Generation and Characterization of ALX-0171, a Potent Novel Therapeutic Nanobody for the Treatment of Respiratory Syncytial Virus Infection


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Human respiratory syncytial virus (RSV) is the most important viral pathogen causing acute lower respiratory tract infections in infants worldwide and is estimated to result in ~3.4 million yearly hospitalizations and ~200,000 deaths globally (1). RSV typically causes its primary infection at the point of entry: apical ciliated epithelial cells that line the nasal cavity and airways (2, 3). Primary infections are usually symptomatic, with clinical signs ranging from mild upper respiratory tract illness to severe lower respiratory tract infections, including pneumonia and bronchiolitis (4). In addition to the acute consequences of infection, the development of long-term recurrent wheezing and asthma has been associated with severe RSV infections in infancy (5, 6). Despite the major clinical importance of RSV, no vaccines or widely accepted antiviral therapies are currently available. The only available drug specific for human RSV is palivizumab (Synagis), a marketed monoclonal antibody that is administered prophylactically before and during the RSV season to infants at high risk of having severe human RSV disease (7–9). Its use is restricted to premature infants (gestational age, <29 weeks), if they have no other underlying morbidities, and infants with chronic lung disease, congenital heart disease, or a compromised immune system during the first year of life (10).

RSV is a member of the Pneumovirus genus of the Paramyxoviridae family and has a linear single-stranded, nonsegmented RNA molecule of negative polarity as its genome. This genome contains 10 genes which encode 11 proteins. The transmembrane glycoproteins F and G are the primary surface antigens of RSV. The attachment (G) protein mediates binding to cell receptors, while the F protein promotes fusion of the viral and cell membranes, allowing virus entry into the host cell cytoplasm (11). The F protein also promotes the fusion of infected cells with adjacent uninfected cells, facilitating the formation of multinucleated cell formations (syncytia), which allow cell-to-cell transmission of the replicated viral RNA and confer additional protection for the virus against host immune responses (12).

On the basis of the antigenic and genetic variability of the G protein, two subgroups of RSV (subgroups A [RSV-A] and B [RSV-B]) have been identified, and these are composed of evolving genotypes (13–17). In contrast to the G protein, the F protein is mostly conserved between RSV subgroups A and B (89% amino acid identity) and is therefore considered the most promising target for the development of viral entry inhibitors.

Nanobodies are therapeutic proteins derived from the heavy-chain variable domains (VHH) that occur naturally in heavy chain-only immunoglobulins of the Camelidae (18, 19). The formatting flexibility of Nanobodies into multivalent constructs, their small size, their stability (which allows delivery through nebulization), and their ease of production make their use against viral targets appealing (20–22).
Here we describe the in vitro and in vivo characterization of ALX-0171, a trivalent Nanobody composed of three monovalent Nb017 moieties linked together with glycine-serine (GS) linkers which is designed to target the RSV F protein for delivery via inhalation. ALX-0171 is currently in clinical development for the treatment of RSV infections in infants (23).

MATERIALS AND METHODS

Generation of RSV-specific Nanobodies. Monovalent RSV F protein-specific Nanobodies were identified from immune libraries of llamas that received repetitive injections with soluble recombinant F protein (rFTR, N protein), inactivated RSV-A (catalog number 8RSV79; HyTest), or a combination of both antigens. The soluble recombinant form of the F protein (derived from the Long strain) was produced with recombinant Sendai virus in embryonated eggs (24). RSV-neutralizing Nanobodies were identified by screening 162 distinct clones in an in vitro microneutralization assay with RSV subtype A Long on HEp-2 cells. RSV-neutralizing Nanobodies were formatted into multivalental constructs by genetic fusion with flexible Gly-Ser (GS) linkers of different lengths. Monovalent RSV Nanobody was routinely generated as C-terminal c-myc-His-tagged proteins in Esherichia coli (22). For the production of untagged monovalent and trivalent Nanobody constructs, a Pichia pastoris X-33 expression system was used (see Fig. S1 in the supplemental material). ALX-0171 is a trimeric Nanobody consisting of three RSV-targeting Nb017 subunits linked by two GS linkers.

