Titel: Reverse design of an influenza neutralizing spiky nano-inhibitor with a dual mode of action

Autoren: Chuanxiong Nie, Badri Parshad, Sumati Bhatia, Chong Cheng, Marlena Stadtmüller, Alexander Oehrl, Yannic Kerkhoff, Thorsten Wolff, and Rainer Haag

Dieser Beitrag wurde nach Begutachtung und Überarbeitung sofort als "akzeptierter Artikel" (Accepted Article; AA) publiziert und kann unter Angabe der unten stehenden Digitalobjekt-Identifizierungsnummer (DOI) zitiert werden. Die deutsche Übersetzung wird gemeinsam mit der endgültigen englischen Fassung erscheinen. Die endgültige englische Fassung (Version of Record) wird ehestmöglich nach dem Redigieren und einem Korrekturgang als Early-View-Beitrag erscheinen und kann sich naturgemäß von der AA-Fassung unterscheiden. Leser sollten daher die endgültige Fassung, sobald sie veröffentlicht ist, verwenden. Für die AA-Fassung trägt der Autor die alleinige Verantwortung.

Zitierweise: Angew. Chem. Int. Ed. 10.1002/anie.202004832

Link zur VoR: https://doi.org/10.1002/anie.202004832
Topography-matching design of an influenza neutralizing spiky nano-inhibitor with a dual mode of action

Chuanxiong Nie, a, b,* Badri Parshad, c Sumati Bhatia, a Chong Cheng, d Marlena Stadtmüller, b Alexander Oehrl, a Yannic Kerkhoff, a Thorsten Wolff, b,* Rainer Haag a,*

Abstract: In this study, we demonstrate the concept of ‘reverse design’ for virus inhibitors. With the current knowledge of influenza A virus (IAV), we designed a nano-inhibitor that has a matched nanotopology to IAV virion and heteromultivalent inhibitory effects on hemagglutinin and neuraminidase. The synthesized nano-inhibitor can neutralize the viral particle extracellularly and block its attachment and entry to the host cells. The virus replication was significantly reduced by 6 orders of magnitude in the presence of the reverse designed nano-inhibitors. Even being used 24 hours after the infection, >99.999% inhibition is also achieved, which indicates such a nano-inhibitor might be a potent antiviral for the treatment of influenza infection.

Besides the recent SARS-CoV-2 pandemic, outbreaks of seasonal or pandemic influenza A virus (IAV) have also been challenged public health due to the high mutation rates of viral glycoprotein genes and the potential for human infection by animal strains. Therefore, a robust virus-neutralizing therapeutic is needed for the lack of universal influenza vaccines. IAV is an enveloped RNA virus, whose membrane anchors two viral proteins that regulate interactions of the virion with the host cells, hemagglutinin (HA) and neuraminidase (NA). For the infection, IAV uses HA to bind to sialic acid on the host cell membrane. After completion of the replication cycle, the viral NA cleaves sialic acid from surface receptors to allow virion release, so called ‘budding’. Recent evidence also demonstrate that NA helps IAV penetration in the mucus by cleaving HA decay receptors, which reveals the crucial role for a balanced HA-NA interplay for the binding behavior. Multivalent sialylated nanostructures are developed to inhibit HA and block viral entry to host cells. However, a heteromultivalent inhibitor engaging both HA and NA is rarely reported.

On the other hand, from a topological viewpoint, the virion of IAV is nano-sized particle around 100 nm with a spiky surface generated by the HA and NA. For an inhibitor, especially nanoparticle-based inhibitors (so-called “nano-inhibitors”), the match of the size and topology to the virion is also essential in order to achieve robust binding to compete with the virus/cell interaction. For this purpose, flexible nanomaterials are favored to afford the viral binding ligands, but they also face the problem of overcoming the internal stress of the scaffold nanomaterials. In our new approach, we use a rigid nanoparticle with a matching nanotopology to the viral particle which binds stronger than the flexible nanomaterials.

