Article type: Communication

Non-Toxic Virucidal Macromolecules Show High Efficacy Against Influenza Virus Ex Vivo and In Vivo

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Keywords: Influenza, virucidal, antiviral, 3'SLN, 6'SLN

Abstract

Influenza is one of the most widespread viral infections worldwide and represents a major public health problem. The risk of new pandemics remains high and it is likely that the development of a vaccine against strongly virulent influenza strains would take too long, hence would not help the initial fight of the pandemic. Antiviral drugs are available but they are far from ideal. Arguably, the ideal antiviral should target conserved viral domains and be virucidal, *i.e.* irreversibly inhibit viral infectivity. Here, we describe a new class of anti-influenza macromolecules that meets these criteria and displays exceedingly low toxicity. These compounds are based on a cyclodextrin core modified on its primary face with long hydrophobic linkers terminated in 6'sialyl-N-acetyllactosamine (6'SLN) or 3'SLN. SLN enables nanomolar inhibition of the viruses while the hydrophobic linkers confer irreversibility to the inhibition. We show that these cyclodextrins are effective against several human or avian influenza strains in vitro, as well as against a 2009 pandemic influenza strain ex vivo and in vivo. Importantly, replacing the hydrophobic linkers with hydrophilic ones results in molecules that are totally ineffective ex vivo, confirming the importance of the virucidal mechanism in the quest of a truly effective antiviral drug.

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Influenza viruses are among the most infective viruses.[1,2] Every year different influenza strains infect a large fraction of both the animal and human population[3] endangering infants, the elderly and immunocompromised people, which are at high risk of hospitalization and death, due to influenza-related complications.[4–8] As a result, seasonal influenza has yearly a remarkable socio-economic impact. Respiratory diseases can cost a significant fraction of the total health expenditures in developed and mainly in developing countries.[9,10] Because influenza mutates so rapidly, the development of a lifelong vaccine is still a major challenge.[11–13] Vaccine development would pose even higher challenges when we focus on the occasional pandemics instead of yearly outbreaks. In such a case, the development time of a new vaccine, which is on average 6 months, would represent a serious risk. Furthermore, even in the presence of a vaccine, reaching reasonable vaccination coverage is far from a foregone conclusion. As a consequence, the risk of a new pandemic, such as the Spanish-flu, is still present and recognised as one of the top threats to global health.[14–16]

Naturally, the second line of defence after vaccines, are antiviral drugs. A number of anti-influenza drugs are currently approved: neuraminidase inhibitors such as zanamivir and oseltamivir, ion channel inhibitors such as amantadine, fusion inhibitors such as umifenovir (only in Russia and China) and polymerase inhibitor such as baloxavir marboxil, that was recently approved in US and Japan. Yet, it is recognized that the efficacy of current drugs is far from ideal. Concerns about these drugs range from significant side effects to the appearance of drug-resistant viruses after a short period of use.[17] Given the importance of this issue, a number of other antivirals are in clinical trials.[18–25] The majority of these drugs are monoclonal antibodies[26,27] that inhibit the fusion of the virus to the host-cell. Although they are promising, it is likely that they will be considerably costly due to their manufacturing processes.

An ideal anti-influenza drug should be broad-spectrum, by targeting a highly conserved part of the virus and, in order to avoid loss of efficacy due to the dilution in body fluids, have an irreversible effect, *i.e.* be virucidal. Obviously, this drug needs to be truly non-toxic. There are quite a few

research lines on the development of molecules that target conserved parts of the virus.[28–40] To the best of our knowledge, these compounds are all reversible in their action and hence could face major hurdles when translating into drugs.

The search for virucidal (*i.e.* irreversible) drugs with limited toxicity has been very challenging. We have previously shown that gold nanoparticles[41] and β-cyclodextrins[42] modified with 11-undecane sulfonic acid display a virucidal mechanism against a wide range of heparan sulfate proteoglycans (HSPGs) binding viruses, with no cellular toxicity. These compounds were capable of exerting forces that ultimately deform the virus particle. The chemical structure of the ligand was shown to be essential in order to achieve such irreversible inhibition.

Here, we adopted a similar strategy in order to target human influenza viruses and avian influenza viruses. 6' sialyl-N-acetyllactosamine (6'SLN) and 3' sialyl-N-acetyllactosamine (3'SLN), that bind to hemagglutinin (HA) trimers of influenza strains with high affinity[43], were grafted onto the primary face of β-cyclodextrins through a series of different linkers. The structure of all modified cyclodextrins discussed in this work is shown in **Figure 1**. Of note, since each HA trimer has three sialic acid binding pockets, we aimed to modify the β-CDs with three trisachharides. Therefore, the cyclodextrins are not fully modified but they all bear, on average, a comparable number of trisaccharides (Figure S1 and S2), determined using ^{1}H Nuclear Magnetic Resonance Spectroscopy (NMR). In vitro dose-response assays against influenza A/Netherlands/602/2009 (H1N1) strain (A/NL/09), were conducted to compare the inhibitory activity of these molecules (Figure 1 and S3). The infection was quantified with immunocytochemical assays at 24 hours post-infection (hpi). β-CDs bearing a sufficiently long, hydrophobic linker and 6'SLN end-group (**C6-6'**, **C11-6'**, and **C14-6'**) showed strong inhibitory activity against infection with influenza A/NL/09, having EC_{50} values in the nanomolar range. On the other hand, the β-CD with a shorter linker, **C1-6'**, poorly protected from the infection. Introducing a sufficiently long linker clearly enhanced the end-group flexibility;

hence the inhibitory concentrations decreased. The EC_{50} was comparable (yet slightly higher) when the hydrophobic linker was replaced with a hydrophilic PEG8 linker (**P8-6'**).

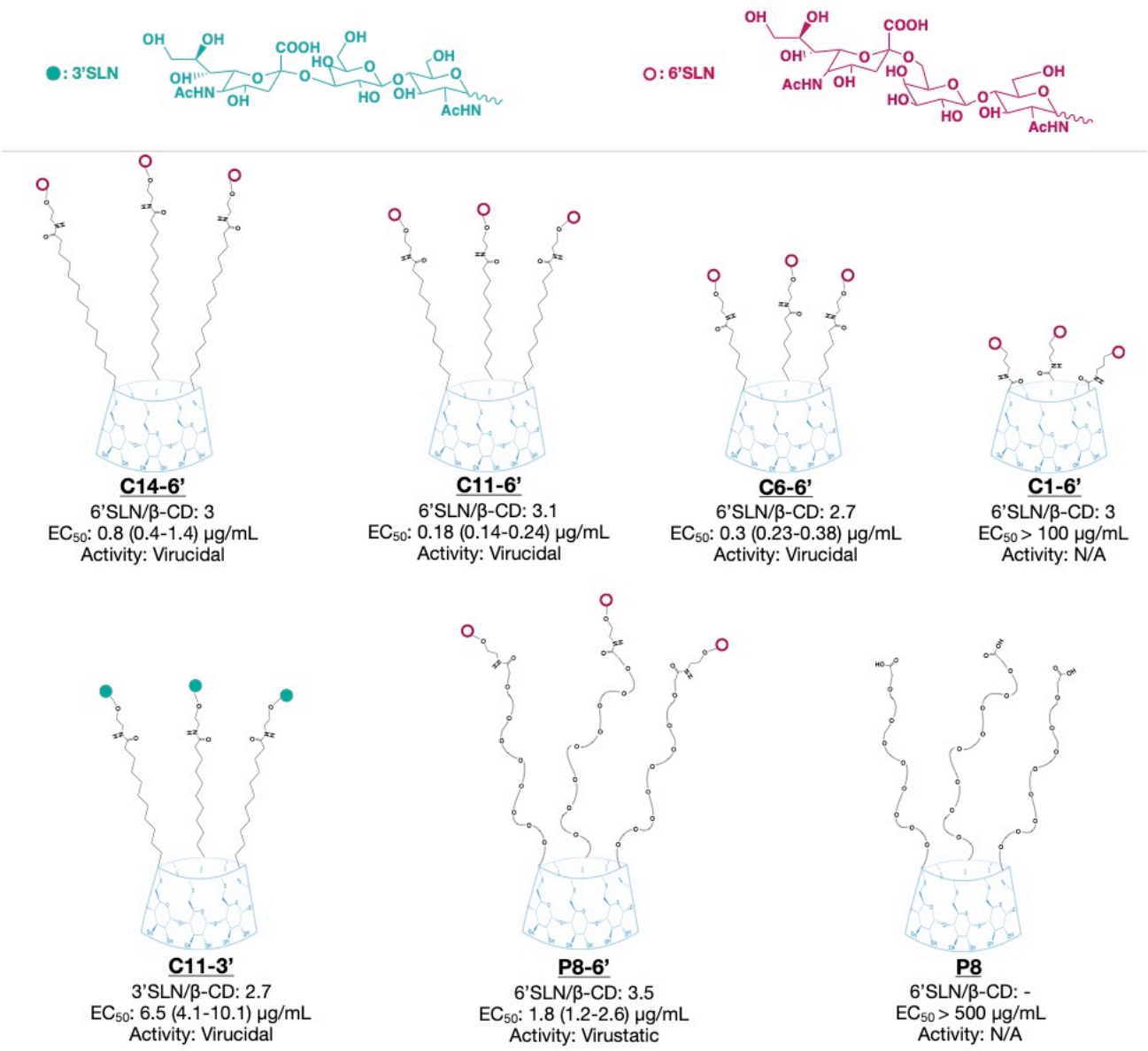

Figure 1: Summary of the modified cyclodextrins. The average number of 6'SLN or 3'SLN per β-CD was calculated by ^{1}H NMR. The representative chemical structures of modified cyclodextrins were constructed based on NMR results. EC_{50} represents the half-effective concentrations on MDCK cells at 24 hpi against A/NL/09 with the respective 95% confidence interval (CI) (Figure S3). N/A: not assessable.