Competition enzyme-linked immunosorbent assay (ELISA). Ninety-six-well microtiter plates (Maxisorp immunoplates; Nunc) were coated overnight with inactivated RSV-A (HyTest) at 3.5 μg/ml in bicarbonate buffer. The plates were blocked for 1 h with SuperBlock 20 (phosphate-buffered saline [PBS] blocking buffer (Pierce)) before being washed 3 times with PBS–0.05% Tween 20. Dilutions of ALX-0171 or palivizumab were mixed with 10 nM biotinylated ALX-0171 (1:1 ratio) and immediately transferred to the plates. After overnight incubation at room temperature (RT) and appropriate washing, a 1/3,000 dilution of peroxidase-labeled streptavidin (Thermo Scientific) was added to the plates for 1 h at RT. Bound streptavidin-horseradish peroxidase was revealed by adding 100 μl/well of enhanced soluble tetramethylbenzidine (EsTMB; SDT Reagents) for 10 min, followed by 1 N HCl. The absorbance was read at 450 nm, and the reference wavelength was 620 nm.

SRR. Affinity constants (the equilibrium dissociation constants [Kd,obs]) of Nb017, ALX-0171, and palivizumab were determined by surface plasmon resonance (SPR) analysis on a Biacore X100 instrument (GE Healthcare). Fab fragments of antigenic site O-specific human monoclonal antibody (MAb) D25 (Fab D25) (25, 26) and antigenic site I-specific mouse MAb 2F (Fab 2F) (27) were used as controls. In brief, anti-His monoclonal antibody (GE Healthcare) was amine coupled to a CM5 sensor chip at a density of 12,000 to 13,000 resonance units (RU) in 10 mM acetate buffer, pH 4.5. Approximately 70 RU of His-tagged fusion F protein was then bound to the anti-His monoclonal antibody. Compounds (ALX-0171, Nb017, Fab D25, Fab 2F, and palivizumab) were injected at 5 different concentrations (1 to 16 nM for ALX-0171, 8 to 128 nM for Nb017, 4 to 64 nM for palivizumab, 1.25 to 20 nM for Fab 2F and Fab D25) at a flow rate of 30 μl/min. The association and dissociation phases were 2 and 13 min, respectively (for Fab D25, the dissociation time was increased to 30 min). The chip was regenerated using 10 mM glycine, pH 1.5. Different concentrations of the compounds were used to generate binding curves, which were fitted to a 1:1 Langmuir binding model for the calculation of the kinetic parameters association rate (kass) and dissociation rate (koff). The Kd was then calculated as the ratio of these two rate constants (kass/koff).

Binding of Nanobodies to RSV escape mutant F protein. HEP-2 cells grown in 100-mm-diameter plates were infected with either wild-type RSV (the RSV Long strain) or previously described mutant viruses resistant to neutralization by monoclonal antibodies (11, 28, 29) (multiplicity of infection, 1 to 3 PFU/cell). After 48 h, the cells were harvested and extracts were made in 500 μl buffer/plate (the buffer was 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, and 1% octyl-glucoside). Equal amounts of each extract (50 μl of a 1:500 dilution in PBS) were used to coat 96-well microtiter plates overnight at 4°C. The wells were saturated with 1% bovine serum albumin in PBS for 1 h, and after washing with PBS, Nanobodies were added to the wells and the plates were incubated for 1 h at RT. After washing, bound Nanobody was detected with a 1:1,000 dilution of rabbit anti-llama IgG antibody, followed by a 1:200 dilution of peroxidase-conjugated donkey anti-rabbit polyclonal antibody. o-Phenylenediamine dihydrochloride (OPD; Sigma) was used as the substrate, and reactions were stopped with 3 N H2SO4 after the absorbance at 490 nm was measured.

