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HEX17(Neumifil): An intranasal respiratory biotherapeutic with broad-acting antiviral activity

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ABSTRACT

Broad-acting antiviral strategies to prevent respiratory tract infections are urgently required. Emerging or re-emerging viral diseases caused by new or genetic variants of viruses such as influenza viruses (IFVs), respiratory syncytial viruses (RSVs), human rhinoviruses (HRVs), parainfluenza viruses (PIVs) or coronaviruses (CoVs), pose a severe threat to human health, particularly in the very young or old, or in those with pre-existing respiratory conditions such as asthma or chronic obstructive pulmonary disease (COPD). Although vaccines remain a key component in controlling and preventing viral infections, they are unable to provide broad-spectrum protection against recurring seasonal infections or newly emerging threats. HEX17 (aka Neumifil), is a first-in-class protein-based antiviral prophylactic for respiratory viral infections. HEX17 consists of a hexavalent carbohydrate-binding module (CBM) with high affinity to sialic acids, which are typically present on terminating branches of glycans on viral cellular receptors. This allows HEX17 to block virus engagement of host receptors and inhibit infection of a wide range of viral pathogens and their variants with reduced risk of antiviral resistance. As described herein, HEX17 has demonstrated broad-spectrum efficacy against respiratory viral pathogens including IFV, RSV, CoV and HRV in multiple *in vivo* and *in vitro* studies. In addition, HEX17 can be easily administered via an intranasal spray and is currently undergoing clinical trials.

1. Introduction

In 2019, the incidence of upper respiratory tract infections, which are mainly caused by viral agents such as influenza viruses (IFVs), parainfluenza viruses (PIVs), human rhinoviruses (HRVs), respiratory syncytial viruses (RSVs) and coronaviruses (CoVs), reached 17.2 billion (Jin et al., 2021). Although such infections are often self-limiting, they can pose a serious threat to certain vulnerable populations, including the very young or old or those with pre-existing respiratory conditions, and, in some cases, lead to harmful secondary bacterial colonization (Huang and Guo, 2022; Matsumoto and Inoue, 2014; Mohan et al., 2010; Nicholson et al., 1993). Virus-induced exacerbations of chronic obstructive pulmonary disease (COPD) and asthma are responsible for significant morbidity, mortality and healthcare costs associated with these conditions (Hewitt et al., 2016). Therefore, there is an unmet need for a broad-acting antiviral prophylactic that can be administered to such vulnerable groups. Furthermore, in situations where effective vaccines are unavailable or where the causative agent is newly emerging, the approach could also be focused towards other vulnerable groups (e.g. front-line healthcare workers) in addition to those with chronic pulmonary conditions.

In order to develop a safe, broad-spectrum antiviral prophylactic with reduced risk of antiviral resistance that is effective against common respiratory viral pathogens (i.e. IFVs, PIVs, HRVs, RSVs and CoVs), we have engineered multivalent carbohydrate-binding domains (mCBMs) with high affinity to sialic acid, a ubiquitous cell surface glycan. Previous studies were carried out using *Sp*2CBMTD – a hexavalent CBM (mCBM) composed of a tandem repeat of CBM domains from *Streptococcus pneumoniae* NanA sialidase fused to a trimerization domain from a

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Abbreviations

BALF	Bronchoalveolar lavage fluid
CBM	Carbohydrate-binding module
COPD	Chronic obstructive pulmonary disease
CoV	Coronavirus
DMEM	Dulbecco minimal essential medium
EC_{50}	50 % effective concentration
HN	Hemagglutinin-neuraminidase
HRV	Human rhinovirus
ICAM1	Intracellular Adhesion Molecule 1
IFV	Influenza virus
LD_{50}	50 % lethal dose
LDLR	Low-Density Lipoprotein Receptor
mCBM	Multivalent carbohydrate-binding module
MDCK	Madin Darby canine kidney
MEM	Minimal essential medium
NEAA	Non-essential amino acids
PIV	Parainfluenza virus
RSV	Respiratory syncytial virus
TD	Trimerization domain

Pseudomonas aeruginosa pseudaminidase, forming an oligomer containing six sialic acid binding sites per molecule (Connaris et al., 2014). The initial target for study was influenza, which uses sialic acid to mediate cell infection – an interaction that could potentially be blocked by a high-affinity sialic acid binding agent. Indeed, intranasal administration of *Sp*2CBMTD was shown to protect mice against a lethal dose challenge with influenza H1N1 or H7N9 (Connaris et al., 2014; Govorkova et al., 2015). Due to the ubiquity of sialic acid in the human respiratory tract, it was reasoned that blocking such sites might inhibit infection by other viruses and pathogens that utilize sialic acid for host binding/entry, in addition to sterically hindering pathogens that are not generally considered to use sialic acid in this manner. Furthermore, the initial set of results with *Sp*2CBMTD, which included excellent efficacy against influenza even when very low doses were administered seven days in advance of infection (Govorkova et al., 2015), suggested that another mechanism, in addition to steric blocking of viral binding, may also contribute to its antiviral effects. Therefore, the approach has since been expanded to test the potential effects of mCBMs against other respiratory viruses.

HEX17 (aka Neumifil) is an mCBM with high (98%) sequence homology to *Sp*2CBMTD that has been modified to reduce its predicted immunogenicity in humans. HEX17 has shown efficacy against SARS-CoV-2 *in vivo* using Syrian golden hamster models of infection (Fell et al., 2022; Knott et al., 2023) and is currently undergoing Phase 2 clinical trials.

In the present study, we have demonstrated the efficacy of mCBMs against a wide range of viruses from distinct viral families with diverse genetic origins and modes of entry that cause seasonal and pandemic respiratory infections. These findings are clinically important, especially for vulnerable groups, and support HEX17 as a preventive approach to providing early and rapid response to the onset of seasonal and pandemic respiratory infections.

