900
124 nM ALX-0171, with a 4.2 log_{10} reduction in mean virus titers, although low virus titers were
125 continually detectable in these cultures until 144 hpi (Figure 1B). Lower, but nonetheless
126 substantial, reductions in RSV Memphis 37 titers were also evident following treatment with 300
127 nM ALX-0171, whereas treatment with 100 nM did not result in substantially reduced viral
128 titers. In contrast, treatment with ALX-0171 concentrations <100 nM did not influence growth
129 kinetics of either RSV strain relative to buffer-treated control cultures under these experimental
130 conditions.

131 Unlike ALX-0171, infectious RSV BT2a was detected following treatment with all palivizumab
132 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 $\log_{10}$ reduction in viral titers. Treatment with 300 or 100 nM palivizumab also resulted in substantial but lower reductions in viral titers compared to buffer-treated controls (Figure 2A). However, treatment with 33, 11 or 4 nM palivizumab had no effect on the growth kinetics of RSV BT2a.

Similar results were also observed in RSV Memphis 37 titers following treatment with palivizumab. Treatment with either 900 or 300 nM palivizumab resulted in substantial reductions in viral titers (Figure 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 900 nM, 300 nM or 100 nM 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 (Figure 3A). The two highest concentrations of ALX-0171 (900 nM and 300 nM) and only the highest concentration for palivizumab substantially reduced the viral titers of RSV Memphis 37 compared to buffer-treated controls. However, treatment with ALX-0171 resulted in greater reductions in viral growth kinetics compared to treatment with palivizumab at equimolar concentrations, except for treatment with 100 nM of ALX-0171 or palivizumab, which showed similar viral titers at each time point (Figure 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} (1090 nM) was significantly higher than the IC_{50} of ALX-0171 (363.6 nM) against Memphis 37. Similarly, the IC_{50} of palivizumab (1048 nM)

155 against BT2a was also significantly higher than that of ALX-0171 (346.9 nM). The IC_{50}
156 calculated from the area under the curves was not significantly different for either ALX-0171
157 ((p-value = 0.751) or palivizumab (p-value = 0.858) against the two RSV strains, indicating that
158 both viruses had comparable sensitivities to neutralisation in the WD-PBEC culture model.

159 A trend towards reduction in RSV BT2a viral loads, as determined by RT-qPCR, was evident
160 following treatment with both ALX-0171 and palivizumab. At 144 hpi, ALX-0171 900 and 300
161 nM doses reduced mean viral loads by 0.8 $\log_{10}$ genome copies (GC)/mL and 1.3 $\log_{10}$ GC/mL,
162 respectively, versus buffer–treated cultures (Figure 4A). At the same timepoint and for the same
163 doses, palivizumab reduced mean viral loads by 0.3 and 0.8 $\log_{10}$ GC/mL versus buffer-treated
164 cultures, respectively. Similarly, reduced viral loads were observed with both ALX-0171 and
165 palivizumab treatment of Memphis 37-infected WD-PBEC cultures. At 144 hpi, 900 and 300 nM
166 ALX-0171 reduced mean viral loads by 0.62 $\log_{10}$ and 0.76 $\log_{10}$ GC/mL, respectively, versus
167 the buffer–treated cultures (Figure 4B). At the same time point and for the same doses,
168 palivizumab reduced mean viral loads by 0.27 and 0.57 $\log_{10}$ GC/mL, respectively, versus buffer-
169 treated control cultures.