RSV clinical strains. Original clinical isolates of RSV-A and RSV-B strains were selected from the RSV bank at the Baylor College of Medicine (Houston, TX). Three additional RSV-A isolates (BT2a, BT3a, BT4a) were isolated from infants hospitalized with bronchiolitis in the Royal Belfast Hospital for Sick Children. This study was approved by The Office for Research Ethics Committees Northern Ireland (ORECNI). Written informed parental consent was obtained. They were all isolated and cultured in HEP-2 cells, as previously described (30, 31).

Antiviral assays. The neutralization activity of the selected Nanobodies and palivizumab was measured in a microneutralization assay, a neutralization assay, and a plaque reduction assay. They were performed using HEP-2 cell monolayers, as described in the supplemental material.

Cotton rat studies. Male and female cotton rats were bred and housed in the Baylor College of Medicine’s vivarium in cages covered with barrier filters and given food and water ad libitum. Cotton rats weighing between 60 to 125 g were lightly anesthetized with isoflurane and intranasally challenged with 100 μl of 1.41 × 106 to 3.5 × 106 PFU of RSV Tracy, depending on the experiment. RSV Tracy stocks were grown in HEP-2 cells. All the experimental protocols were approved by the Baylor College of Medicine Investigational Animal Care and Use Committee.

ALX-0171 was administered either intranasally or by nebulization. For intranasal administration, cotton rats were lightly anesthetized with isoflurane and inoculated intranasally with 100 μl of ALX-0171.

For nebulization, an Akita® Apixneb nebulizer (Vectura GmbH, Germany) was used for whole-body exposure (see Fig. S2 in the supplemental material).

Cotton rats were euthanized with CO2 at 4 days postinfection. The large left lobe and one of the large right lobes of the lungs were removed, rinsed in sterile water, and weighed. The left lobe was then transepturally lavaged using 3 ml of Iscove’s medium with 15% glycerol mixed with 2% fetal bovine serum (FBS)–minimal essential medium (MEM) (1:1, vol/vol) in a 3-ml syringe with a 26-gauge by 3/8-in. needle and injected at multiple sites to totally inflate the lobe. Subsequently, the lavage fluid was recovered by gently pressing the inflated lobe flat and further used to transepturally lavage the right lobe following the same technique. The lavage fluid was collected and stored on ice until titrated. To obtain nasal washes, the jaws were disarticulated, the head was removed, and 1 ml of Iscove’s medium with 15% glycerol mixed with 2% FBS–MEM (1:1, vol/vol) was pushed through each nare (total, 2 ml). The effluent was collected from the posterior opening of the pallet and stored on ice until titrated. RSV Tracy lung lavage titers and nasal wash titers were determined by plaque assay as described in the supplemental material.

Statistical analysis. A one-way analysis of variance (ANOVA) followed by a post hoc Dunnett’s test for pairwise comparison was performed to compare the treated groups and the buffer-treated groups. A P value of <0.05 was considered statistically significant. Statistical analysis was performed with GraphPad Prism (version 5) software.

RESULTS

Generation and production of ALX-0171. The development of a Pichia pastoris strain for the production of ALX-0171 delivered a stable recombinant strain suitable for the manufacture of the multivalent trimeric ALX-0171 Nanobody. Further streamlining of the typical Pichia pastoris fermentation process resulted in an

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TABLE 1 Affinities of ALX-0171, Nb017, and palivizumab toward RSV prefusion F protein measured by SPR