2. Results

2.1. HEX17 inhibits influenza infection in vitro and in vivo

In vitro administration of HEX17 to MDCK cells prior to infection with H1N1 or H3N2 influenza reduced plaque formation, with EC_{50}s of 0.75 and 1.32 mg/mL, respectively (Fig. 1). *In vivo*, intranasal administration of BALB/c mice with a single 100 μg dose of HEX17 or *Sp*2CBMTD given one day pre-infection protected animals against a lethal challenge ($3XLD_{50}$) with H1N1 influenza (Fig. 2). The greatest efficacy was seen with HEX17 (100% survival), followed by *Sp*2CBMTD (80% survival) (Fig. 2a). There was a trend in improved clinical scores in the HEX17-treated animals in comparison with *Sp*2CBMTD and control-treated mice (Fig. 2b). In addition, administration of HEX17 or *Sp*2CBMTD in the absence of infection confirmed that the CBM compounds did not cause any morbidity or mortality (results not shown).

2.2. HEX17 is effective against RSV in vitro and in vivo

In a cell-based model of RSV-A2 infection, treatment of HEp2:Npro cells with HEX17 reduced viral titre in a dose-dependent manner when added prophylactically, therapeutically, or simultaneously with virus (Fig. 3). In an *in vivo* model, untreated RSV-A2-infected mice showed a significant increase in lung viral load four days post-infection. This was accompanied by significant pulmonary inflammation, as demonstrated by increased cell numbers in bronchoalveolar lavage fluid (BALF), especially neutrophils and lymphocytes. Pro-inflammatory cytokines were also significantly elevated in the BALF. All mice that were treated with HEX17, including therapeutic-only dosing groups, showed statistically significant reductions in viral load compared to the untreated control group ($p < 0.0001$) (Fig. 4). Prophylactic treatment with HEX17 in RSV-A2-challenged mice also reduced both inflammatory cell influx (Fig. S1) and cytokine concentrations in BALF (Fig. S2). Group 4 (combined prophylactic and therapeutic treatment) demonstrated a significant reduction in neutrophils (Fig. S1b), while lymphocyte counts were reduced in all animals receiving prophylactic treatment (Fig. S1c). Compared to the untreated control, levels of the inflammatory cytokines IFN-γ, IL-6, IL-1α, IL-1β, TNFα, IL-12, IP-10, MCP-1, GM-CSF, MIP-1α, RANTES, G-CSF, IL-2, KC, MIP2 and VEGF were significantly reduced in the BALF of treated animals (Fig. S2). The greatest effect was seen in mice that received a regimen of three doses of HEX17 on day −3, day −1 and 1 h pre-challenge. No overt clinical signs or symptoms were observed in any of the infected groups.

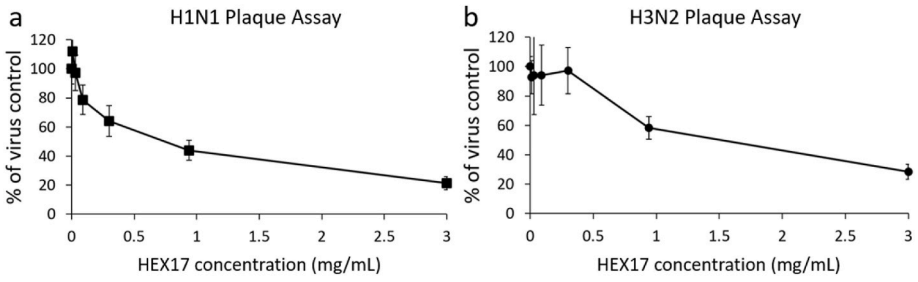

Fig. 1. MDCK cells were treated with different concentrations of HEX17 for 1 h prior to infection with H1N1 or H3N2 influenza virus. Plots show plaque number (mean of triplicates) as a percentage of the untreated control. (**a**) H1N1, (**b**) H3N2. Error bars indicate the standard deviations.