C11-6', the molecule that showed the best inhibitory activity against A/NL/09, displayed strong antiviral activity against human influenza strains from both the A (H1N1 and H3N2) and the B type (Table 1 and Figure S4). Importantly, it inhibited very recent A (H1N1) and B clinical strains (from

the 2017/2018 influenza season), isolated from patients in the University Hospital of Geneva and passaged only once in MDCK cells. **C11-6'** did not show any antiviral activity against HSV-2, an HSPG-binding virus, indicating specificity of the compound for sialic acid dependent viruses.

Table 1: Inhibitory activity of C11-6' and C11-3' against different influenza strains.

	Compound	CC_{50} (µg/mL)	EC_{50} (µg/mL)	EC_{50}† (nM)
A/Netherlands/602/2009 (H1N1)	C11-6'	>100	0.18 (0.14 - 0.24)	42
	C11-3'	>100	6.5 (4.1-10.1)	>1000
A/Switzerland/8337/2018-MDCK1 (H1N1)	C11-6'	>100	0.5 (0.4 - 0.67)	125
A/Singapore/37/2004 (H3N2)	C11-6'	>100	0.23 (0.16 – 0.34)	56.5
B/Wisconsin/01/2010*	C11-6'	>100	2.2 (1.49 – 3.42)	500
B/Switzerland/3849/2018-MDCK1*	C11-6'	>100	20 (10.5 – 28.7)	>1000
A/turkey/Turkey/2005 (H5N1)	C11-3'	>100	4.1 (2.55-6.7)	931
	C11-6	>100	N/A	N/A
A/turkey/Italy/1999 (H7N1)	C11-3'	>100	8.8 (3.2-26)	>1000
HSV-2 (Control)	C11-6'	>100	N/A	N/A

* B Yamagata lineage

† Molar concentrations were determined based on the modified cyclodextrins.

CC_{50}: Half-cytotoxic concentration.

EC_{50}: Half-effective concentration with 95% CI in brackets

N/A: not assessable

6'SLN is known to be specific to human influenza strains, whereas 3'SLN is preferred by avian influenza strains as a primary attachment point.[44] To prove the generality of our approach, especially against influenza strains that are known to have the ability of crossing the species barrier, we synthesized and tested **C11-3'** (Figure 1) against avian influenza strains. We show that **C11-3'** inhibits both H5N1 and H7N1 avian strains, at 4.1 and 8.8 µg/ml concentrations respectively (see Table 1). *De facto*, these results confirm the antiviral strategy adopted against human strains. We additionally tested whether **C11-3'** could inhibit the human strain A/NL/09 and whether **C11-6'** would also be active against the avian strain, H5N1. **C11-3'** displayed a good inhibitory activity against A/NL/09 (Figure 1 and table 1), whereas **C11-6'** did not show any activity against H5N1

(table 1 and Figure S5). These results are in line with previous literature comparing the binding affinities of avian and human strains to the different types of sialic acids[44,45]. Avian influenza strains (particularly H5N1 strains) preferentially bind to alpha -2,3 linked sialic acid, which has a thin and straight trans conformation. On the other hand, the wider sialic acid binding site of human strains can accommodate both the bulky cis conformation of alpha -2,6 linked sialic acid and the narrower -2,3 linked sialic acid[44,45].

The synthesis of similar compounds sharing the β-cyclodextrin core and the 6'SLN moiety but different linkers allowed us to highlight a structural feature conferring irreversible inhibitory activity i.e. virucidal action (**Figure 2** and S6). This feature differs from the one described in our previous work where only the length of the linkers was compared[41]. Here, we hypothesized that one of the key components of an irreversible viral inhibition is that the binding moiety (here 6'SLN) is borne by a sufficiently long hydrophobic linker. A hydrophilic linker such as PEG should not be capable of generating forces that permanently inactivate the virus. To test this hypothesis, we compared **C11-6'** and **P8-6'**. These two compounds differ solely in the hydrophobicity of the linker and show comparable inhibitory activity against A/NL/09, as shown on the left in figure **2a** and **2b**. Virucidal assays were conducted as previously described[41] to compare the mechanism of inhibition of these compounds, *i.e.*, virucidal (irreversible) or virustatic (reversible). Briefly, amounts of the compounds that provide complete protection (10 µg of **C11-6'** and 50 µg of **P8-6'**) were incubated with the virus for 1h. Serial dilutions of the inocula were conducted followed by evaluation of the infectivity. In the case of **P8-6'**, the right graph in **2a** shows that while at the initial concentration complete protection was present, upon dilution the difference with the infectivity of the control sample (virus alone) was lost, *i.e.* the inhibitory effect was found to be reversible (virustatic). In the case of **C11-6'** the right graph in **2b** shows that complete protection was kept upon dilution and the graphs in **2c** show that this property was conserved against a number of different strains.