<table>
<thead>
<tr>
<th>Compound</th>
<th>$k_{\text{on}}$ (M$^{-1}$ s$^{-1}$ [10$^9$])</th>
<th>$k_{\text{off}}$ (s$^{-1}$ [10$^{-4}$])</th>
<th>$K_D$ (nM)</th>
<th>$R_{\text{max}}$ (RU)</th>
<th>$\chi^2$ (RU$^2$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nb017</td>
<td>1.305</td>
<td>233.5</td>
<td>17,880</td>
<td>14.73</td>
<td>0.713</td>
</tr>
<tr>
<td>ALX-0171</td>
<td>4.091</td>
<td>4.604</td>
<td>0.113</td>
<td>24.30</td>
<td>0.46</td>
</tr>
<tr>
<td>Palivizumab</td>
<td>0.084</td>
<td>0.739</td>
<td>0.879</td>
<td>56.2</td>
<td>0.0947</td>
</tr>
<tr>
<td>Fab D25</td>
<td>0.488</td>
<td>0.331</td>
<td>0.068</td>
<td>51.78</td>
<td>0.158</td>
</tr>
<tr>
<td>Fab 2F</td>
<td></td>
<td></td>
<td>&gt;20</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

ALX-0171 upstream process in which more than 7.5 g/liter ALX-0171 was secreted in the fermentation broth. After clarification of the broth, the downstream process consisted of capture, intermediate purification, and polish chromatography steps and was followed by a final formulation step. A range of ALX-0171 concentrations was tested in combination with a series of buffers and excipients to provide the highest stability after storage, nebulization, freeze-thaw, etc. These formulation and (stressed) stability studies resulted in the formulation of ALX-0171 as a stable nebulizer solution.

Characteristics of ALX-0171 binding to RSV F protein. The kinetics of Nb017 and ALX-0171 binding to the RSV F protein in its prefusion conformation was assessed by SPR analysis. The conformational integrity of the F protein was confirmed with the antigenic site Ø-specific Fab D25, which binds only to the prefusion conformation, whereas the antigenic site 1-specific Fab 2F did not bind to the prefusion conformation, as expected. The binding affinity of palivizumab was 0.88 nM on the prefusion conformation, which is in a range similar to that of previously reported data (22, 32) (Table 1). Although Nanobody Nb017 was originally selected for binding to the RSV F protein in its postfusion conformation, as this was the only tool available at the time, both ALX-0171 and Nb017 were shown to bind the prefusion conformation of the F protein. The formatting of Nb017 into trimeric ALX-0171 increased the binding affinity by $\sim$160-fold ($K_D$, 0.113 nM) compared to that of the monovalent Nb017 ($K_D$, 17.88 nM) (Table 1).

Formatting to ALX-0171 greatly increased in vitro potency against RSV-A and RSV-B. Microneutralization assays were used to investigate whether trimeric formatting of Nb017 (ALX-0171) would improve the neutralization capacity. The monovalent and trivalent formats inhibited the replication of the RSV-A (Long) and RSV-B (18537) strains in a dose-dependent manner (Fig. 1.). Formatting of the monovalent building block into a trivalent compound greatly increased the potency by roughly 6,000-fold against RSV-A Long and $>10,000$-fold against RSV-B 18537. ALX-0171 was 126- and 6-fold more potent than palivizumab against RSV-A Long and RSV-B 18537, respectively (Table 2). This increased potency of ALX-0171 over the potencies of the monovalent Nb017 and palivizumab was considerably more than anticipated on the basis of affinity differences. Similarly, the conversion of palivizumab Fab to full-length IgG resulted in an improved potency of $\sim$200-fold with only a marginal $<3$-fold increase in $K_D$ (22, 33).

ALX-0171 neutralizes a wide panel of clinical RSV isolates. The neutralization capacity of ALX-0171 toward RSV clinical isolates was tested. For this evaluation, 3 RSV-A strains isolated at Queen’s University Belfast, Belfast, Northern Ireland, United Kingdom, and 3 RSV-B strains isolated at the Baylor College of Medicine, Houston, TX, were selected. The 50% virus neutralization titers (as determined by endpoint dilution assays) are shown in Table 2. ALX-0171 was $\approx$180-fold more potent than palivizumab against the RSV-A clinical isolates tested (range, 180- to 409-fold) and $\approx$12-fold more potent than palivizumab against the RSV-B clinical isolates tested (range, 11.5- to 647.5-fold), and this potency difference was in the same range as that determined for the prototypic RSV-A Long and RSV-B 18537 strains (177- and 17-fold, respectively) using the same assay.