170

171 **Discussion**

172 The aims of this study were two-fold: to assess the efficacy of ALX-0171 as an anti-RSV
173 therapeutic; and to evaluate the WD-PBEC model for use in pre-clinical studies. Despite
174 extensive research into RSV pathogenesis and mechanisms of disease, vaccines and treatments
175 have remained elusive. Ablynx nv has developed ALX-0171 to address the need for a RSV
176 treatment option. In this study we used our RSV/WD-PBEC model to assess the therapeutic

177 potential of ALX-0171 or palivizumab to neutralise two different clinical strains of RSV.
178 Palivizumab is the only licensed neutralising anti-RSV monoclonal antibody. It is administered
179 prophylactically and reserved for infants deemed at high risk of severe RSV infection, in large
180 part because of its high cost. However, the majority of children hospitalised due to severe RSV
181 infection are not classified as high-risk and, as such, do not receive palivizumab prophylaxis. A
182 therapeutic intervention that can be administered after the onset of symptoms would reduce the
183 huge economic burden of RSV and potentially reduce the number and/or duration of
184 hospitalisations.

185 Growth kinetics for both RSV Memphis 37 and BT2a followed a similar pattern in the WD-
186 PBECs. Both strains reached similar peak viral titers between 72 and 96 hpi. RSV BT2a and
187 Memphis 37 demonstrated similar susceptibilities to neutralisation by ALX-0171 or
188 palivizumab. The highest concentration of ALX-0171 (900 nM) reduced viral titers to near or
189 below the limit of detection by 24 h post-treatment (~5 $\log_{10}$ reduction), whereas palivizumab
190 treatment at the same concentration was less effective (~3 $\log_{10}$ reduction). These differences
191 were reflected in the respective IC_{50} values for both molecules.

192 The RT-qPCR data demonstrated that ALX-0171 or palivizumab treatment resulted in a trend
193 toward reduced viral replication for both RSV BT2a and Memphis 37 but this did not reach
194 significance. However, the differences were much less marked than the $TCID_{50}$ data. When the
195 $TCID_{50}$ and RT-qPCR data were considered together they suggested that both ALX-0171 and
196 palivizumab treatment resulted in efficient neutralization of RSV released from the WD-PBEC
197 cultures ($TCID_{50}$ results) but had a limited effect on intracellular virus replication. Interestingly,
198 a similar effect was seen following motavizumab administration to infants hospitalised with
199 RSV; a significant reduction in infectious viral titers was reported coincident with a much lower

200 reduction in virus copy numbers(28). However, the RT-qPCR assay does not distinguish between
201 released virus that was neutralized and virus that remained infectious, thereby masking the effect
202 of treatment. Similarly, it was shown that the natural rate of viral load decline is less steep when
203 using a RT-qPCR method than when using quantitative infectivity culture and that this can
204 confound antiviral efficacy determination of test compounds targeting RSV replication(29).

205 The respiratory system of human infants and young lambs have similarities, suggesting that
206 lambs provide interesting models for asthma, drug delivery, lung development and vaccine
207 efficacy studies(30). A neonatal lamb-RSV Memphis 37 model was also used to assess the
208 efficacy of ALX-0171. There are several similarities in the results from the neonatal lamb model
209 and the WD-PBEC model. Both models showed peak viral titers between 72 to 96 hpi. ALX-
210 0171-treated lambs also demonstrated reduced clinical signs of disease and diminished lung
211 pathology. Importantly, similar IC_{50} values for inhibition of viral growth kinetics following
212 ALX-0171 treatment were obtained from WD-PBECs and neonatal lambs.

213 The highest Memphis 37 titers reached in the lamb model and the WD-PBEC model in the
214 current study were 4.83 log_{10} FFU/mL(27) and 7.05 log_{10} $TCID_{50}$/mL for the buffer-treated
215 cultures, respectively. As such, RSV evidently reached much higher peak viral titers in the WD-
216 PBEC model compared with the lamb model under these experimental conditions. However,
217 RSV infectivity titers in nasal and/or tracheal aspirates from hospitalised infants were reported to
218 range from ~10^1 to ~10^7 pfu/mL, suggesting that virus replication in both models may reflect
219 virus growth kinetics in infants(14). In the lamb model viral titers in the lungs were markedly
220 reduced by day 8. However, persistent RSV infection was reported in a WD-PBEC model over a
221 3 month period, with limited damage to the culture(31). Despite these differences, both the WD-
222 PBEC culture model and neonatal lamb model provided similar IC_{50} values. As the reduction in