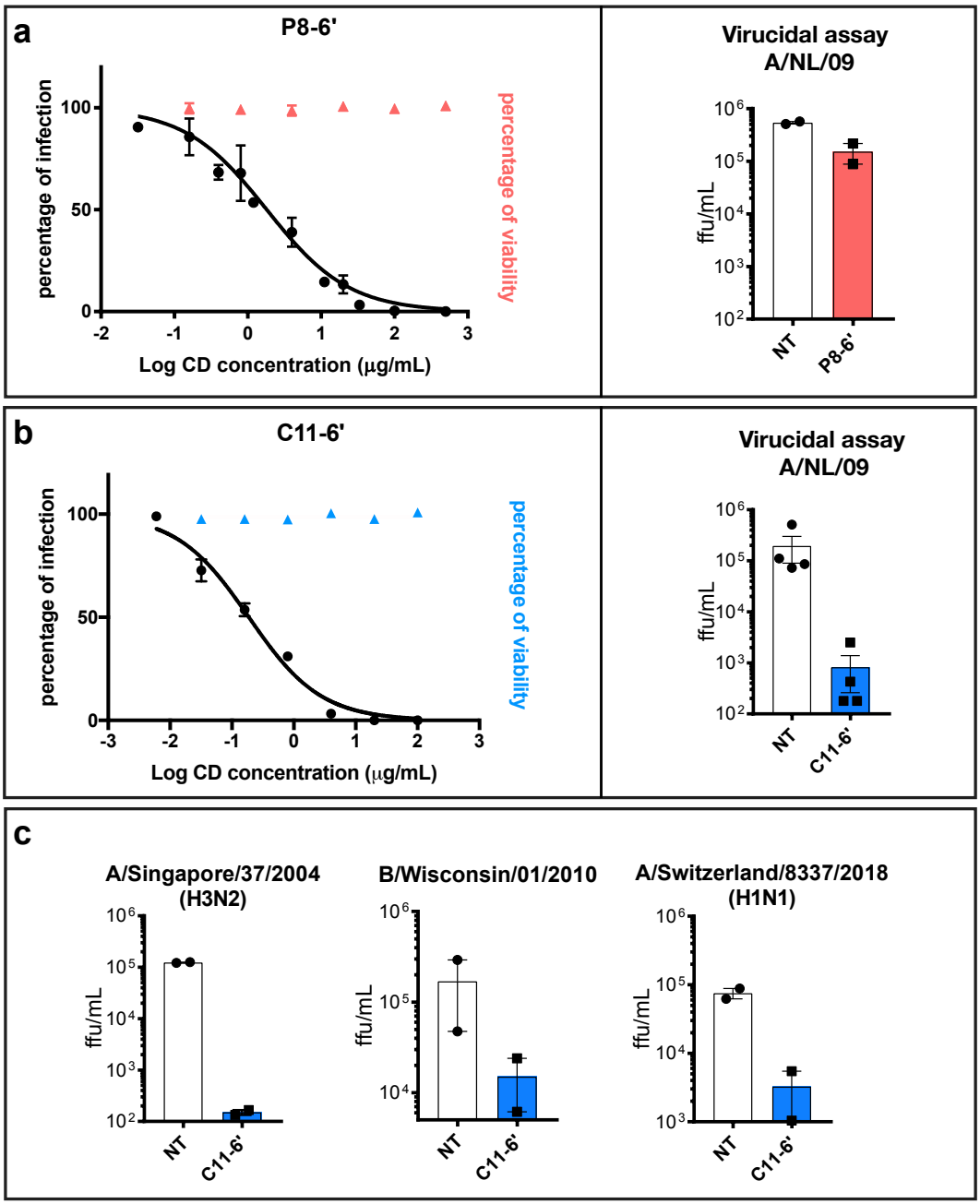

Figure 2: **Antiviral activity comparison of C11-6' and P8-6' in vitro**. Panels (**a**) and (**b**) show on the graphs on the left the inhibitory activity of each compound against A/NL/09, superimposed with the results of the cell viability assays. Both of the compounds inhibit the virus in the dose-response assay. In the virucidal assays on the right, **C11-6'** reduced the virus titer by 1000 times, whereas the infection was fully recovered in the case of **P8-6'**. Hence **C11-6'** has an irreversible inhibitory effect on the virus while the effect of **P8-6'** is reversible. Virucidal activity of **C11-6'** against other influenza strains was further investigated confirming its irreversible activity independently of the strain (**c**).

Note that in the figure's axes **ffu** stands for focus forming units and **NT** for non-treated. In (**c**) the following viral strains were tested: A/Singapore/37/2004 (H3N2), B/Wisconsin/01/2010 and A/Switzerland/8337/2018-MDCK1 (H1N1) (**c**). Results are mean and SEM of 2 independent experiments performed in duplicate.

To further compare **C11-6'** and **P8-6'**, we performed ex vivo experiments in MucilAir®, a 3D model of human airway reconstituted epithelia. These air-liquid interface cultures perfectly mimic both the pseudostratified architecture (basal, ciliated and goblet cells) and the barrier defence mechanism (i.e. the mucociliary clearance and epithelial cell immunity) of the human upper respiratory epithelium, the main site of influenza virus replication in humans. Ex vivo experiments were conducted with clinical H1N1 pandemic 09 strain (A/Switzerland/3076/16) that has not been passaged in cells to exclude any adaptation bias. **C11-6'** or **P8-6'** (50 µg/tissue) and the virus (10^4 RNA copies/tissue) were first added simultaneously on the apical surface of the tissues, without prior incubation. After four hours, the inocula were removed, the tissues were washed and the progress of the infection was monitored on a daily basis with qPCR performed on viruses isolated from the apical washes of the tissue, without any re-addition of the molecule. **C11-6'** completely prevented virus replication throughout the entire course of the experiment, while **P8-6'** slightly reduced viral replication the first two days post-infection (dpi) but not thereafter (**Figure 3**a).

Moreover, in the tissues treated with **C11-6'**, the inhibition of viral replication was also reflected by the absence of infected cells and the undisturbed morphology of the treated tissues, strikingly different from the untreated or **P8-6'**-treated tissues (Figure 3b). Immunofluorescence images and the lack of lactate dehydrogenase (LDH) release in the apical washes demonstrated that the ciliated cell layer as well as the physiological cilia beating and tissue integrity were preserved (Figure 3b and S7). In stark contrast, the untreated tissue or the **P8-6'**-treated controls, presented reduced thickness due to alteration of the ciliated cell layer, and presence of infected cells (Figure 3b). To exclude that the residual viral level detected by qPCR in the treated tissues was related to active replication, we kept the tissues in culture for 23 days but never observed an increase in viral titer over time, while the untreated tissues were persistently shedding virus as previously reported[46] (Figure S8). Importantly, ex vivo experiments were conducted also in more stringent post-treatment conditions in which **C11-6'** (30 µg/tissue) was administrated every 24 h and for 4 days, starting at 1 dpi to mimic a therapeutic administration. Also in these conditions, the compound showed a remarkable inhibitory activity,

proving its potential as a therapeutic agent (Figure 3c). In the same ex vivo model we also evaluated the biocompatibility of high doses of **C11-6'**, administered daily. **C11-6'** did neither show any cytotoxic nor pro-inflammatory activity in the above described conditions (Figure S9).

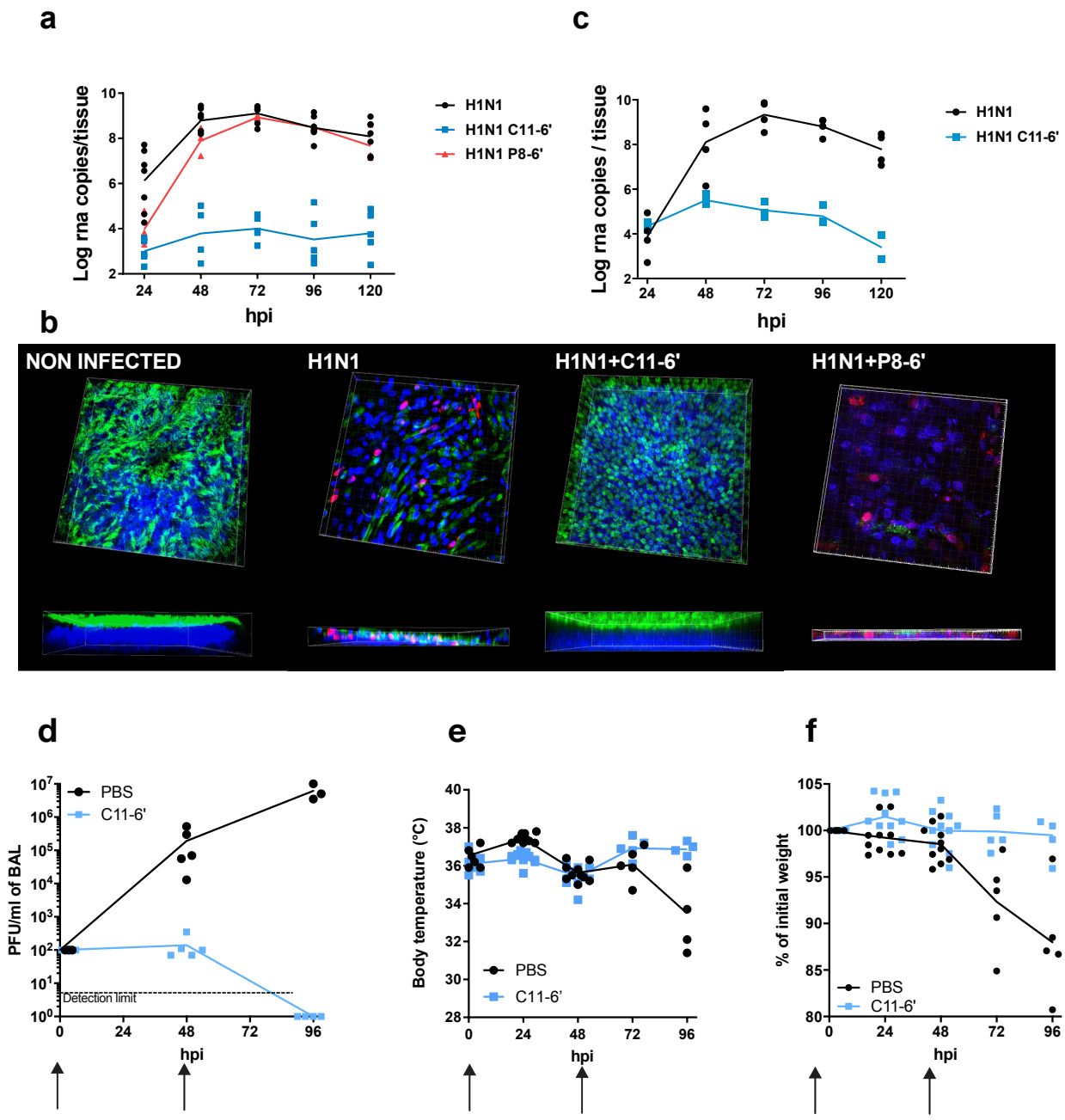

Figure 3: Ex vivo (a to c) and in vivo (d to f) inhibitory activity of C11-6'. **Ex vivo**, C11-6' provided a full protection against clinical H1N1 pandemic 09 strain in co-treatment condition, whereas P8-6' only provided a minor protection in the beginning of the infection (**a**) Immunofluorescence at 7 days post-infection (co-treatment condition) confirms the protection provided by C11-6'. Red: monoclonal antibody influenza A, blue: DAPI, green: β-IV-tubulin (marker of ciliated cells). The thickness of each tissue is shown at the bottom of the corresponding image (**b**). C11-6' also showed high efficacy in post-treatment condition (**c**). Results of (a) and (c) are mean and SEM of 2 to 4 independent experiments

with intra-experimental duplicates. Images of (b) are representative of 10 images taken for each condition. **In vivo**, mice were intra-nasally treated with PBS or C11-6' and immediately infected with A/NL/09. A second treatment was administered 2 days later. Viral loads were quantified 48- and 96-hours post-infection (**d**) and morbidity [loss of temperature (**e**) and weight (**f**)] of infected mice was monitored daily. Results are expressed as single individuals with a line connecting the means. Arrows indicate the treatment times.