To provide a more comprehensive understanding of the relative neutralization capacity of ALX-0171 and palivizumab against a large panel of RSV clinical isolates, 61 RSV isolates, including the RSV Tracy and RSV-B 18537 strains (see Table S1 in the supplemental material), were tested for inhibition by ALX-0171 and palivizumab at a single concentration of 40 µg/ml in a semiquantitative plaque reduction assay. This concentration represents the mean 30-day trough serum concentration of palivizumab after the first intramuscular injection (32). ALX-0171 and palivizumab reduced the viral titers by $2 \log_{10}$ for 97% and 85% of viruses tested, respectively (Table 3). For RSV-A isolates, this proportion was 94% and 85%, respectively.
100% with ALX-0171 and 84.4% with palivizumab (P = 0.053), and for RSV-B isolates, this proportion was 93.1% with ALX-0171 and 86.2% with palivizumab (P = 0.67). These results are consistent with published data demonstrating that palivizumab neutralized 75/77 (97%) clinical isolates tested (32). Importantly, ALX-0171 demonstrated a broad RSV strain neutralization capacity that was at least as extensive as that of palivizumab when looking at 2 log_{10} reductions.

Our data were the most striking when the complete suppression of RSV replication was compared between ALX-0171 and palivizumab. When they were tested at equivalent concentrations, inhibition of RSV replication was compared between ALX-0171 and palivizumab, which is likely the consequence of the higher potency of ALX-0171 compared with that of palivizumab (as shown in Table 2) and the lower molecular weight of palivizumab, although only partially (Fig. 3 and data not shown). These results provide further evidence that Nb017 and, consequently, ALX-0171 target antigenic site II of the F protein and that its epitope is close to or overlaps that of palivizumab.

**ALX-0171 retains physicochemical properties following nebulization.** As the intended route of ALX-0171 administration is by inhalation, it is important that ALX-0171 withstand the nebulization process. An Akita® Apixnub nebulizer was used to nebulize ALX-0171, and the collected aerosol was characterized for product stability by size-exclusion (SE) high-pressure liquid chromatography (HPLC) (to determine purity/impurity), reverse-phase (RP)-HPLC (to determine purity), and ELISA (to determine potency). These analytical methods were suitable for assessing higher-molecular-weight product-related variants (i.e., dimeric, trimERIC, and higher-multimer forms of ALX-0171), protein fragmentation, and potential changes in potency. During product development, no physicochemical changes were observed by RP-HPLC analysis after nebulization. SE-HPLC analysis showed a small increase in higher-molecular-weight species after nebulization. The formation of higher-molecular-weight species by nebulization was monitored during long-term stability studies and remained minimal, not exceeding 2% after nebulization.