viral titers was similar in the neonatal lamb model and the WD-PBEC model, and 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 pre-clinical 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 hospitalised with RSV(13, 27). Motavizumab, an affinity-matured derivative of palivizumab, which was not approved for prophylactic use, was assessed as a parenterally administered therapeutic following RSV infection. However, there is 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 hospitalisation(32). In pre-clinical studies motavizumab and ALX-0171 was 16.8 fold (33) and 126 fold (26)more potent, respectively, than palivizumab. Studies of G-specific antibodies administered post infection have shown 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, provided the IC_{50} is sufficient. Route of administration is an important consideration. In a study of an adenovirus-based RSV vaccine, intranasal, 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 IC_{50} values and the routes of

246 administration may explain why ALX-0171 appears to have greater therapeutic efficacy *in vivo*
247 than previously developed anti-RSV antibodies.

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

259 Interestingly, the neonatal lamb model also resulted in a smaller reduction in virus copy numbers
260 in the airways compared to viral titers following ALX-0171 treatment, despite a significant
261 reduction in clinical severity scores(27). This suggests that the viable virus titer is more
262 indicative of disease severity than the viral load detected by RT-qPCR. Although viral load has
263 been correlated with disease severity in both the human challenge model and infants hospitalised
264 with RSV(41, 42), it may be more appropriate to measure replication competent virus as an
265 indicator of disease severity.

266 The development of RSV pharmaceuticals presents several challenges. These challenges relate
267 primarily to the fact that much of the pathology associated with RSV infection is thought to be

268 caused by the inflammatory immune responses to the virus infection, rather than direct viral
269 cytopathogenesis. It is imperative, therefore, that RSV antivirals result in both virus
270 neutralisation and modulation of the pro-inflammatory immune responses induced by infection.
271 As has been demonstrated for influenza virus antivirals, such as oseltamivir, early treatment with
272 potent RSV antivirals delivered at sufficiently high doses to the site of infection is likely to be
273 required for effective disease therapy. Using the WD-PBEC model to preform pre-clinical and
274 dose adjustment studies on antiviral treatments offers several benefits. The costs associated with
275 large animal *in vivo* studies is very high and, although WD-PBECs are more expensive than
276 monolayer cell line assays, they can be cultured in a routine class II safety laboratory. Several
277 parameters can be tested in parallel with WD-PBECs and experiments can be carried out in
278 duplicate on cultures from multiple donors. Furthermore, ethical considerations for the use of the
279 neonatal lamb model must include a comprehensive rationale for numbers of animals to be used.

280 The translation of pre-clinical model data to the clinic has so far proved elusive for RSV
281 therapeutic drugs. The similarity between our RSV/WD-PBEC and the neonatal lamb RSV
282 infection model data suggests that use of our morphologically- and physiologically-authentic
283 RSV/WD-PBEC therapeutic model may provide a basis for predicting drug efficacy in clinical
284 trials that is currently not possible. As such, ALX-0171 may have therapeutic potential against
285 RSV in young infants and our data supports further clinical development.

286 Materials and Methods

287 Well-differentiated primary pediatric bronchial cell culture

288 The generation of WD-PBEC cultures was described previously(20). Briefly, primary pediatric
289 epithelial cells obtained from Lonza were expanded in collagen-coated flasks until almost

290 confluent and transferred onto collagen-coated semi-permeable (6 mm diameter, 0.4 μm pore
291 size) Transwells (Corning). When confluent, the apical medium was removed and an air-liquid
292 interface (ALI) was established to promote differentiation. Cells were maintained in ALI for a
293 minimum of 21 days. Cultures were only used when hallmarks of excellent differentiation were
294 evident, including, no holes in the cultures, extensive coverage of beating cilia and obvious
295 mucus production.