Lastly, in vivo experiments were conducted with BALB/c mice administered first with **C11-6'** (25 µg/mouse) and immediately after with A/NL/09 (100 infectious particles/mouse) via the intranasal route. The body temperature and the weight of the mice were measured daily in order to estimate the impact of **C11-6'** administration on the infected-animal's physiological condition. At 2 dpi, half of the mice were randomly euthanized and the rest of the mice retreated with **C11-6'**. The second group of mice was euthanized at 4 dpi. Viral titers were quantified from broncho-alveolar lavages (BAL) (Figure 3d). Significant decrease of viral titers was observed at day 2 and 4 post-infection in treated mice (Figure 3d). The antiviral activity of the **C11-6'** also significantly diminished mice morbidity with a significant preservation of both body weight and body temperature compared to untreated mice (Figure 3e and 3f). Collectively, these results suggest a potent capacity of the **C11-6'** compound to prevent in vivo infection.

In summary, we present here a new design rule to produce effective, non-toxic, virucidal compounds against influenza virus. To dissect the relationship between the antiviral mechanism of action and the structure of our newly designed virucidal we compare, for the first time, its efficacy with that of a highly similar compound displaying a virustatic activity. We show that if the inhibitory effect is reversible, i.e. virustatic, the antiviral efficacy is lost when moving from in vitro to ex vivo. On the other hand, the virucidal counterpart of the same molecule keeps its efficacy is maintained from in vitro all the way to in vivo. Therefore, we believe that our approach to design non-toxic virucidal macromolecules has an outstanding potential for the prevention and the treatment of not only human, but also avian influenza infections.

Experimental Section

Detailed information on the experimental procedures can be found in the Supporting Information.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

Acknowledgements

The work was supported by the Leenaards Foundation (grant 4390 to L.K., C.T. and F.S.) and the Swiss National Science Foundation (Sinergia grant CRSII5_180323 to F.S. and C.T.).

Author contributions

Conceptualization, O.K., V.C., C.T. and F.S.; Methodology, O.K., V.C. and R.L.G. Investigation, O.K. and Y.Z. synthetized the cyclodextrins; O.K., V.C., J.B., J.M. and C.M. performed the in vitro experiments; V.C. performed the ex vivo experiments; L.S., B.D.C. and R.L.G. performed the in vivo experiments. Writing – Original Draft, O.K., V.C., C.T. and F.S. Writing – Review & Editing, all authors. Funding Acquisition, C.T., L.K. and F.S.; Resources, S.H. and S.C. Supervision, W.L.J.H., A.H., R.L.G., C.T., F.S.

Declaration of interests

O.K., V.C., C.T. and F.S. are inventors on patent number EP18192559.5. All the other authors declare no conflict of interests.

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Supporting Information

1.Supplemantary figures

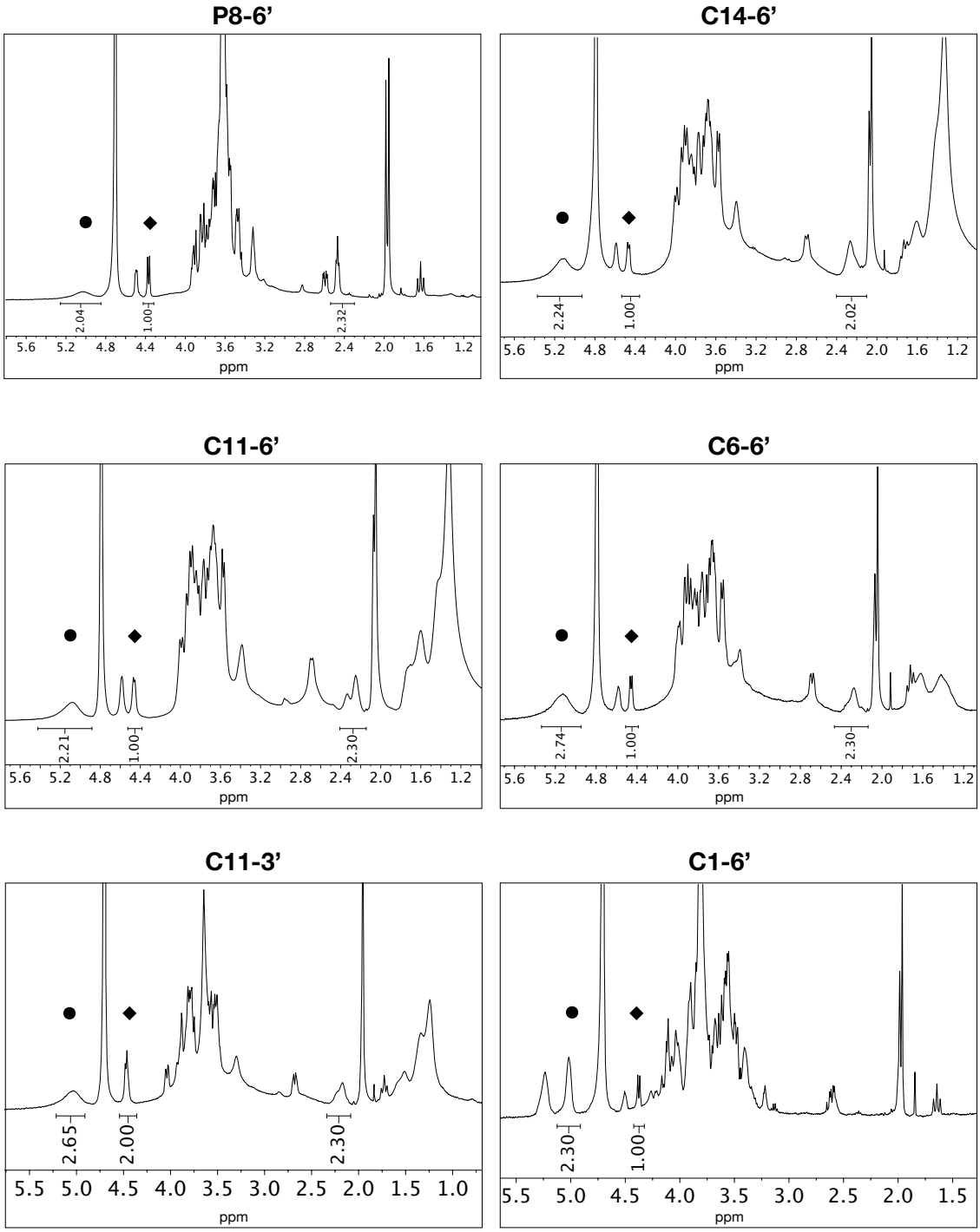

Figure S1: ^{1}H NMR studies were conducted in order to characterize the modified cyclodextrins. Average number of 6'SLN or 3'SLN per ß-cyclodextrin was calculated by comparing a distinctive peak from the trisaccharide (♦) to the one from ß-cyclodextrin (•). Both peaks correspond to 1H.

	Number of spacers/CD	Number of 6'SLN/CD	Molecular weight (kDa)
P8-6'	4	3.5	5.7
C14-6'	3.1	3	4.1
C11-6'	3.6	3.1	4.1
C6-6'	2.8	2.7	3.4
C11-3'	3	2.7	3.9
C1-6'	-	3	3.5

Figure S2: Average number of 6'SLN or 3'SLN per ß-cyclodextrin determined by ^{1}H NMR and corresponding molecular weights.