### TABLE 2 Neutralization of RSV prototypic and clinical isolates

<table>
<thead>
<tr>
<th>RSV strain</th>
<th>IC_{50} (Mean ± SD IC_{50} (nM))</th>
<th>Fold difference</th>
<th>VN_{50} (Mean ± SD VN_{50} (nM))</th>
<th>Fold difference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ALX-0171</td>
<td>Palivizumab</td>
<td>Palivizumab</td>
<td>ALX-0171</td>
</tr>
<tr>
<td>RSV-A</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Long</td>
<td>0.1 ± 0.07 (n = 3)</td>
<td>12.6 ± 5.4 (n = 3)</td>
<td>126</td>
<td>0.03 ± 0.02 (n = 3)</td>
</tr>
<tr>
<td>BT2a(^b)</td>
<td>0.011 ± 0.006 (n = 3)</td>
<td>4.06 ± 1.39 (n = 3)</td>
<td>380</td>
<td></td>
</tr>
<tr>
<td>BT3a(^b)</td>
<td>0.01 ± 0.005 (n = 3)</td>
<td>3.95 ± 0.61 (n = 3)</td>
<td>409</td>
<td></td>
</tr>
<tr>
<td>BT4a(^b)</td>
<td>0.006 ± 0.006 (n = 3)</td>
<td>1.14 ± 0.91 (n = 3)</td>
<td>180</td>
<td></td>
</tr>
<tr>
<td>RSV-B</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18537</td>
<td>0.4 ± 0.2 (n = 20)</td>
<td>2.4 ± 1.2 (n = 5)</td>
<td>6</td>
<td>0.42 ± 0.66 (n = 6)</td>
</tr>
<tr>
<td>BTX-60567(^b)</td>
<td>0.24 ± 0.22 (n = 3)</td>
<td>2.8 ± 0.8 (n = 3)</td>
<td>11.5</td>
<td></td>
</tr>
<tr>
<td>BTX-61406(^b)</td>
<td>0.028 ± 0.026 (n = 3)</td>
<td>2.0 ± 1.1 (n = 3)</td>
<td>72.3</td>
<td></td>
</tr>
<tr>
<td>BTX-79233(^b)</td>
<td>0.003 ± 0.001 (n = 3)</td>
<td>1.7 ± 2.2 (n = 3)</td>
<td>647.5</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) IC_{50}, 50% inhibitory concentration; VN_{50}, 50% virus neutralization titer; n, number of isolates.

\(^b\) These viruses have been reported previously (30, 31).

### TABLE 3 Comparative inhibition of *in vitro* replication of a panel of RSV clinical isolates by palivizumab and ALX-0171 by ≥100-fold or completely

<table>
<thead>
<tr>
<th>RSV group</th>
<th>GMT(^a) (log_{10})</th>
<th>No. of isolates with ≥100-fold reduction/total no. of isolates tested (%)</th>
<th>P value(^c)</th>
<th>No. of isolates with complete inhibition/total no. of isolates tested (%)</th>
<th>P value(^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RSV-A</td>
<td>4.9 ± 0.4</td>
<td>27/32 (84.4)</td>
<td>0.053</td>
<td>0/32 (0)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>RSV-B</td>
<td>4.7 ± 0.4</td>
<td>25/29 (86.2)</td>
<td>0.67</td>
<td>11/29 (37.9)</td>
<td>0.003</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>52/61 (85.2)</td>
<td>0.054</td>
<td>11/61 (18)</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

\(^a\) GMT, geometric mean titer of buffer controls.

\(^b\) Differences between groups were analyzed using Fisher’s exact test for comparison of proportions. Significance was defined at a P value of <0.05.

\(^c\) Complete virus inhibition was defined as no detectable virus plaques. The limit of virus detection in the plaque reduction neutralization assay was 5 PFU/ml.
The difference in F-protein expression. Nb017 was used at 0.2 \( \mu \text{g/ml} \) on days 2 and 3 after RSV challenge resulted in significant viral load reductions in the lungs ranging from \(~50\)-fold (1.66 \( \log_{10} \)) to \(~2,000\)-fold (3.3 \( \log_{10} \)) for the highest dose tested (68 mg/kg) \((P < 0.0001)\). Nasal RSV titers were also significantly reduced for all doses and schedules tested \((P < 0.0001)\), except for the day 2 treatment at 4 mg/kg \((P = 0.7)\).