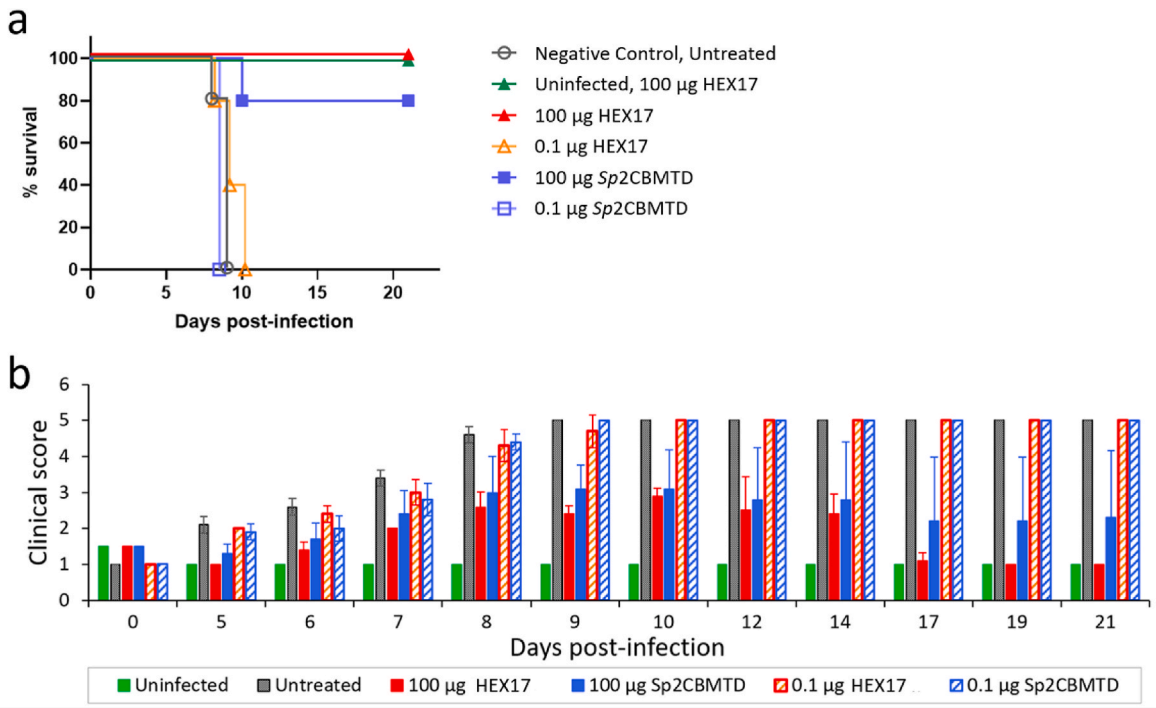

Fig. 2. (**a**) Survival curve of BALB/c mice showing effects of 0.1 or 100 µg of HEX17 or *Sp*2CBMTD when intranasally administered on day −3 and −1 (for 0.1 µg dosing) and day −1 (100 µg dosing) before infection with 3xLD_{50} A/PR/8/34 influenza; (**b**) Clinical scoring showing effects of HEX17 and *Sp*2CBMTD when administered on day −1 (100 µg doses) or day −3 and −1 (0.1 µg doses) before infection on day 0. Animals scored from 1 (healthy) to 5 (dead) – see text for details. Error bars indicate the standard deviations.

2.3. HEX17 reduces infection against multiple major and minor group HRVs

Prevention of HRV cell recognition using CBMs would require targeting the host cell receptors, rather than the virus, as the HRV capsid is not glycosylated. Initial ELISA binding tests demonstrated that HEX17 was able to interact with the receptor proteins Intracellular Adhesion Molecule 1 (ICAM-1) and Low-Density Lipoprotein Receptor (LDLR) of major and minor group HRVs, respectively (results not shown). *In vitro* efficacy against HRV was tested using an H1–HeLa cell infection model. HEX17 and *Sp*2CBMTD reduced the formation of plaques by HRV-14 (a major group HRV) in a dose-dependent manner, with mean EC_{50}s of 0.051 ± 0.014 mg/mL and 0.065 ± 0.019 mg/mL, respectively (Fig. 5a and b). A further experiment, testing *Sp*2CBMTD against a minor group HRV strain (HRV-1A) showed similar efficacy (EC_{50} = 0.067 ± 0.003 mg/mL), indicating that the effect is not HRV strain-specific (Fig. 5c).

2.4. HEX17 interacts with MERS spike protein and human DPP4 in vitro

Previous *in vivo* data showed efficacy of HEX17 against SARS-CoV-2 Victoria/01/2020 (Fell et al., 2022). We demonstrated that HEX17 could form direct interactions with the virus's main human receptor protein, ACE2, and viral spike proteins corresponding to variants of interest and variants of concern. In this study, initial testing was performed to test HEX17's interaction with the host and viral proteins involved in MERS-CoV infection. As shown in Fig. S3, HEX17 binds to the positive control (SARS-CoV-2 spike) with an EC_{50} of 296 ng/mL, which is consistent with our previous data (Fell et al., 2022). The binding profile and EC_{50} of the HEX17/MERS-CoV spike protein interaction are similar to the control binding and indicate that the CBM interacts with MERS-CoV spike with high affinity. HEX17 also directly binds to the MERS receptor protein human DPP4; the EC_{50} for the interaction is 102 ng/mL (Fig. S3).

2.5. A high-affinity sialic acid binding mCBM protects against PIV strains in vivo

Another type of sialic acid-binding mCBM, based on the carbohydrate-binding domain from *Vibrio cholerae* sialidase, has undergone preliminary testing for efficacy against parainfluenza viruses. Promising initial plaque assay data were obtained against PIV2, PIV3 and PIV5 (results not shown). *Vc*4CBM, which contains a repeat of four CBM domains, was then tested in a non-lethal PIV3 cotton rat infection model. *Vc*4CBM (1 mg) was administered intranasally 1 h prior to infection with PIV3. The data were normally distributed as determined by the Shapiro–Wilk test and demonstrated a significant ($p < 0.01$) reduction in lung viral titres using an unpaired parametric T-test (Fig. S4).

3. Discussion

Upper respiratory tract infections in humans are mainly caused by viruses – particularly influenza, RSV, rhinoviruses, and coronaviruses (Grief, 2013; Jin et al., 2021). Respiratory pathogens employ a wide range of mechanisms to infect the host. Identifying the pathogen responsible is often challenging: many produce overlapping symptom profiles and specific diagnostics tests are often not practical or possible, particularly in the case of a newly emerging virus or variants. There is an urgent need for a product that could be administered to patients who are at particular risk of infection-induced exacerbation of conditions such as COPD, bronchiectasis and asthma (Paules and Subbarao, 2017). A broadly effective antiviral agent would also be of value against the emergence of new pathogens (or variants) that have developed resistance to antiviral drugs or for which vaccines are unavailable.