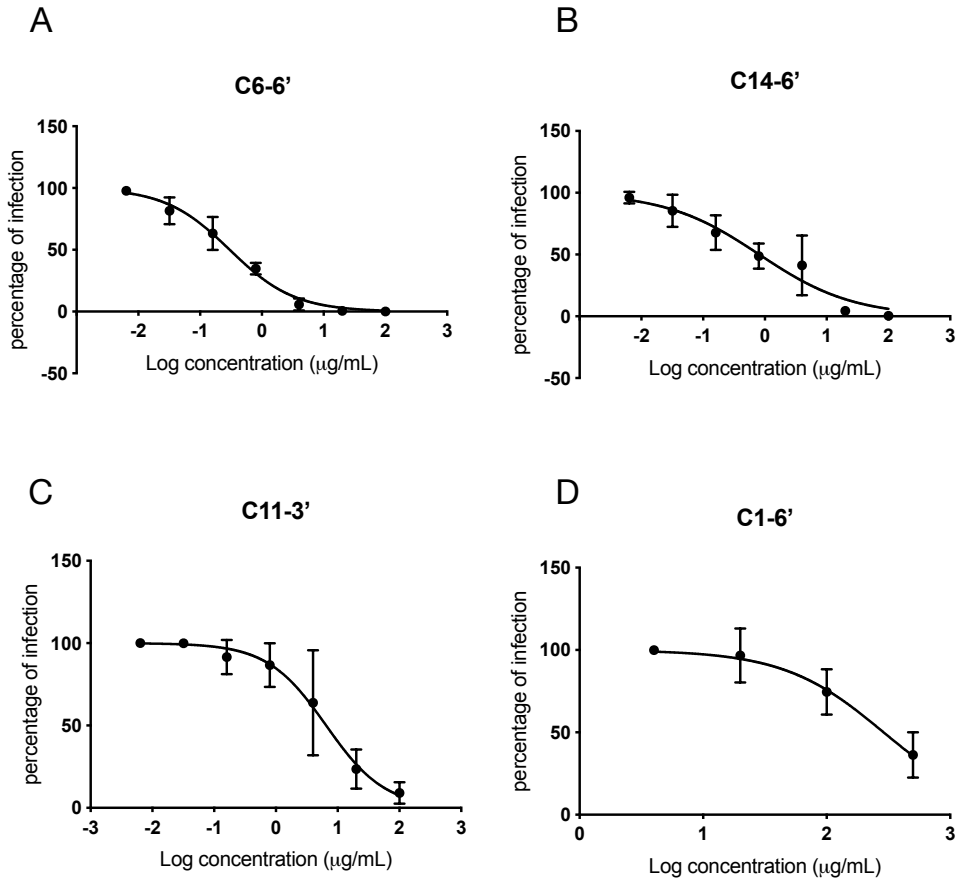

Figure S3: Dose-response curves demonstrating the antiviral activity of C6-6' (A), C14-6' (B), C11-3' (C) and C1-6' (D) against A/Netherlands/2009 H1N1.

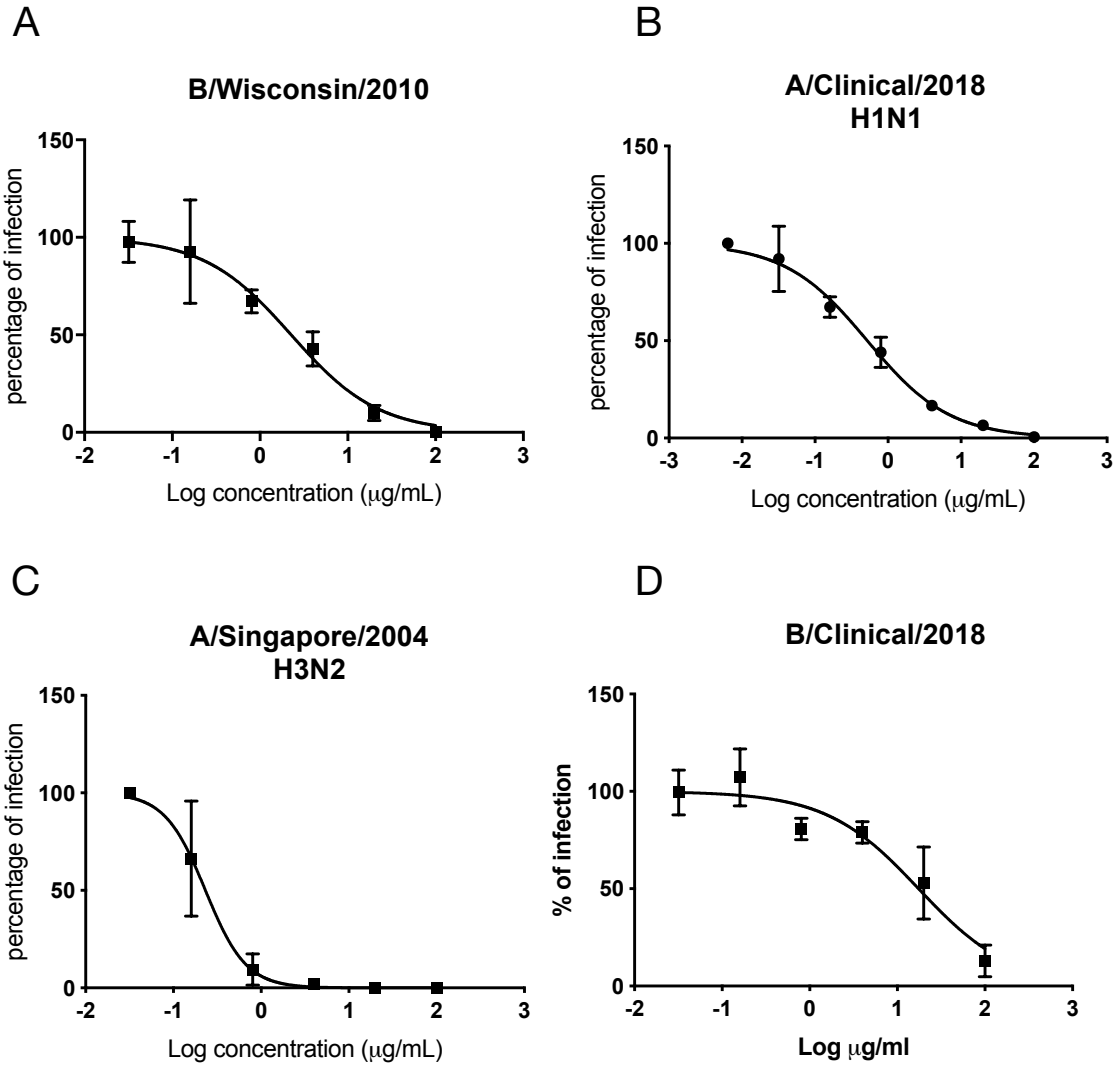

Figure S4: Dose-response curves demonstrating the antiviral activity of C11-6' against B/Wisconsin/2010 (A), A/Clinical/2018 H1N1 (B), A/Singapore/2004 H3N2 (C) and B/Clinical/2018 (D)

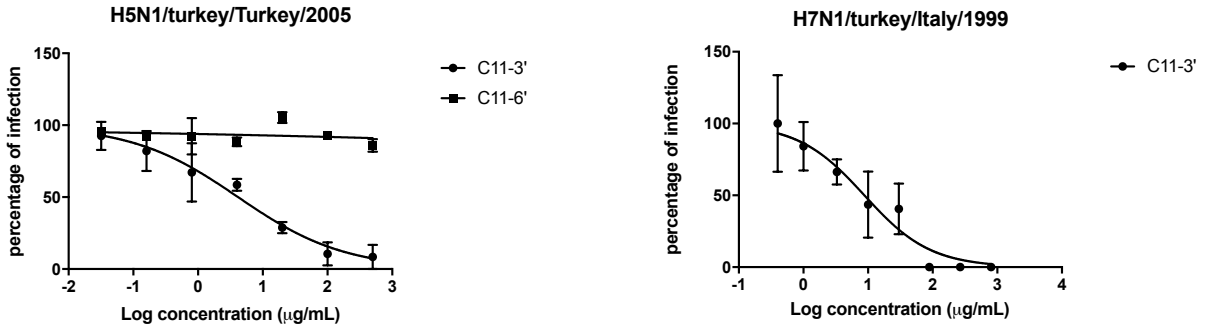

Figure S5: Dose-response curves demonstrating the antiviral activity of C11-3' against avian strains A/turkey/Turkey/2005 H5N1 (A) and A/Turkey/Italy /977/1999 H7N1 (B). A comparison experiment was also conducted with C11-6' in the case of A.

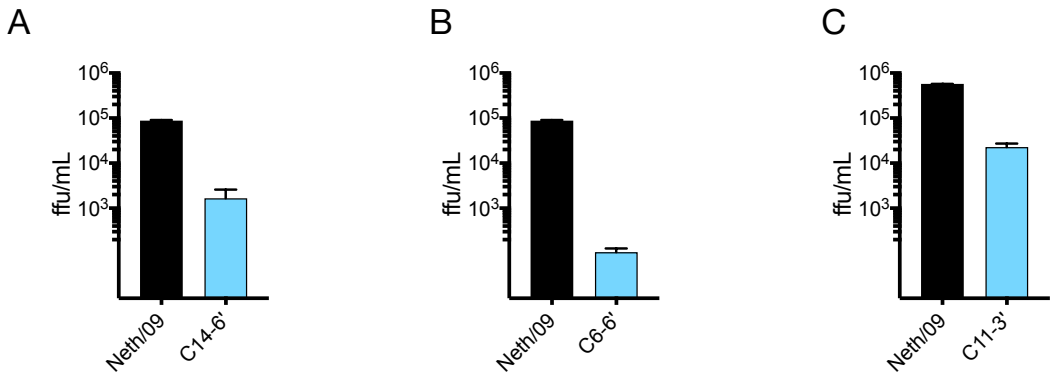

Figure S6: Virucidal activity of the C6-6' (A), C14-6' (B) and C11-3' (C) against A/Netherhands/2009 (H1N1). The experiments were performed with a compound concentration of 100 μg/mL.