As the intended route of delivery of ALX-0171 is by nebulization, we performed a proof-of-concept experiment to confirm that nebulization did not affect the antiviral activity of ALX-0171. For this experiment, prophylactic delivery of ALX-0171 via nebulization was chosen as the most straightforward approach. In total, 4 groups of 6 cotton rats received estimated delivered doses of 0.3 mg/kg, 0.8 mg/kg, and 2 mg/kg 1 h before RSV Tracy challenge (Table 5). A dose-dependent reduction in viral titers was seen in the nose, which was significant for the two higher doses \((P = 0.01\) and 0.006 for doses of 0.8 mg/kg and 2 mg/kg, respectively). Furthermore, RSV replication was almost completely blocked in the lungs, even at the lowest dose tested \((>3.67 \log_{10} \text{reduction}, P < 0.0001)\).

**DISCUSSION**

ALX-0171 is a novel therapeutic biologic in development for the treatment of RSV infections in infants. ALX-0171 is a trimeric Nanobody that binds an epitope in antigenic site II of RSV F protein with subnanomolar affinity. This epitope partially overlaps the palivizumab epitope.

The formatting of the monovalent Nb017 into the trivalent Nanobody ALX-0171 increased the potency of neutralization (>6,000-fold) of both RSV-A and RSV-B strains. Although formatting clearly increased the binding affinity for the biologically relevant prefusion conformation of the F protein \((~160\)-fold\)), it does not fully explain the observed large increase in the virus neutralization capacity of ALX-0171. Our results are consistent with those of a previous report demonstrating that a bivalent Nanobody construct specific for F-protein antigenic site II had a 4,000-fold improved neutralization capacity compared to that of the monovalent construct against RSV-A Long (22). Likewise, the difference in potency between palivizumab Fab and the full-length antibody is 100-fold, while there is only a minimal improvement

**TABLE 4** Binding reactivity of Nb017 to membrane extracts of cells infected with distinct RSV-A Long escape mutants

<table>
<thead>
<tr>
<th>RSV mutant</th>
<th>Amino acid substitution(s)</th>
<th>Antigenic site</th>
<th>% Nb017 binding&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>R47F/4</td>
<td>N262Y</td>
<td>II</td>
<td>1.4 ± 1.6</td>
</tr>
<tr>
<td>R47F/7</td>
<td>N268I</td>
<td>II</td>
<td>61.1 ± 17.2</td>
</tr>
<tr>
<td>RAK13/4</td>
<td>N216D/N262Y</td>
<td>II</td>
<td>0.6 ± 0.8</td>
</tr>
<tr>
<td>R7C2/11</td>
<td>K272T</td>
<td>II</td>
<td>19.5 ± 16.8</td>
</tr>
<tr>
<td>R7C2/1</td>
<td>K272E</td>
<td>II</td>
<td>0.9 ± 1.1</td>
</tr>
<tr>
<td>R7.936/1</td>
<td>IV</td>
<td>119.9 ± 37.3</td>
<td></td>
</tr>
<tr>
<td>R7.936/4</td>
<td>IV</td>
<td>122.3 ± 40.5</td>
<td></td>
</tr>
<tr>
<td>R7.936/6</td>
<td>IV</td>
<td>106.5 ± 15.9</td>
<td></td>
</tr>
<tr>
<td>R9.432/1</td>
<td>IV</td>
<td>121.4 ± 20.0</td>
<td></td>
</tr>
<tr>
<td>RRA3</td>
<td>N262Y/R429S</td>
<td>II and IV</td>
<td>1.6 ± 3.2</td>
</tr>
</tbody>
</table>

<sup>a</sup> Absorbance results were normalized to those for a reference Nanobody recognizing antigenic site I (191C7) with preserved binding to all depicted mutants to account for the difference in F-protein expression. Nb017 was used at 0.2 \( \mu \text{g/ml} \).

<sup>b</sup> Data represent the percent binding to that of the reference RSV-A Long wild-type strain ± standard deviation. Shading and boldface indicate where the binding of Nb017 was >75%, shading indicates where the binding of Nb017 was 25 to 75%, and no shading indicates where the binding of Nb017 was <25%.