The aim of this study is to develop a nano-inhibitor with the principle of topography-matching design. Not only with dual inhibitory effects on HA and NA, the inhibitor should also exhibit a matched nanotopology to IAV virion, which is expected to increase the contact area and enhance the binding. To achieve that, a virus-like nanoparticle (VLNP) with nano-spikes was firstly synthesized according to an earlier report. Except the difference of the morphology, the VLNP was the same as its smooth control nanoparticle for size and density (Figure S2, Supporting Information). Then, the nano-inhibitor was synthesized via the functionalization of VLNP with linear polyglycerol-sialyllactose (PG-SAL) and LPG-zanamivir (LPG-Zan) via copper-free SPAAC click reactions as shown in Figure 1a. Despite the different subtypes of HA, human IAV strains tend to bind the 6-sialyllactose for the entry into host cells. In this study, 6-sialyllactose was conjugated onto the nanoparticles via LPG linker in a multivalent manner as shown in Figure 1b, as the polyglycerol based multivalent structures has been proved to be efficient to block virus infection in our former studies. In order to inhibit NA, an approved NA inhibitor, zanamivir, was also conjugated to the VLNP similarly in a multivalent manner. The resulted heteromultivalent structure is expected to bind IAV virion strongly as shown in Figure 1c.
studied by XPS analysis, as shown in Figure 1e-f. From the XPS N1s scan, the emerge of the N1s single for VLNP-SAL/Zan indicated that the LPG-SAL and LPG-Zan have been conjugated to the VLNP. For N1s scan three characteristic peaks were detected as shown in Figure 1e. Overall, the above results of HR-TEM and XPS revealed that the VLNP has been successfully functionalized with LPG-SAL and LPG-Zan.

The binding with the virus was studied by a centrifuge-western blot method as shown in Figure 2a-b and Figure S4, Supporting Information, whereas the band intensity of the viral nucleoprotein (viral NP) revealed the amount of virus binding to the nanoparticles. For the LPG functionalized smooth nanoparticles (Figure S4, Supporting Information), no binding was detected. But VLNP-LPG showed a weak binding with the virus, which should be due to the spiky nanostructures on the surface. All the SAL nanoparticles showed robust binding to influenza virus and VLNP-SAL/Zan was better than VLNP-SAL. This should be attributed to the additional interactions between Zan and NA, which not only added extra binding sites to the nanoparticles, but also enhanced the SAL-HA interaction.\(^{[13]}\) It was also clear that the nanospikes on the surface have a positive effect on the viral binding. Cryo-TEM image was acquired to show the effect of the nanospikes on the viral binding as shown in Figure 2c. From the images, it was noticed that the surface proteins can insert into the gaps of the nanospikes, which increased the contact area to benefit the binding. Besides, aggregates of the VLNP-SAL/Zan with the virus particles were also noticed.

The NA inhibition was studied by MU-NANA assay (Figure 2d). The nanoparticles with zanamivir all showed inhibition to NA and the IC\(_{50}\) values for the VLNP-SAL/Zan, NP-SAL/Zan and VLNP-Zan are 5.38 ± 1.37, 40.34 ± 10.82, 20.54 ± 1.31 \(\mu\)g/mL, respectively. By comparing the results of VLNP-SAL/Zan and NP-SAL/Zan, an enhancement by the spiky nanostructures was also noticed with a factor of 8, which can be attributed to the increased virus binding by the spiky nanostructures.

We then investigated if the binding of IAV virions to the MDCK II cells can be blocked by the nano-inhibitors, to prove the acting mechanism as binding decoys (Figure 3a-c). In this test, the virions of A/X31 (H3N2) were labelled with octadecyl rhodamine B chloride (R18) and incubated with the nano-inhibitors for 45 minutes.\(^{[14]}\) After being incubated for 45 minutes with the virus/inhibitor mixture, the cells were washed with PBS to remove free viruses, and then fixed for fluorescent microscopy (Figure 3a) or harvested for flow cytometry (Figure 3b). The results clearly showed that the number of virions binding to the cells was reduced by the nano-inhibitors with SAL functionalization. For VLNP-SAL/Zan, there was nearly no signal for virus being detected by CLSM, and the fluorescent intensity profile for the cells was identical to the control sample as shown in flow-cytometry, which proved our hypothesis that the spiky nano-inhibitors can block virus binding to the host cells effectively.