Previously, we have demonstrated that intranasal administration of an mCBM (*Sp*2CBMTD) with high affinity to sialic acid was able to protect 100% of mice against a lethal challenge with H1N1 influenza (Connaris et al., 2014). Notably, in a separate study, *Sp*2CBMTD did not affect the virus-specific adaptive immune response, which was sufficient

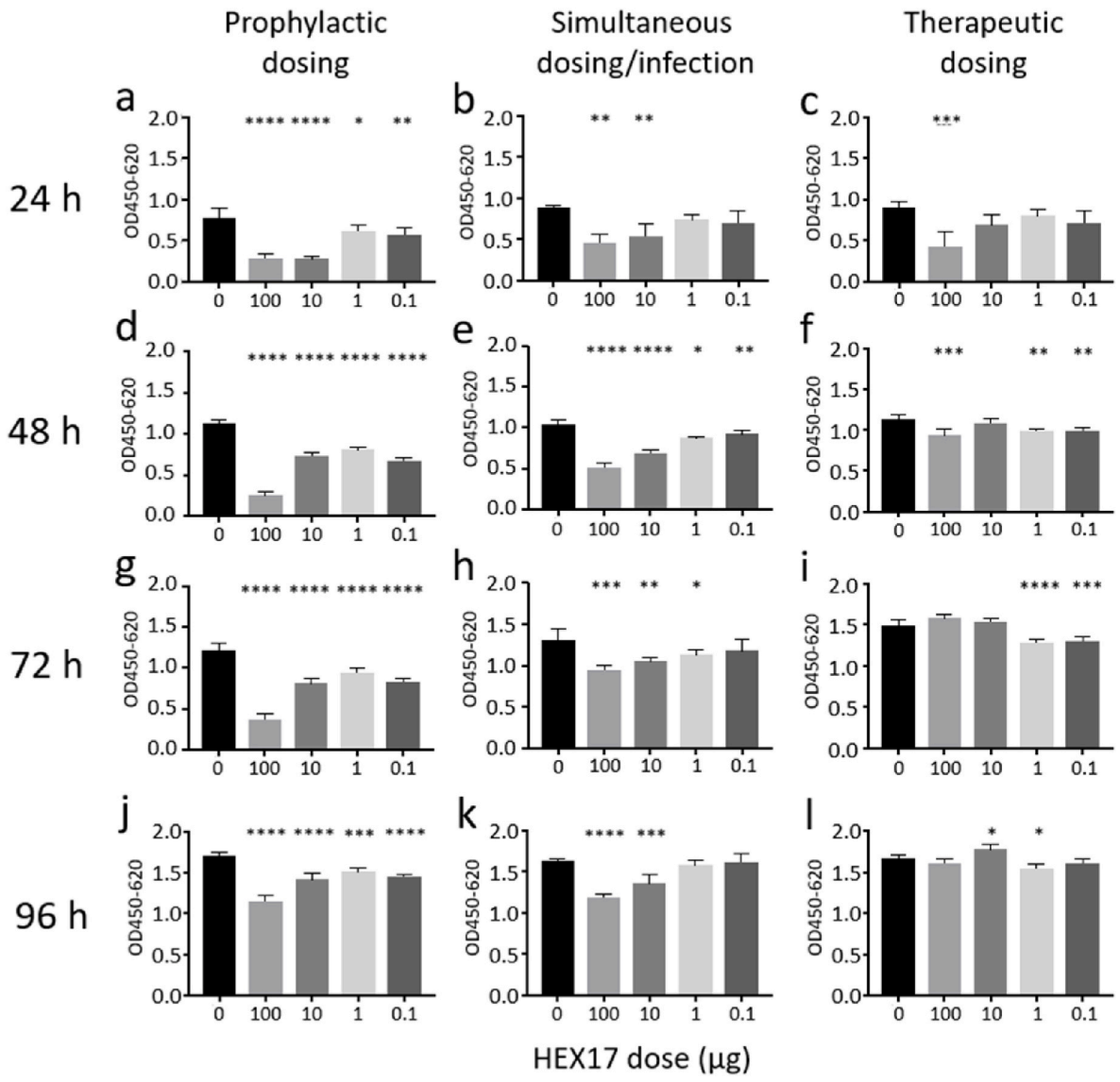

Fig. 3. Effect of HEX17 treatment on respiratory syncytial virus (RSV) infection in HEp2:Npro cells. (**a, d, g** and **j**) Cells were pre-treated with HEX17 before addition of RSV; (**b, e, h, k**) HEX17 was premixed with RSV before incubating with cells for 1 h; (**c, f, i, l**) Cells were incubated with RSV for 1 h then treated with HEX17. The cells were analyzed after 24 h (**a-c**); 48 h (**d-f**); 72 h (**g-i**); or 96 h (**j-l**). Each column represents the group mean and each bar represents the standard error of the mean (SEM) of n = 4. Changes in each group were compared to RSV-infected cells treated with HEX17 using Dunnett's one-way analysis of variance. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

to protect against reinfection with a higher dose of homologous H7N9 virus or heterologous H5N1 virus (Govorkova et al., 2015). Using HEX17 (a form of *Sp*2CBMTD modified to reduce its immunogenicity in humans), we have further studied the potential of mCBMs to protect against other respiratory pathogens, including viruses that have not been described as directly binding and/or infecting the host via sialic acid receptors. The rationale behind this is that binding of HEX17 to sialic acid – ubiquitous on respiratory cell surfaces and present on some viruses – could sterically hinder viral attachment to adjacent receptors. Furthermore, our earlier results indicated that very low levels of mCBM administered seven days in advance of infection were able to reduce infection, suggesting that a secondary effect in which the mCBM triggers an antiviral state may be in play, although this aspect of the mechanism has not been studied in HEX17 and requires further investigation.

To assess the efficacy of HEX17 against different viruses, subtypes and strains, we performed initial testing in ligand binding and cell-based assays and progressed to *in vivo* testing once *in vitro* efficacy and/or positive binding to viral surface proteins or receptors was demonstrated. Consistent with our earlier *Sp*2CBMTD data, *in vivo* testing confirmed that HEX17 retained the excellent protective effect of its parent molecule, protecting 100% of mice against a lethal H1N1 influenza challenge.

RSV is well established as a significant respiratory pathogen that contributes substantially to morbidity and mortality in infants, young children and older adults, particularly those with pre-existing conditions such as COPD (Li et al., 2022; Ramaswamy et al., 2009; Zwaans et al., 2014). Following positive *in vitro* data, HEX17 was tested in a non-lethal mouse model of RSV, which demonstrated significant reductions in lung viral load of HEX17-treated mice. This was accompanied by a significantly reduced inflammatory response and a reduction in immune cell counts compared to the untreated group. Although the greatest effect against RSV was produced with prophylactic administration of HEX17, a significant reduction in viral titre was also observed in the group receiving HEX17 after infection.