296 **Cell lines and viruses**

297 RSV BT2a was originally isolated from a 4-month old infant hospitalized with bronchiolitis in
298 Belfast, UK. It was cultured in HEp-2 cells as previously described(43) and passaged a total of
299 three times before use. RSV Memphis 37 was originally isolated from a four-month old male in
300 Memphis, USA who presented with bronchiolitis. The virus was isolated and passaged in FDA-
301 approved Vero cell cultures as previously described(44). RSV Memphis 37 was further passaged
302 seven times on HEp-2 cells. RSV titers in biological samples were determined by a tissue culture
303 infectious dose 50 ($TCID_{50}$) assay, as previously described (45), or by RT-qPCR (see below).

304 **Infection and treatment**

305 WD-PBECs were infected apically in duplicate (2 wells per condition per patient) for 2 h at
306 37°C. Apical rinses were carried out by adding low glucose DMEM and gently pipetted up and
307 down several times. The recovered DMEM was added to cryovials and snap frozen and stored in
308 liquid nitrogen. Following the 24 hpi apical wash the cultures were treated apically with 100 μL
309 of either ALX-0171 or palivizumab at the indicated concentrations, or buffer control, for 1 h at
310 37°C. To maintain the air-liquid interface, the treatment was removed and replaced with 10 μL of
311 the same concentration of ALX-0171 or palivizumab and incubated for 24 h, until the next apical

312 rinse. After each subsequent apical rinse 10 µL of the indicated concentration of ALX-0171 or
313 palivizumab were added to the apical surface. This was repeated every 24 h for 6 days.

314

315 **Virus Quantification**

316 RSV titers in apical washes were determined on HEp-2 cells as previously described(45). To
317 determine the viral load by RT-qPCR RNA was extracted from apical washes (High Pure Viral
318 RNA kit, Roche). cDNA was prepared using 10 µL RNA (High Capacity cDNA Reverse
319 Transcription kit, ABI). The Light Cycler 480 probe master kit (Roche) was used to amplify
320 RSV cDNA. Primers and probes specific for RSV L-gene were designed with Mega6 software
321 based on alignment of multiple RSV L-gene sequences derived from GenBank representing both
322 clinical and prototypic strains of RSV A and B subgroups (Table 2). Standard curves were
323 generated using a plasmid containing the RSV-A2 genome in 10-fold dilutions.

324

325 **Statistical Analysis**

326 . The change in $TCID_{50}$ values over time was summarized per donor using the area under the
327 curve (AUC) for each compound and concentration. Area under the viral load curves (AUC)
328 were computed using the trapezoid rule in Graphpad Prism where the lower limit of detection of
329 $TCID_{50}$ was used as a baseline for calculation. Subsequently, the dose response of average AUC
330 values was fitted using a 4PL with the bottom constrained to 0 to calculate the IC50 values.
331 Since the variances were not assumed to be equal, a Welch's t-test was performed and the
332 degrees of freedom were approximated using the Welch-Satterthwaite equation. Two-sided t-
333 tests were performed at a 5% significance level based on the IC50 from the 4PL model.

334

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512 Figure 1. Duplicate WD-PBEC cultures (n=3 donors) were infected apically with either RSV
513 BT2a (A) or RSV Memphis 37 (B) (MOI=0.1) for 2 h at 37°C and then washed 5 times. The fifth
514 wash was retained as the 2 hpi time point for virus titrations. At 24 hpi (and every 24 h
515 thereafter) apical washes were undertaken and harvested for virus titrations. Following the apical
516 washes at 24 hpi (and every 24 h thereafter) the cultures were apically treated with 100 µL ALX-
517 0171 at the indicated concentrations. After 1 h the treatment was removed and replaced with 10
518 µL ALX-0171 at the same concentration, which remained on the apical surfaces for the duration
519 of the interval between washes. All apical washes were titrated on HEp-2 cells to determine viral
520 titers, which were reported as $\log_{10}$ $TCID_{50}$/mL ± SEM (LOD = limit of detection).