With the proof of binding decoy, we then investigated if the nano-inhibitors could inhibit influenza virus infection at the entry step. Immune-fluorescent staining of viral NP for the infected cells was carried out. The total cells were marked by DAPI while the infected cells were marked by viral NP as shown in Figure 4a. In control cultures, 42.2 ± 6.6% of the cells expressed viral antigen. The pre-treatment by the nano-inhibitors clearly reduced the infection. Without SAL or Zan, there was no significant inhibition of viral infection, which excluded the effects of underivatized LPG for the viral inhibition (Figure S7, Supporting Information). For the cells treated with VLNP-SAL-Zan and VLNP-Zan, the infections were 6.2 ± 2.8% and 22.5 ± 2.9%, respectively. Little-to-no infected cells (0.1 ± 0.1%) were detected upon treatment with VLNP-SAL/Zan, corresponding to an inhibition ratio higher than 99.9%.
The potent inhibitory effects indicated that the viruses might be neutralized by the nano-inhibitors in the medium. For verification, plaque reduction assays were carried out (Figure 4c-e). The dose-dependent plaque reduction (PR) curves were obtained by varying the nano-inhibitor concentrations (Figure 4d) and the IC50 values were then estimated (Figure 4e). VLNP-SAL/Zan showed the best plaque reduction performance, for which the IC50 is 1.33 ± 0.14 µg/mL. The tendencies for VLNP-SAL and NP-SAL/Zan were similar to it for VLNP-SAL/Zan, but with higher IC50 values. For VLNP-Zan, only a slight reduction of the viral activity was noticed. This also indicated for an efficient neutralizing of the virus, heteromultivalent engagement with both HA and NA was essential.

A potent influenza inhibitor should show robust inhibitory activity even being used after the infection. Herein, we evaluated the inhibition of post-infection viral replication by using a multicyclic viral replication assay for the nano-inhibitors. In this assay, the MDCK II cells were infected by the virus at the MOI of 0.01, and then cultured in the medium supplied with the nano-inhibitors for 24 hours. Afterwards, the active virus in the medium was titrated by plaque assay to investigate the viral replication. We firstly investigated the inhibitory effects on A/X31 (H3N2) for the inhibitors at different concentrations from 50 to 1000 µg/mL as shown in Figure 4a. A potent inhibition with 6 orders of magnitudes reduction, corresponding to 99.9999% inhibition was achieved by VLNP-SAL/Zan at the cellular nontoxic dose, which support a therapeutic window for the inhibitor. With only 50 µg/mL VLNP-SAL, 5 orders of magnitudes reduction were also achieved. NP-SAL/Zan showed potent inhibition at high dose, at 50 µg/mL, there were only 2.5 orders of magnitudes reduction, supporting the idea that the spiky nanostructures could enhance the viral binding and inhibition. Similar inhibitory effects were obtained for A/PR/8/34 (H1N1) and A/Panama/2007/1999 (H3N2) infection (Figure 4b), two other typical human IAV strains, which indicated that the VLNP-SAL/Zan might be broadly active in human IAV strains.

We also evaluated the inhibition of the viral replication at late stage of infection with high viral load (Figure S9, Supporting Information). In this case, the nano-inhibitors were used 24 hours post infection. Compared with the experimental settings for Figure 4a-b, there were 2.5-fold more replication cycles with approx. 10^6 PFU/mL virions in the medium before using the inhibitors. Reduction of viral titres was also achieved by VLNP-SAL/Zan, for which, 5 orders of magnitudes reduction were achieved, which was slightly lower but in the same level as its performance at the early stage inhibition. However, in this case, VLNP-SAL only showed a moderate inhibition of viral replication with 2 orders of magnitudes reduction.

In conclusion, in this study, we demonstrated our concept of ‘topology-matching design’ of an IAV inhibitor that has a matching surface topology towards the IAV particle and dual inhibitory effects towards the two key proteins for the viral binding. The synthesized nano-inhibitor is able to neutralize IAV virions extracellularly and block their binding to the host cells. As a result, robust inhibition (>99.9999%, 6 orders of reduction) of viral replication is achieved. We also envision that the idea of topology-matching design can be forwarded to the inhibition of other viruses, especially the ones with a spiky morphology such as coronaviruses.