HRVs are the most prevalent respiratory viruses in humans, causing more than 50% of cold-like illnesses and posing a serious risk to vulnerable patients (Mäkelä et al., 1998; Thibaut et al., 2016). With over

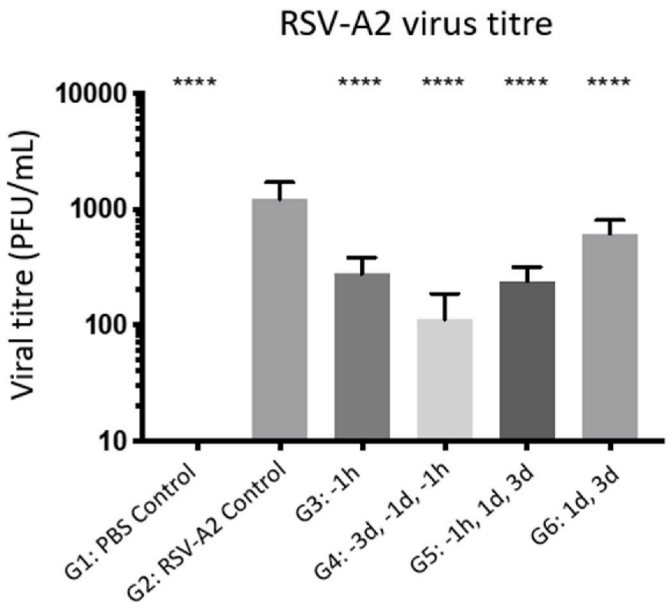

Fig. 4. Respiratory syncytial virus-A2 (RSV-A2) titre in lung homogenates of mice infected with RSV-A2 and treated with HEX17 (100 μg, i.n). Groups represent vehicle (PBS) control (G1), vehicle (RSV-A2) control (G2), and HEX17-treated groups dosed 1 h before infection (G3); 3 days, 1 day and 1 h before infection (G4); 1 h before infection, 1 day and 3 days after infection (G5); 1 day and 3 days post-infection (G6). Each column represents the group mean and each bar represents the standard error of the mean (SEM) of n = 8 animals. Changes in each treatment group were compared to RSV-infected animals treated with vehicle (G2) using Dunnett's one-way analysis of variance. ****p < 0.0001.

100 recognised serotypes that provide little or no cross-protection to infected individuals, control of HRV by vaccination is not feasible (Savolainen et al., 2002). Their lineages fall into major and minor groups that use different host receptors (ICAM-1 and LDLR, respectively) to infect cells (Bella and Rossmann, 2000; Hofer et al., 1994). Initial ELISA binding experiments confirmed that HEX17 can form a direct interaction with recombinant forms of both of these molecules. Furthermore, the mCBMs HEX17 and *Sp*2CBMTD reduced plaque formation in H1–HeLa cells infected with major and minor group HRVs. *In vivo* efficacy of mCBMs against HRV has not yet been tested; as HRV infections only cause mild/no symptoms in most animal models, a suitable intranasal challenge model is unavailable. However, our *in vitro* protein binding and cell-based experiments with influenza, SARS-CoV-2 and RSV have successfully translated into *in vivo* efficacy, strengthening the likelihood that HEX17 would also produce an anti-HRV effect in animals.

Following on from previous demonstrations of *in vivo* efficacy against SARS-CoV-2 (Fell et al., 2022; Knott et al., 2023), we have also obtained preliminary interaction data related to another coronavirus, MERS-CoV. The MERS-CoV spike glycoprotein mediates entry into host cells by attachment to human DPP4 (Raj et al., 2013). The HEX17 data show that HEX17 can form high-affinity interactions with MERS-CoV spike protein and human DPP4, suggesting that binding of MERS-CoV to the host cell could potentially be disrupted by targeting viral and/or host sialic acid.

Parainfluenza viruses are another important cause of respiratory disease in infants, young children, the immunocompromised and the elderly (Henrickson, 2003). PIV infection is initiated by binding of the viral hemagglutinin-neuraminidase (HN) to sialic acid on cell surface receptors and is, therefore, another promising target for a host-targeted, sialic acid-binding therapeutic. Although HEX17 has not yet been tested against PIVs, a preliminary experiment using a different mCBM with high-affinity sialic acid binding (*Vc*CBM4) showed a significant reduction in hPIV3 replication in cotton rats, indicating that such an approach has potential against PIVs. In a somewhat related approach, DAS181 (a sialidase fusion protein) has shown *in vitro* and *in vivo* efficacy against PIVs (Malakhov et al., 2006; Moscona et al., 2010; Roth et al., 2009). DAS181 functions by enzymatically cleaving sialic acid from cells to prevent attachment by pathogens that require these glycans for cell binding, and has also shown efficacy against multiple strains of influenza (Belser et al., 2007).