![FIG 2](image_url) Crystal structure representation of the F protein in its prefusion conformation. Ribbon representation of one prefusion F-protein protomer is shown in red, and the other two protomers are shown in surface representation in blue and green. The residues listed are those that were mutated in the tested RSV escape mutants and are shown in yellow. The figure was prepared by using ICM Molsoft (46) and was derived from the sequence with PDB accession number 4JHW (26).

![FIG 3](image_url) Results of competitive-binding ELISAs. The inhibition concentration-response curves obtained when biotinylated ALX-0171 was incubated with increasing concentrations of either unlabeled ALX-0171 or palivizumab are shown. The results shown depict the means of triplicate values ± SEs.
in the \( K_T \) (33). However, the basis of this observation is not yet understood, and the precise mechanisms involved remain under investigation.

Like palivizumab, Nanobodies Nb017 and ALX-0171 bind to both the postfusion and prefusion conformation of the F protein and, as a result, likely inhibit the conformational changes related to F-protein activation, as suggested for other Nanobodies and monoclonal antibodies specific for antigenic site II (21, 34–36). This hypothesis is supported by a model of the RSV F-protein trimer (35). As such, interactions between the binding epitopes may prevent structural changes in the three subunits within a single F-protein trimer (20, 21), as the length of the GS linkers was specifically designed to allow such interactions. Further crystallography studies are needed to elucidate the mechanisms involved in this increased neutralization capacity of ALX-0171.

**In vivo**, ALX-0171 was shown to be highly efficient at reducing and/or blocking RSV replication in the lung and nose, similar to what was reported for motavizumab, an affinity-matured version of palivizumab (39, 40), whereas no effect on viral load in the nose was demonstrated for palivizumab at doses of \( \leq 15 \text{ mg/kg} \) (40, 41). There remains the possibility that the residual ALX-0171 present in the wash fluid interfered in the plaque assay. Nonetheless, despite this caveat, we believe that it remains the most valid assessment, as it reflects the fact either that less virus is present or that any virus still present is effectively neutralized. Importantly, as ALX-0171 is devoid of an Fc, its therapeutic index likely relies entirely on direct antiviral activity, in contrast to what has been reported for palivizumab (42). In addition and in contrast to the route of delivery of palivizumab and motavizumab, which were administered systemically in therapeutic trials in infants and demonstrated conflicting effects on nasal/tracheal viral loads (39, 41, 43), ALX-0171 is delivered straight to the site of infection. Direct administration to the airways is likely to provide faster and more robust antiviral activity in the respiratory tract, which may be critical for the treatment of an acute disease like RSV infection. Indeed, the therapeutic effect of topical administration of purified human immunoglobulins, screened for high RSV neutralization activity, was shown to be effective (44).
activity, resulting in a decrease in viral load in cotton rats was shown to be 160 times greater than that by administration by the parenteral route (44). In addition to the greater effectiveness of the ALX-0171 administration route, the neutralization threshold of ALX-0171 (i.e., the expected lung concentration needed to exert the full antiviral effect) is also anticipated to be lower than that of palivizumab. This is particularly important in a clinical setting where the administered dose is a limiting factor.

In summary, ALX-0171 represents a novel, highly potent anti-viral with broad specificity toward a large panel of RSV clinical isolates and may have significant potential for therapeutic use. Viral with broad specificity toward a large panel of RSV clinical isolates and may have significant potential for therapeutic use. Furthermore, direct delivery of ALX-0171 to the airways/lungs by nebulization proved an effective mode of drug delivery, as even the lowest dose of only 1 mg/kg delivered intranasally still showed antiviral efficacy. As nebulization has been shown to result in fast and efficient drug delivery to the principal site of RSV infection (45), i.e., the upper and lower respiratory tract, this mode of drug delivery may provide major therapeutic advantages for ALX-0171 in treating RSV-infected patients.

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