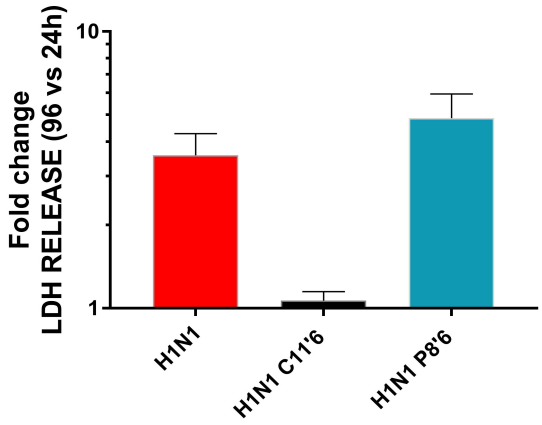

Figure S7: LDH release from infected tissues. Tissues were infected and treated with C11-6' (50 µg) or P8-6' at the time of infection. Apical washes performed at 96 and 24 hpi were subjected to LDH measurement. Results are mean and SEM of triplicates.

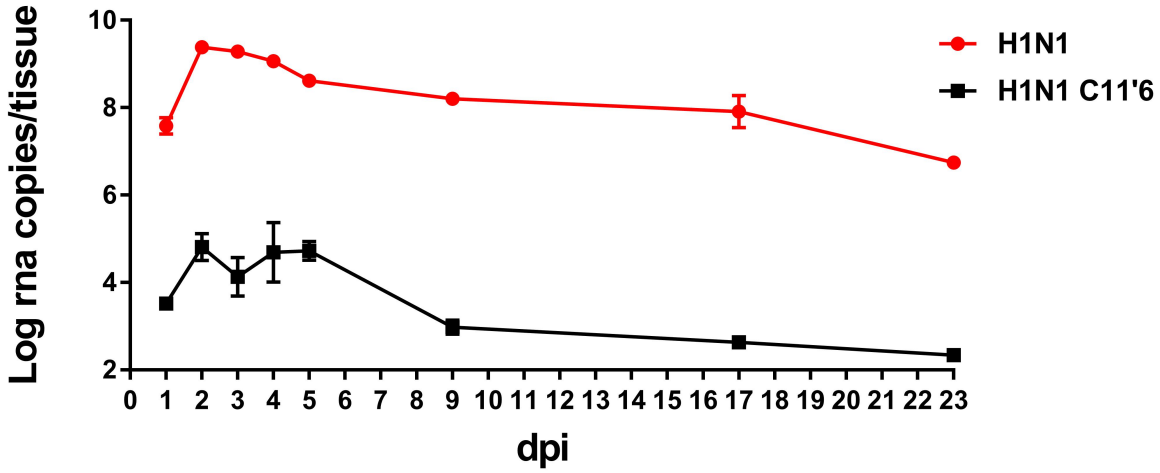

Figure S8: Long co-treatment experiment. Tissues were treated and infected with C11-6' (50 µg) at the time of infection. Daily apical washes were collected for the first 5 days, subsequently at 9, 17 and 23 days, with a wash the previous day in order to evaluate daily virus production. Results are mean and SD of a single experiment performed in duplicate.

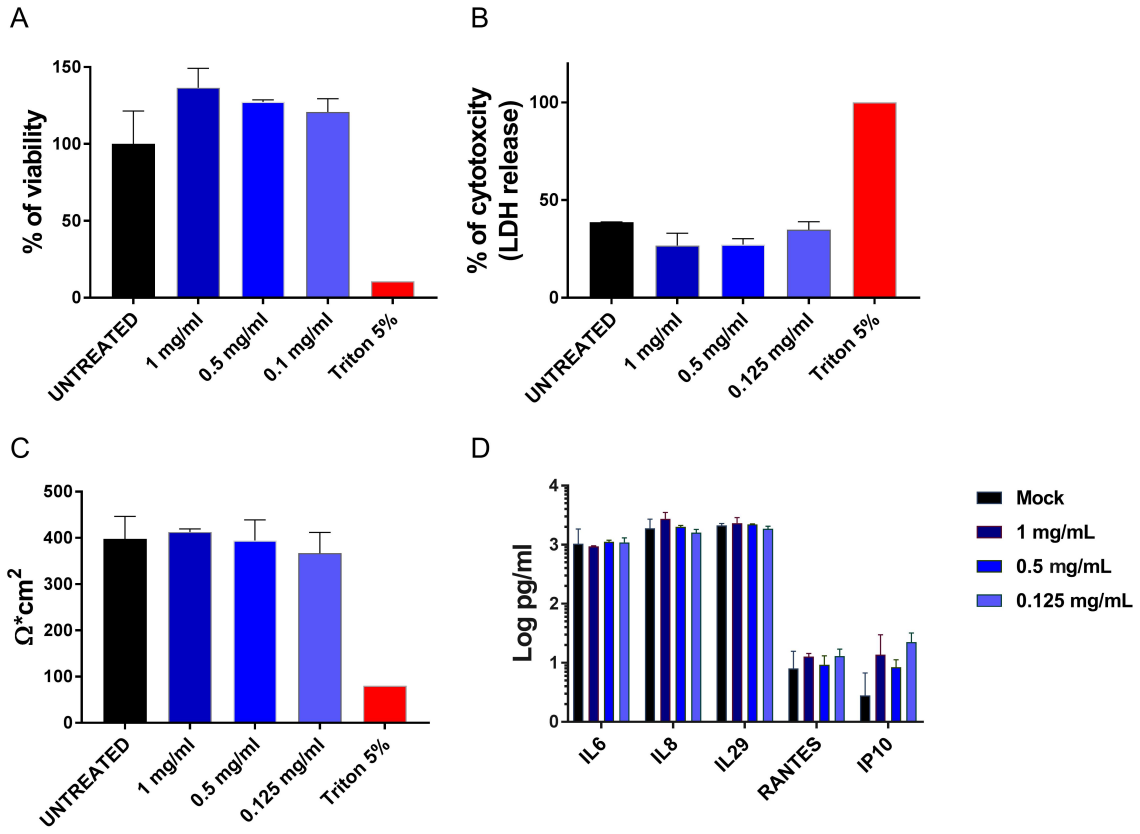

Figure S9: Ex vivo toxicity. Tissues were treated with different doses of C11'6 or equal volume of medium or triton 5% with daily addition. At 96 hours post treatment tissues were subjected to: A) MTT assay, B) LDH assay in which the viability of the tissues was evaluated, C) trans epithelial resistance evaluation and D) ELISAs assay to evaluate the release of pro-inflammatory cytokines. LDH and ELISA were performed on collected basal medium was collected and subjected to: B) LDH assay and D) The experiments are mean and SEM of 2 independent experiments performed in duplicates.

2.Methods

Synthesis of Modified Cyclodextrins

Chemicals: Neu5Acα(2,6)-Galβ(1-4)-GlcNAc-β-ethylamine and Neu5Acα(2,3)-Galβ(1-4)-GlcNAc-β-ethylamine was purchased from TCI chemicals. Heptakis-(6-deoxy-6-mercapto)-beta-Cyclodextrin and carboxymethyl-beta-cyclodextrin sodium salt was purchased from Cyclodextrin-Shop. 11-dodecenoic acid was purchased from abcr GmbH. 14-pentadecenoic acid was purchased from Larodan AB. Maleimide-PEG_8-CH_2CH_2COOH was purchased from PurePEG. All the other chemicals and solvents were purchased from Sigma-Aldrich.

Methods: Cyclodextrin derivatives to target influenza virus were synthesized in three steps. The first step was the conjugation of the spacer molecules onto the β-cyclodextrin. The second step was N-Hydroxysuccinimide (NHS) activation of the –COOH end group of the spacer molecule. The last step was 6'SLN grafting onto the spacer molecules.

Step1: Modification of β-cyclodextrin with the spacer molecules

0.04 mmol Heptakis-(6-deoxy-6-mercapto)-beta-Cyclodextrin was stirred with 0.28 mmol of bi-functional molecules bearing an allyl and a carboxylic acid end-groups (6-heptenoic acid, 11-dodecenoic acid or 14-pentadecenoic acid) in 5 mL of DMSO, in the presence of UV lamp (250 W), overnight. The resulting cyclodextrin derivative was precipitated from DCM-diethyl ether mixture by centrifugation and dried under vacuum.