As demonstrated in this study and previous work, HEX17 is effective at the tested concentrations of 5–6 mg/kg *in vivo* against influenza, RSV and SARS-CoV-2. When comparing the potency of HEX17 (in terms of effective mg/kg doses in small animal models) with that of other biological therapies, it should be noted that other such approaches are largely designed to target specific families of viruses and use a different mechanism of action to HEX17 (often with a different route of administration). Other protein-based strategies against respiratory pathogens include antibody-based therapeutics (most notably anti-influenza HA monoclonal antibodies) and host-directed therapies (e.g. DAS181, described above). Examples of highly potent influenza antibodies include KPF1, which showed unusually high *in vitro* potency (down to 1 μg/mL) against influenza, and protected mice against lethal challenge with multiple H1 influenza strains when administered prophylactically (via intraperitoneal injection) at a dose level of 1 mg/kg (Nogales et al., 2018). Intranasally dosed anti-influenza antibodies include CR9114, which was effective against multiple different influenza subtypes with effective doses as low as 0.2 mg/kg (Beukenhorst et al., 2024). A number of anti-influenza antibodies, such as VIS410 (Hershberger et al., 2019) and MHAA4549A (McBride et al., 2017) have progressed to clinical studies and shown protective efficacy (Kumari et al., 2023; Sun et al., 2024). There is also increasing interest in multi-specific antibody-based constructs, which potentially provide broader activity within influenza

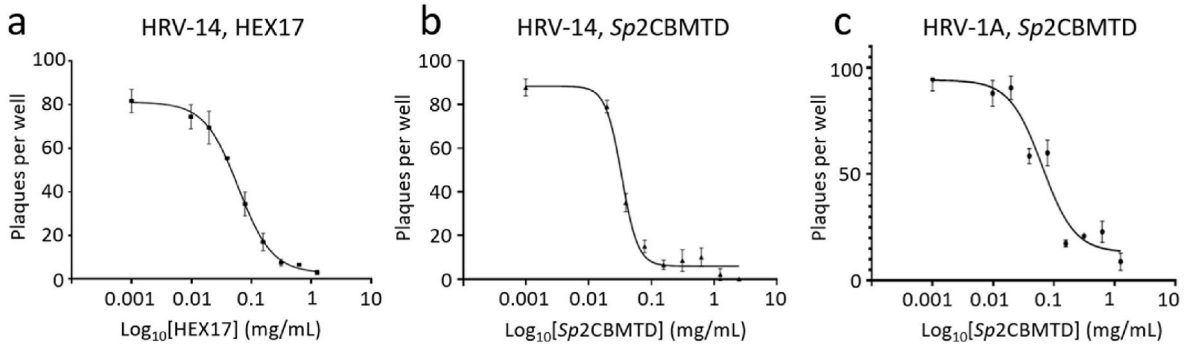

Fig. 5. Representative non-linear regression plots of plaque number vs CBM concentration against challenge with human rhinovirus (HRV). The points represent the mean number of plaques per concentration and the error bars indicate the standard error of the mean (SEM). Three replicate experiments were performed (n = 2 in each; n = 6 in total). The plots show dose-dependent protective effects of (**a**) HEX17 against HRV-14, (**b**) *Sp*2CBMTD against HRV-14 and (**c**) *Sp*2CBMTD against HRV-1A.

virus species (Sedeyn and Saelens, 2019). Examples include MD3606, a multidomain antibody which protected mice against influenza A or B viruses at a dose of 5 mg/kg (Laursen et al., 2018); and BiFlu, which contains two enzymatically linked IgG molecules that can neutralize both group 1 and group 2 influenza viruses, and was effective *in vivo* against H1N1 at a dose of 2 mg/kg (Wagner et al., 2014). Another strategy, reported by Liu et al., is the conjugation of zanamivir, a neuraminidase inhibitor, to an anti-mouse kappa light chain nanobody to trigger effector functions against virus-infected cells (Liu et al., 2023). A single 1 mg/kg dose protected mice against both influenza A and B viruses, while a dose of 10 mg/kg was effective when given three days after infection. These alternative antibody-based approaches have yet to be assessed clinically. For the treatment of SARS-COV-2, antibodies targeting the viral spike protein have shown clinical efficacy, although many have been rendered obsolete by the emergence of variants. However, some neutralizing antibodies have shown preclinical promise against multiple variants; intranasal administration of antibody F61 alone or in combination with antibody H121 produced significant protective effects against different SARS-CoV-2 variants in a lethal mouse model of infection, including significant efficacy against the delta variant with the lowest dose tested (1.25 mg/kg) (Lu et al., 2022). In an *in vivo* RSV infection model, intramuscularly dosed antibody MEDI8897 (which targets the prefusion conformation of the RSV F protein) reduced the viral loads of cotton rats infected with either RSV A or RSV B at dose levels down to 1–2 mg/kg (Zhu et al., 2017) and has produced positive results in Phase 2 b and 3 clinical trials. DAS181 has shown efficacy *in vivo* against hPIV3 and multiple subtypes of influenza down to 0.3 mg/kg (Belser et al., 2007; Jones et al., 2013). *In vivo* HEX17 studies demonstrating efficacy with dose levels of 5–6 mg/kg were not designed to determine the minimum effective dose; however, given that (i) HEX17 and *Sp*2CBMTD provided comparable potency when tested alongside each other in a lethal influenza mouse model and (ii) *Sp*2CBMTD has previously demonstrated anti-influenza efficacy with single dosing down to 0.5 mg/kg (Govorkova et al., 2015), it is likely that lower doses of HEX17 would also be effective. HEX17 has completed preclinical toxicity testing and is undergoing Phase 2 clinical trials. These data will be described elsewhere. The drug is delivered via nasal administration using a spray pump, which provides various advantages such as lower systemic exposure, rapid delivery to the site of action and ease of use for the patient.

By targeting a ubiquitous host carbohydrate that is also present on some viruses, we have shown that a single compound, HEX17, can prevent and treat infection by a diverse range of unrelated viruses that are key causative agents of respiratory infectious diseases. In addition to producing a broad-acting effect, the dual host- and viral-targeted approach greatly reduces the likelihood of the development of viral resistance.