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523 Figure 2. Duplicate WD-PBEC cultures (n=3 donors) were infected apically with RSV BT2a (A)
524 or RSV Memphis 37 (b) (MOI=0.1) for 2 h at 37°C and then washed 5 times. The fifth wash was
525 retained as the 2 hpi time point for virus titrations. At 24 hpi (and every 24 h thereafter) apical

526 washes were undertaken and harvested for virus titrations. Following the apical washes at 24 hpi
527 (and every 24 h thereafter) the cultures were apically treated with 100 µL palivizumab at the
528 indicated concentrations. After 1 h the treatment was removed and replaced with 10 µL
529 palivizumab at the same concentration, which remained on the apical surfaces for the duration of
530 the intervals between washes. All apical washes were titrated on HEp-2 cells to determine viral
531 titers, which were reported as $\log_{10}$ $TCID_{50}$/mL ± SEM. (LOD = limit of detection)

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535 Figure 3. WD-PBEC cultures (n=3 donors and duplicated cultures for each conditions) were
536 infected apically with RSV BT2a (A) or RSV Memphis 37 (B) (MOI=0.1) for 2 h at 37°C and
537 then washed 5 times. The fifth wash was retained as the 2 hpi time point for virus titrations. At
538 24 hpi (and every 24 h thereafter) apical washes were undertaken and harvested for virus
539 titrations. Following the apical washes at 24 hpi (and every 24 h thereafter) the cultures were
540 apically treated with 100 µL of either ALX-0171 or palivizumab at the indicated concentrations.
541 After 1 h the treatment was removed and replaced with 10 µL of either ALX-0171 or
542 palivizumab at the same concentration, which remained on the apical surfaces for the duration of
543 the intervals between washes. All apical washes were titrated on HEp-2 cells to determine viral
544 titers, which were reported as $\log_{10}$ $TCID_{50}$/mL ± SEM. (LOD = limit of detection).

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547 Figure 4. WD-PBEC cultures (n=3 donors and duplicated cultures for each conditions) were
548 infected apically with RSV BT2a (A) or RSV Memphis 37 (B) (MOI=0.1) for 2 h at 37°C and
549 then washed 5 times. The fifth wash was retained as the 2 hpi time point. At 24 hpi (and every 24

550 h thereafter) apical washes were harvested. Following the apical washes at 24 hpi (and every 24
551 h thereafter) the cultures were apically treated with 100 µL of ALX-0171 or palivizumab at the
552 indicated concentrations. After 1 h the treatment was removed and replaced with 10 µL of either
553 ALX-0171 or palivizumab at the same concentration, which remained on the apical surfaces for
554 the duration of the intervals between washes. RNA was extracted from apical washes and RT-
555 qPCR performed. Data were plotted as $\log_{10}$ genone copies/mL ± SEM.

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563 **Table 1: IC_{50} for ALX-0171 and palivizumab towards RSV BT2a or RSV Memphis 37**

RSV Memphis 37	**IC_{50}* [95% Confidence Intervals] in nM**
ALX-0171	363.6 [276.3-472.9]
Palivizumab	1090 [727.2-2126]
P-value (palivizumab v ALX)	0.0038
RSV BT2a	**IC_{50}* [95% Confidence Intervals] in nM**

ALX-0171	346.9 [262.2-468.3]
Palivizumab	1048 [869-1337]
P-value (palivizumab v ALX)	0.0001

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567 **Table 2: RT-qPCR primer and probe sequences**

hRSV L-gene	Forward primer 3	5'- GATTGCAATGATCATAGTTTACC
	Reverse Primer 4 degenerate	5'- TAAANTTTGCNGAACCTATNAG
	Hydrolysis Probe 12	5'- GACCATTCCTGCTACAGATG

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