To modify the β-cyclodextrin with the PEG_8 spacer, 0.04 mmol Heptakis-(6-deoxy-6-mercapto)-beta-Cyclodextrin was stirred with 0.28 mmol of Maleimide-PEG_8-CH_2CH_2COOH in the 5 mL of

phosphate buffer, pH: 6.8, overnight. The modified β-cyclodextrin was purified with dialysis and dried with lyophilization.

Step2: NHS activation reactions

0.04 mmol of cyclodextrin derivative obtained in step 1 was stirred with 1.12 mmol N-hydroxysuccinimide (NHS), 0.56 mmol -Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC-hcl) and 0.02 mmol 4-dimethylaminopyridine pyridine (DMAP) in 5 mL of DMSO, overnight. The resulting cyclodextrin was firstly precipitated from ice-cold water. Then washed with acetonitrile and diethyl ether. The product was dried under vacuum.

To obtain C1-6', step 1 was skipped and NHS activation reaction was directly conducted. Carboxymethyl-beta-Cyclodextrin sodium salt directly (0.04 mmol) was activated with 1.12 mmol NHS 0.56 mmol EDC-hcl and 0.02 mmol DMAP was added. The activation reaction was conducted overnight. Resulting cyclodextrin was first precipitated DCM-diethyl ether mixture and further washed three times. The product was dried under vacuum. 6'SLN grafting was conducted in the same way as step 3.

Step3: Trisachharide grafting

5.6 μmol Neu5Acα(2,6)-Galβ(1-4)-GlcNAc-β-ethylamine Neu5Acα(2,3)-Galβ(1-4)-GlcNAc-β-ethylamine in the case of C11-3') was mixed with 1.6 μmol of the cyclodextrin derivative obtained in the step 2. 15 μmol triethylamine (TEA) was added and the reaction was conducted overnight in 1 mL DMSO. The reaction product was diluted with 0.01 M phosphate buffer (pH: 7.5) and concentrated using amicon filters (MWCO: 3k). The resulting material was further washed with distilled water and dried with lyophilization. The grafting of the trisaccharide onto the spacer-modified cyclodextrin was confirmed with ^{1}H and DOSY NMR studies (S10 and S11).

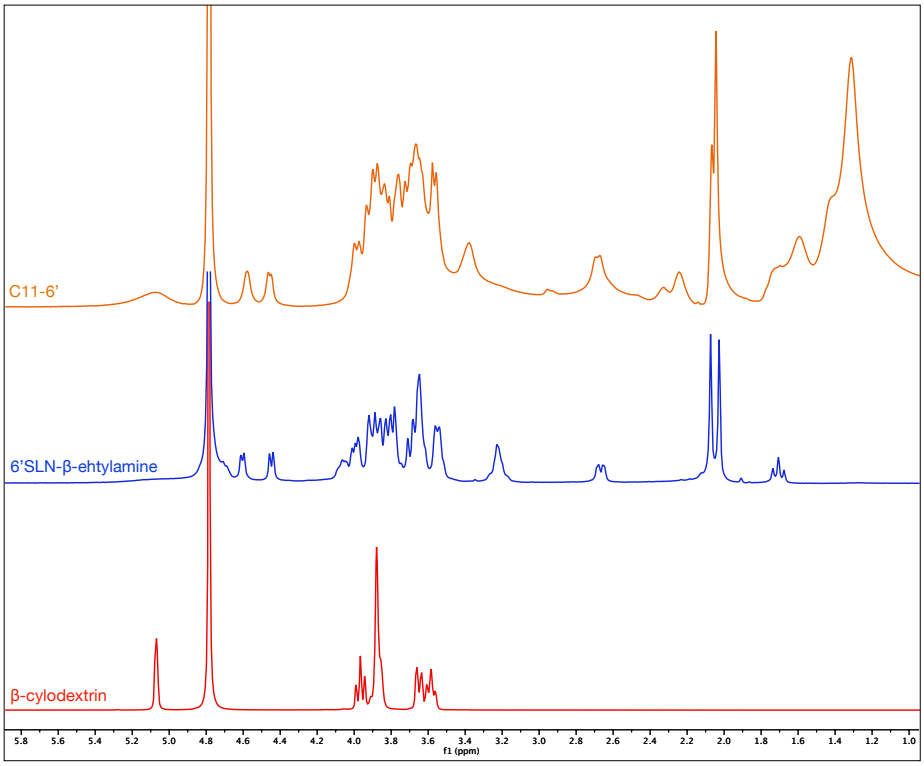

Figure S10: Stacked ^{1}H NMR spectrum of ß-clycodextrin, 6'SLN-ß-ethylamine and C11-6'.

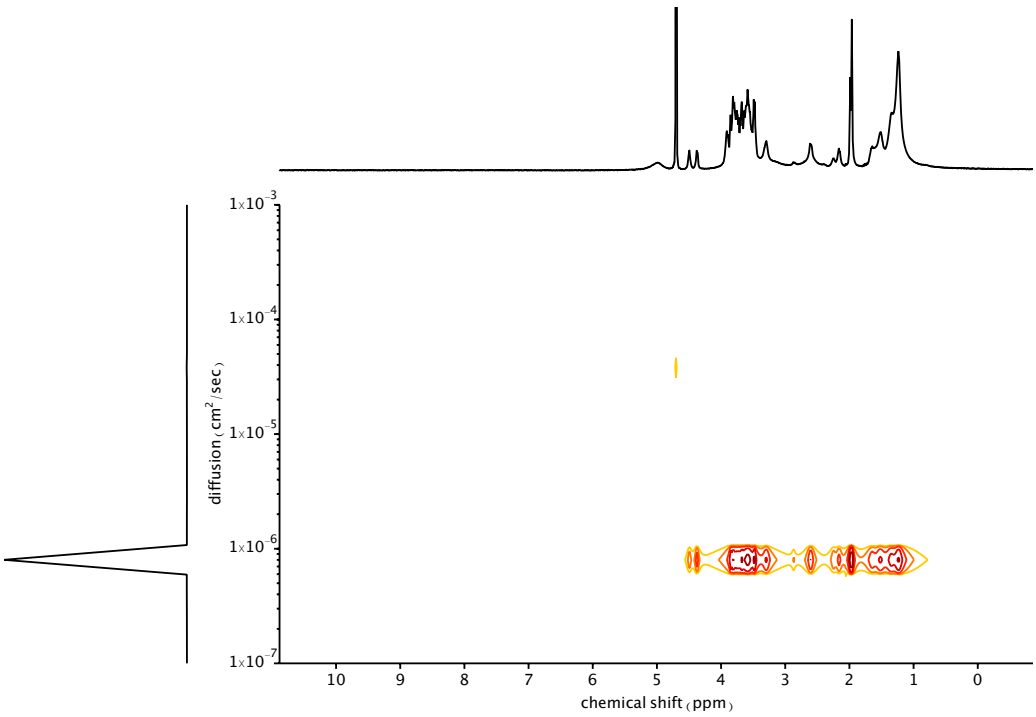

Figure S11: DOSY spectrum of the C11-6' demonstrates that the resulting compound is free from unbound trisaccharides.

Biological Assays

Materials: DMEM– Glutamax medium was purchased from Thermo Fischer Scientific. Tween 20® for washing buffer and 3,3'-diaminobenzidine (DAB) tablets were purchased from Sigma Aldrich. Primary antibody (Influenza A monoclonal antibody) was purchased from Light Diagnostics. Secondary antibody (Anti-mouse IgG, HRP-linked antibody) was purchased from Cell Signaling Technology®. The CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay that contains a tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2Htetrazolium, inner salt; MTS] and an electron coupling reagent (phenazine ethosulfate; PES) was purchased from Promega.

Cell Culture:

MDCK (Madin-Darby Canine Kidney Cells) cell line, was purchased from ATCC (American Type Culture Collection, Rockville, MD). The cells were cultured in Dulbecco's modified Eagle's medium with glucose supplement (DMEM+ GlutaMAX™) containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. MDCK cell lines was grown in humidified atmosphere with CO_2 (5%) at 37°C.

Viral Strains:

A clinical isolate of HSV-2 was originally provided by Prof. M. Pistello, (University of Pisa, Italy) and was propagated and titrated by plaque assay on Vero cells. H1N1 Neth09 and B Yamagata were a kind gift from Prof M. Schmolke (University of Geneva). Avian strain NIBRG-23 (prepared by reverse genetics using A/turkey/Turkey/1/2005 H5N1 surface proteins and A/PR/8/34 (H1N1) backbone) was obtained from National Institute for Biological Standards and Controls,

Potters Bar, United Kingdom and was grown further in 10 days old embryonated chicken eggs followed by virus purification and characterization. Clinical samples were provided from the Geneva University Hospital from anonymized patients. All influenza strains were propagated and titrated by ICC on MDCK cells in presence of TPCK-treated trypsin (0.2 mg/ml)

Cell viability assay

Cell viability was measured by the MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] assay. Confluent cell cultures seeded in 96-well plates were incubated with different concentrations of nanoparticles or ligand in triplicate under the same experimental conditions described for the antiviral assays. Cell viability was determined by the CellTiter 96 Proliferation Assay Kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. Absorbance was measured using a Microplate Reader (Model 680, BIORAD) at 490 nm. The effect on cell viability at different concentrations of nanoparticles or cyclodextrins was expressed as a percentage, by comparing the absorbance of treated cells with the one of cells incubated with culture medium alone. The 50 % cytotoxic concentrations (CC_{50}) and 95 % confidence intervals (CIs) were determined using Prism software (Graph-Pad Software, San Diego, CA).