4. Materials and methods

4.1. Cells and viruses

Madin Darby canine kidney (MDCK), H1–HeLa cells, LLC-MK2 cells and HEp2:Npro cells were from ATCC. MDCK cells were cultured in Dulbecco minimal essential medium (DMEM) supplemented with 5% (v/v) FBS, 2 mM L-glutamine, 50 μg/mL gentamycin and 0.1 mM non-essential amino acids (NEAA). H1–HeLa cells were propagated in minimum essential media, (MEM) supplemented with 10% (v/v) FBS, 2 mM L-glutamine and 0.1 mM NEAA. HEp2:Npro cells were cultured in DMEM supplemented with 10% (v/v) FBS and 1% (v/v) penicillin-streptomycin (Pen-Strep; GIBCO). For the *in vitro* studies, the virus strains (influenza H1N1 [A/PR/8/34], influenza H3N2 [A/HK/8/68], human RSV-A2, HRV-1A and HRV-14) were all from ATCC. Viruses were propagated in MDCK cells (for influenza testing), HEp2:Npro cells (RSV) or H1–HeLa cells (HRV). For *in vivo* studies, influenza virus strain PR8 (A/Puerto Rico/8/34, H1N1) was sourced from Virapur, RSV-A2 was from Pharmidex and human PIV3 (strain C243) was from ATCC.

4.2. Compounds

*Sp*2CBMTD and HEX17 were produced as described previously (Connaris et al., 2014; Fell et al., 2022). Briefly, *Sp*2CBMTD is a recombinant construct composed of a tandem repeat of a CBM from *Streptococcus pneumoniae* NanA sialidase and a trimerization domain (TD) from *Pseudomonas aeruginosa* pseudaminidase and was expressed in *E. coli* BL21 (DE3). HEX17 is a version of *Sp*2CBMTD, containing the mutations V239A, V249G, A162P, S342D and R403K, which were introduced to reduce the predicted immunogenicity. The two mCBMs share 98% sequence identity. *Vc*4CBM, which was used in parainfluenza efficacy testing, contains four repeats of the sialic acid-binding CBM from *Vibrio cholerae* NanH sialidase and has been described previously (Connaris et al., 2014).

4.3. Efficacy testing of HEX17 against influenza in vitro plaque reduction assays

In influenza plaque assays, each plate contained cell control wells (uninfected, untreated wells), virus control wells (infected, untreated cells) and treatment wells (infected, treated cells) at six concentrations in triplicate. HEX17 was evaluated using a highest test concentration of 3 mg/mL and five serial half-logarithmic dilutions in assay medium (DMEM supplemented with 2 mM L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin), in triplicate. HEX17 was incubated at 37 °C on MDCK cell monolayers for 1 h in 250 μL of assay medium per well, before the addition of 100 μL of 250 pfu/mL H1N1 (A/PR/8/34) or H3N2 (A/HK/8/68) influenza virus. After 1 h, the virus was removed and cells were washed in PBS. Overlay medium (1:1 2% (v/v) Avicel and 2× assay medium, supplemented with 0.22% (w/v) sodium bicarbonate, 0.12% (w/v) BSA, 1 μg/mL TPCK-treated trypsin, 100 U/mL penicillin, 100 μg/mL streptomycin, 2 mM L-glutamine and 1 mM sodium pyruvate) was added to each well in a volume of 500 μL. Following incubation at 37 °C for 24 h, the overlay was removed and washed with PBS before fixation with 70% (v/v) ethanol for 1 h at 4 °C. Plaques were immunostained using FITC-conjugated anti-NP (VirosStat; 1:100) as the primary antibody, and goat anti-mouse IgG peroxidase (Merck; 1:1000) as the secondary antibody. Each antibody was diluted in PBS containing 0.05% (v/v) Tween 80 and 10% (v/v) horse serum (Sigma) and was incubated on the cells for 1 h at 37 °C. The plates were washed with PBS and 250 μL of TrueBlue substrate (KPL) was added to the wells for 1 h. Plates were dried and the plaques were counted.

4.4. Efficacy of HEX17 against RSV in vitro

HEX17 was serially diluted 10-fold, from 1000 μg/mL to 1 μg/mL. Human RSV-A2 was diluted to 3×10^4 pfu/mL (MOI~0.08) with serum-free DMEM containing 1% (v/v) Pen-Strep and kept on ice until use. Confluent HEp2:Npro cells in 96-well plates were washed three times with pre-warmed PBS before treatment and/or infection. In pretreatment wells, cells were treated with 100 μL of serially diluted HEX17 for 1 h, before it was removed and 100 μL RSV-A2 was added for 1 h. For simultaneous CBM treatment/RSV infection, equal volumes of HEX17 and RSV-A2 were mixed and added to cells (200 μL per well) for 1 h at 37 °C/5% CO_2. In therapeutic conditions, cells were incubated with 100 μL RSV-A2. Following 1 h incubation, the virus was removed and 100 μL of serially diluted HEX17 was added for 1 h at 37 °C/5% CO_2. The supernatant was removed and cells were overlaid with 1.2% Avicel, 2% (v/v) FBS, 1× DMEM and 1% (w/v) Pen-Strep and incubated at 37 °C/5% CO_2 for 24, 48, 72 or 96 h. The overlay medium was removed and cells were fixed with 100 μL of 10% (v/v) formaldehyde for 10 min at room temperature. Plates were washed three times with PBS. 100 μL mouse anti-RSV fusion protein (BioRad) was added into each well for 1 h, followed by washing and the addition of 100 μL goat anti-mouse

secondary antibody (Cell Signaling Technology) for 1 h. Wells were washed and 50 μL TMB was added to each well. After 10 min in the dark, the reaction was stopped by the addition of 50 μL of 1 M HCl per well. Absorbances at 450 nm were recorded, along with reference values at 620 nm.