Acknowledgements
The authors gratefully acknowledge financial support from Deutsche Forschungsgemeinschaft (DFG) through grants within the Collaborative Research Center (SFB) 765. C. N. acknowledges the support from China Scholarship Council (CSC). We would like to acknowledge the assistance of the Core Facility BioSupraMol supported by the DFG.

Keywords: topology-matching design • influenza inhibition • heteromultivalent inhibitor • virus binding decoy

References

[1] a) D. Gatherer, J. Clin. Virol. 2009, 45, 174-178; b) G. J. D. Smith, D. Vijaykrishna, J. Bahl, S. J. Lycett, M. Worobey, O. G. Pybus, S. K. Ma, C. L. Cheung, J. Raghwani, S. Bhatt, J. S. M. Peiris, Y. Guan, A. Rambaut, Nature 2009, 459, 1122-1125; c) V. N. Petrova, C. A. Russell, Nat. Rev. Microbiol. 2018, 16, 47-60.

[2] a) M. Kanekiyo, M. G. Joyce, R. A. Gillespie, J. R. Gallagher, S. F. Andrews, H. M. Yassine, A. K. Wheatley, B. E. Fisher, D. R. Ambrozak, A. Creanga, K. Leung, E. S. Yang, S. Boyoglu-Barnum, I. S. Georgiev, Y. Tsybovsky, M. S. Prabhakaran, H. Andersen, W.-P. Kong, U. Baxa, K. L. Zephr, J. E. Ledgerwood, R. A. Koup, P. D. Kwong, A. K. Harris, A. B. McDermott, J. R. Mascola, B. S. Graham, Nat. Immunol. 2019, 20, 362-372; b) J. Wang, P. Li, Y. Yu, Y. Fu, H. Jiang, M. Lu, Z. Sun, S. Jiang, L. Lu, M. X. Wu, Science 2020, 367, eaau0810; c) C. I. Paules, S. G. Sullivan, K. Subbarao, A. S. Fauci, N. Engl. J. Med. 2017, 376, 7-8.

[3] a) R. E. Amaro, P. U. Leong, G. Huber, A. Dommer, A. C. Steven, R. M. Bush, J. D. Durrant, L. W. Votapka, ACS Cent. Sci. 2018, 4, 1570-1577; b) H.-L. Yen, C.-H. Liang, C.-Y. Wu, H. L. Forrest, A. Ferguson, K.-T. Choy, J. Jones, D.-Y.-D. Yang, P. P.-H. Cheung, C.-H. Hsu, O. T. Li, K. M. Yuen, R. W. Y. Chan, L. L. M. Poon, M. C. W. Chan, J. M. Nicholls, S. Krauss, C.-H. Wong, Y. Guan, R. G. Webster, R. J. Webby, M. Peiris, Proc. Nat. Acad. Sci. 2011, 108, 14264-14269.

[4] F. Krammer, Nat. Rev. Immunol. 2019, 19, 383-397.

[5] a) M. D. Vahey, D. A. Fletcher, eLife 2019, 8, e43764; b) P. H. Hamming, N. J. Overeem, J. Huskens, Chem. Sci. 2020, 11, 27-36; c) R. Wagner, T. Wolff, A. Henwig, S. Pleschka, H.-D. Klent, Journal of Virology 2000, 74, 6316-6323.