Inhibition assays

MDCK cells were pre-plated 24 h in advance in 96-well plates. Increasing concentrations of materials were incubated with the influenza virus (MOI: 0.02 or 0.01 for H5N1 and 0.1 for other viruses) at 37 °C for one hour and then the mixtures were added to cells. Following the virus adsorption (1 h at 37 °C), the virus inoculum was removed, the cells were washed and the fresh medium was added. After 24 h of incubation at 37°C, the infection was analyzed with immunocytochemical (ICC) assay. The cells were fixed and permeabilized with methanol. Then the primary antibody (1:100 dilution) was added and incubated for 1 hour at 37°C. The cells were washed with wash buffer (DPBS + Tween

0.05%) three times; then secondary antibody (1:750 dilution) was added. After 1 hour the cells were washed and the DAB solution was added. Infected cells were counted and percentages of infection were calculated comparing the number of infected cells in treated and untreated conditions.

In the case of H5N1, flow cytometry-based inhibition assays were conducted additionally. MDCK cells were pre-plated 24 h advanced in a 24- well plate (75,000 cells/well). Increasing concentrations of materials were incubated with the influenza virus (MOI: 0.04) at 37 °C for one hour and then the mixtures were added to cells. Following the virus adsorption (1 h at 37 °C), the virus inoculum was removed, the cells were washed and the fresh medium was added. After 5 h of incubation at 37°C, the infection was analyzed with flow cytometry. Briefly cells were trypsinized and fixed using IC fixation buffer (Thermo Fisher Scientific, Netherlands) for 15 minutes at room temperature followed by permeabilization using 1X IC permeabilization buffer (Thermo Fisher Scientific, Netherlands) for 15 minutes at 4°C. Cells were stained with Anti-Influenza A Virus Nucleoprotein mouse monoclonal antibody [D67J] (FITC) (Abcam ab210526, The Netherlands) for 30 minutes at 4°C. Antibody dilution was 1:80 in permeabilization buffer and 50 µl per tube was added. FACS analysis was carried out using FACS Calibur 3 software. The concentration producing 50 % reduction in the number of infected cells (effective concentration (EC_{50})) was determined using the Prism software.

H7N1 infectivity was evaluated through Luciferase activity. MDCK cells were seeded at $5x10^4$ on 96-wells plates. After 24h, the medium was replaced by serum-free medium. Increasing concentrations of C11-3' were incubated with 100 pfu of H7N1 A/Turkey/Italy/977/1999 encoding the NanoLuciferase. The mixture was incubated 1h at 37°C before to be added to the cells for another 1h incubation at 37°C (100 µL per well). Cells were washed and medium replaced by serum-free medium with 1 µg/mL TPCK-Trypsin. Twenty-four hours post-infection, cells were washed and then lysed with 40 µl per well of Nano Glo® Luciferase Assay Buffer (Promega) diluted 1/2 in PBS. Luciferase activity was measured in the cell lysates using a Tecan Infinite M200PRO plate reader: 15

µl of Nano Glo® Luciferase Assay Substrate (Promega) diluted 1/5000 in PBS were added to 15 µl of lysate for each well.

Virucidal Assays

Viruses (focus forming unit (ffu):10^5/mL) and the materials (EC_{99} concentration, figure S12) were incubated for 1 hour at 37°C. Serial dilutions of the virus-material complex together with the non-treated control were conducted and transferred onto the cells. After 1 hour, the mixture was removed and the fresh medium was added. Next day, viral titers were evaluated with ICC assay. For the ICC assay, the same procedure described above was followed.

Figure S12: The material concentrations at which the in-vitro virucidal assays were performed.

	Material	Concentration (µg/mL)
A/Netherlands/2009 H1N1	C6-6'	100
	C11-6'	100
	C14-6'	100
	P8-6'	500
	C11-3'	500
	NPs C15-6'SLN	100
	NPs PEG4-6'SLN	500
A/Singapore/2004 H3N2	C11-6'	100
B/Wisconsin/2010	C11-6'	200

Data analysis

All results are presented as the mean values from three independent experiments performed in duplicate. The EC_{50} values for inhibition curves were calculated by regression analysis using the

program GraphPad Prism version 5.0 (GraphPad Software, San Diego, California, U.S.A.) to fit a variable slope-sigmoidal dose–response curve.

Ex vivo

Co-treatment: Human airway epithelia reconstructed *in vitro,* MucilAir tissues, (Epithelix Sàrl, Geneva, Switzerland) were cultured at the air-liquid interface from a mixture of nasal polyp epithelial cells originating from healthy donors. Influenza H1N1 pdm 2009 clinical strain (1e4 rna copies/tissue) and C11-6' (50 µg/tissue) were transferred onto tissues without pre-incubation, together with non-treated control. After 4 hours of incubation time, the tissues were washed twice. On a daily basis, the basal medium was changed. To conduct daily qPCR measurements, 200 uL of medium was added onto tissues and then collected 20 minutes later. RNA extracted with EZNA viral extraction kit (Omega Biotek) was quantified by using qPCR with the QuantiTect kit (#204443; Qiagen, Hilden, Germany) in a StepOne ABI Thermocycler.

Post treatment: MucilAir tissues were infected with H1N1 pdm 2009 (1e4 copies/tissue) after 4 h the inoculum was removed and tissues were washed. After 20 h an apical wash was done for 20' and subsequently C11-6' (30 µg/tissue), or an equal volume of medium in the untreated tissues, were added apically. Everyday after the 20' apical wash new addition of C11-6' was performed. RNA was then extracted and qPCR done as described above. A similar procedure in the absence of virus was conducted for toxicity studies.

Immunofluorescence

Influenza infected cells were detected by direct with Influenza A antibody (Light Diagnostic) and beta tubulin primary rabbit antibody (Abcam) was used as a marker of ciliated cells. The Alexa 488-goat anti-rabbit Ab and the Alexa 594-goat anti-mouse Ab (Life technologies) were used as secondary Ab and nuclei were stained with DAPI. Images were acquired with Zeiss LSM 700 Meta confocal microscope and processed by Imaris.

Lactate dehydrogenase assay (LDH)

LDH release in basal medium was measured with the Cytotoxicity Detection Kit (Roche 04744926001).

MTT assay

MTT solution was diluted in MucilAir medium (1 mg/ml) and 300 µl were added basally in a 24 well plate. After 4 hours incubation at 37°C the tissues were transferred in a new plate and lysed with 1 ml of DMSO. The supernatant was read at 570 nm. Percentages of viability were calculated comparing treated and untreated tissues.

ELISA

Interleukin-6 (IL-6), CXC motif chemokine 10 (CXCL10 or IP-10), CC motif chemokine5 (CCL5 or RANTES), interleukin-8 (IL-8 or CXCL-8) and interferon lambda (IL-29 /IL-28B) were measured in the basal medium by ELISA (R&D DY206-05, DY266-05, DY278-05, DY208-05 and DY1598B-05) following everyday treatment with different concentrations of CD.

In vivo

Four groups of five BALB/c mice were treated at day 0 with 50 µl of PBS or C11-6' (25 µg in 50 µl) and immediately inoculated with A/NL/09 (10^2 ffu). On a daily basis, body temperature and weight of the mice were measured. Two days post-infection, 2 groups of mice were killed (one group pbs and the other C11-6' treated). Lung homogenate and nasal mucosa and bronchoalveolar lavage were collected to quantify the viral titer through qPCR measurements. The C11-6' treated group was retreated with the same amount of the compound. Two days after, all the remaining mice were killed, lung homogenate and nasal mucosa were collected. After the tissue disruption, the RNA was extracted

with Trizol and quantified by using qPCR while BAL were subjected to plaque assay. Two independent experiments were performed.

Ethics statement

This study was carried out in accordance with INRA guidelines in compliance with European animal welfare regulation. The protocols were approved by the Animal Care and Use Committee at "Centre de Recherche de Jouy-en-Josas" (COMETHEA) under relevant institutional authorization ("Ministère de l'éducation nationale, de l'enseignement supérieur et de la recherche"), authorization number: 2015100910396112v1 (APAFIS#1487). All experimental procedures were performed in a Biosafety level 2 facility.