4.5. Efficacy of Sp2CBMTD and HEX17 against HRV in vitro

For efficacy testing against major and minor-group HRVs, a range of HEX17 concentrations – a two-fold dilution series from 2.5 mg/mL – was applied to near-confluent H1–HeLa cells in a volume of 400 μL per well and incubated for 1 h at 37 °C, 5% CO_2. The experiment was performed in duplicate six-well plates on three separate occasions. Following two washes with PBS, cells were incubated with 400 μL of virus solution at a concentration of 80 pfu/mL (or serum-free medium) per well for 1 h at 37 °C. The virus solution was removed and 2 mL of agarose overlay was applied to each well and allowed to set. 2 mL of complete MEM was added on top of the set agarose. Plates were incubated at 35 °C, 5% CO_2 for 48 h (HRV-14) or 72 h (HRV-1A). 2 mL of 10% (v/v) formalin was added to each well and allowed to incubate at 22 °C for 15 min to fix the cells. Formalin and agarose were removed and the cells were washed gently with 2 mL PBS. To visualize the plaques, 2 mL of 1% (w/v) crystal violet solution was added to each well. The stain solution was aspirated after 15 min at 22 °C and the plaques were counted.

4.6. In vivo influenza infection model

For the influenza model, female 7-week-old BALB/c mice (Charles River Laboratories) were randomly allocated to cages (five mice per cage). Mice were monitored and acclimatised to the facility for seven days. Potable water and food were available ad libitum. Cage enrichment was present and daily care of the animals was performed. The experiment was approved by and carried out in strict accordance with the local Consumer and Veterinary Affairs department (ethical approval number VD3111). On dosing days, the mice (five animals per group) were anaesthetised with isofluorane and intranasally administered with either (i) 0.1 μg mCBM (HEX17 or *Sp*2CBMTD) on days −1 and −3 before lethal viral challenge on day 0, or (ii) 100 μg mCBM on day −1 only. Following a specified period, the animals were anaesthetised by intraperitoneal injection with 9.75 mg Xylasol and 48.75 mg Ketasol per kg body weight and each animal received 50 μL virus solution (3xLD_{50}/50 μL of A/PR/8/34 [H1N1]) by intranasal inoculation. The toxicity control group animals were uninfected but treated with 100 μg HEX17, while the infection control group animals were infected but untreated. Mice were monitored for weight and clinical signs (scores: 1, healthy mouse; 2, signs of malaise, including slight piloerection, slightly changed gait, periods of inactivity; 3, strong piloerection, constricted abdomen, changed gait, periods of inactivity; 4, enhanced characteristics of the previous group, but showing little activity and becoming moribund; 5, dead mouse). Mice were euthanized and scored dead when they either reached a clinical score of 4 or a temperature of less than 32 °C.

4.7. In vivo efficacy testing of HEX17 against RSV

HEX17 efficacy against RSV was tested in an *in vivo* non-lethal mouse model of RSV-A2 infection. Female BALB/c mice, weighing 15–20 g on arrival (Charles River Laboratories), were acclimatised for seven days. Mice were housed in ventilated cages of four, and conditions of light/dark, temperature and humidity were maintained within Home Office guidelines. Mice had access to standard chow ad libitum. For infection, mice (eight per group) were anaesthetised with isoflurane and infected intranasally with RSV-A2 in both nostrils (total volume 50 μL of 5×10^6 pfu). Mock-infected mice received virus diluent (DMEM, 2% (v/v) FCS, 12.5% (w/v) sucrose). The mice were treated with different dosing regimens: HEX17 (100 μg [5 mg/kg]) was administered (i) prophylactically 1 h pre-RSV-A2 infection, (ii) on day −3, day −1 and 1 h pre-infection, (iii) therapeutically on day 1 and day 3 post-RSV-A2 infection, or (iv) with a combined prophylactic and therapeutic regimen: 1 h pre-RSV-A2 infection and 1 day and 3 days post-infection. The uninfected vehicle (PBS) control group received PBS on days −1, 0, day +1 and day +3. The infected, untreated control group received PBS on days −1, +1 and + 3 with infection on day 0. To assess the effect of HEX17 in this model, body weight and clinical signs were monitored and lung viral titres were measured by plaque assay. In addition, the concentrations of inflammatory mediators (GM-CSF, IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, TNFα, IL-1α, IL-13, IL-17, IP-10, KC, MCP-1, G-CSF, MIP-1α, VEGF, RANTES, MIP-2, MIP-1β, IL-33) of mouse BALF supernatant were measured in duplicate using a Mouse Cytokine Magnetic 23-plex panel assay (Biotechne) as per the manufacturer's instructions. Levels were measured using a MAGPIX system (Luminex Corp). The study was performed under the UK Home Office Project Licence P8AE03703. Data are reported as the total and differential number of cells per mL of BALF, cytokine concentration (pg/mL) or plaque forming units (pfu) per treatment group ± standard error of the mean (SEM). Inter-group deviations were statistically analyzed by one-way analysis of variance (ANOVA). In the case of significant differences in the mean values among the different levels of treatment, comparisons versus the vehicle group were carried out using Dunnett's test. In case the equal variance test failed, a Kruskal-Wallis one-way ANOVA on ranks followed by a Dunn's test was used. P values < 0.05 were considered statistically significant.

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Jane A. Potter: Writing – original draft, Visualization, Supervision, Investigation, Formal analysis, Conceptualization. **Angus Aitken:** Writing – review & editing, Visualization, Methodology, Investigation, Formal analysis. **Lei Yang:** Writing – review & editing, Visualization, Methodology, Investigation, Formal analysis. **Jennifer Hill:** Methodology, Investigation, Formal analysis. **Antoni Tortajada:** Methodology, Investigation, Formal analysis. **Julia L. Hurwitz:** Supervision, Formal analysis, Conceptualization. **Bart G. Jones:** Visualization, Investigation, Formal analysis. **Nadiawati Alias:** Writing – review & editing, Visualization, Investigation, Formal analysis. **Mingkui Zhou:** Writing – review & editing, Visualization, Supervision, Formal analysis, Conceptualization. **Helen Connaris:** Writing – original draft, Visualization, Validation, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

All data supporting the findings of this study are included in the article and/or Supporting information.

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Appendix A. Supplementary data

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