[6] a) S.-J. Kwon, D. H. Na, J. H. Kwak, M. Douaisi, F. Zhang, E. J. Park, J.-H. Park, H. Youn, C.-S. Song, R. S. Kang, J. S. Dordick, K. B. Lee, R. J. Linhardt, Nat. Nanotechnol. 2017, 12, 48-54; b) S. Tang, W. B. Puryear, B. M. Seifried, X. Dong, J. A. Runstadler, K. Ribbeck, B. D. Olsen, ACS Macro Lett. 2016, 5, 413-418; c) S. Bhatia, D. Lauster, M. Bardua, K. Ludwig, S. Angioletti-Uberti, N. Popp, U. Hoffmann, F. Paulus, M. Budi, M. Stadtmüller, T. Wolff, A. Hamann, C. Böttcher, A. Herrmann, R. Haag, Biomaterials 2017, 138, 22-34; d) D. Lauster, M. Glanz, M. Bardua, K. Ludwig, M. Hellmund, U. Hoffmann, A. Hamann, C. Böttcher, R. Haag, C. P. R. Hackenberger, A. Herrmann, Angew. Chem. Int. Ed. 2017, 56, 5931-5936; e) M. A. Sparks, K. W. Williams, G. M. Whitesides, J. Med. Chem. 1993, 36, 778-783; f) D. Lauster, S. Klein, K. Ludwig, S. Nojoumi, S. Behren, L. Adami, M. Stadtmüller, S. Saenger, S. Zimmer, K. Hünzke, L. Yao, U. Hoffmann, M. Bardua, A. Hamann, M. Witzenthurn, L. E. Sander, T. Wolff, A. C. Hocke, S. Hippenstein, S. De Carlo, J. Neudecker, K. Osterrieder, N. Bülisa, R. R. Netz, C. Böttcher, S. Liese, A. Herrmann, C. P. R. Hackenberger, Nat. Nanotechnol. 2020.

[7] a) A. Harris, G. Cardone, D. C. Winkler, J. B. Heymann, M. Brecher, J. M. White, A. C. Steven, Proc. Nat. Acad. Sci. 2006, 103, 19123-19127; b) L. J. Calder, P. B. Rosenthal, Nat. Struct. Mol. Biol. 2016, 23, 853-858.

[8] a) P. Dey, T. Bergmann, J. L. Cuellar-Camacho, S. Ehrmann, M. S. Chowdhury, M. Zhang, I. Dahmani, R. Haag, W. Azab, ACS Nano 2018, 12, 6429-6442; b) I. S. Donskii, W. Azab, J. L. Cuellar-Camacho, G. Guday, A. Lippitz, W. E. S. Unger, K. Osterrieder, M. Adeli, R. Haag, Nanoscale 2019, 11, 15804-15809.

c) S. Bhatia, L. C. Camacho, R. Haag, J. Am. Chem. Soc. 2016, 138, 8654-8666; b) J. Voormiem, S. Liese, C. Kuehne, K. Ludwig, J. Diederdeck, C. Böttcher, R. R. Netz, R. Haag, J. Am. Chem. Soc. 2015, 137, 2572-2579.

[9] W. Wang, P. Wang, X. Tang, A. A. Elzatahry, S. Wang, D. Al-Dahyan, M. Zhao, C. Yao, C.-T. Hung, X. Zhu, T. Zhao, X. Li, F. Zhang, D. Zhao, ACS Cent. Sci. 2017, 3, 839-846.

[10] M. Imai, Y. Kawaoka, Curr. Opin. Virol. 2012, 2, 160-167.

[11] J.-J. Shie, J.-M. Fang, P.-T. Lai, W.-H. Wen, S.-Y. Wang, Y.-S. E. Cheng, K.-C. Tsai, A.-S. Yang, C.-H. Wong, J. Am. Chem. Soc. 2011, 133, 17959-17965.

[12] a) J. L. McAuley, B. P. Gilbertson, S. Trifkovic, L. E. Brown, J. L. McKimm-Breschkin, Front. Microbiol. 2019, 10; b) F. Wen, X.-F. Wan, Trends Microbiol. 2019, 27, 477-479.

[13] M. Müller, D. Lauster, H. H. K. Wildenauer, A. Herrmann, S. Block, Nano Lett. 2019, 19, 1875-1882.
By the principle of ‘topology-matching design’, we report a potent influenza A virus nano-inhibitor with matched topology and heteromultivalent inhibiting of the receptor-binding proteins. Such an inhibitor is capable of neutralizing influenza A virus extracellularly and block its interaction with the host cell receptors. This idea also shows the potential for the inhibiting of other viruses with spiky surface.