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COMMUNICATION

Biotin-, fluorescein- and 'clickable' conjugates of phospha-oseltamivir as probes for the influenza virus which utilize selective binding to the neuraminidase[†]

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The synthesis of conjugates of phospha-oseltamivir to the well established reporter groups fluorescein and biotin and an approach to multimeric inhibitors is described. We report powerful inhibition of the influenza neuraminidase by these probes and quantify fluorescence quenching during binding of the fluorescein conjugate through titration with the neuraminidase. Thus, we show that they could be useful tools to efficiently inhibit, detect and quantify the virus and the neuraminidase in biological systems.

Virtually each year, the influenza virus succeeds in keeping the WHO and national health authorities on high alert because of the fear of a possible pandemic, coupled with high mortality rates in a worst case scenario.¹ This is particularly true for influenza type A viruses, many of which are avian viruses that can infect humans and are thus the most likely source for new, highly pathogenic strains. While in the past decades, most winter epidemics in the UK were caused by the influenza A (H3N2) virus, it has for the time being apparently been replaced in this role by the H1N1 ('swine flu', 'Mexican flu') virus, the source of the 2008 pandemic.² In addition, the less contagious (for humans), but highly pathogenic H5N1 ('bird flu') virus remains a threat and there is little doubt that all are here to stay as potential sources for dangerous recombinations.

The development of anti-influenza drugs based on inhibition of the viral neuraminidase (NA) has added new options to combat influenza.^{3,4,5} Oseltamivir carboxylate 1,⁶ available in the form of its ethyl ester prodrug Tamiflu[™], zanamivir⁷ (marketed as Relenza[™]) and peramivir⁸ are mimetics of the sialic acid substrates of influenza NA and inhibit the enzyme in the subnanomolar range.^{3,4} So far, carbocyclic oseltamivir 1 has been the most frequently used in the field due to its advantages in formulation and bioavailability. The main features of the inhibitor beside the cyclohexene scaffold are the pentyloxy group mimicking the glycerol sidechain of sialic acid, the acetamide, the amino group providing important ionic bonds with the enzyme and the carboxylate group (Fig. 1). The negative charge of the latter is indispensable and consequently present in all three inhibitors to allow for ionic bonding between the carboxylate and an arginine triad in the active site of the NA which is an essential feature for strong binding of sialic acids and sialylmimetics.^{9,10,11,12}

We have argued that these three powerful inhibitors are quite simply also the strongest binding low molecular weight ligands for the virus.^{13,14} For instance, they bind NA much stronger than anything that has been discovered to bind to the sialic acidrecognising influenza virus hemagglutinin (HA), at least as long only monovalent ligands are considered. Consequently, they could serve as efficient, selective anchors in tools designed to detect, quantify or otherwise investigate the virus or the neuraminidase. In particular oseltamivir has a pharmacophore with reduced analogy to sialic acids that should display very little cross-reactivity with other sialic acid binding proteins in complex systems.¹⁵

The problem to chemically link oseltamivir without impairing the pharmacophore has been solved by us through replacement of the carboxylate by phosphonate monoesters, which retain the negative charge crucial for binding under physiological conditions.^{13,14,16}

In this contribution we have chosen an ω-ethynylbutyl- and an ω -azidohexyl-derivative (**3a** and **3b**) as they also allow immobilisation/conjugation through Huisgen-1,3-dipolar cycloaddition ('click-chemistry') (Fig. 1).17 We have demonstrated the latter by 'clicking' 3b with 1.4-diethynylbenzene to afford a dimeric inhibitor $(\rightarrow 3c)$ as example for a class of compounds with known improved bioavailability.¹⁸ As reporter groups to be conjugated with 3b, we have chosen biotin $(\rightarrow 3d)$ and fluorescein $(\rightarrow 3e)$ for the following reasons: Biotin is universally used to immobilise or conjugate bioactive compounds, normally via its high-affinity interaction with avidin or streptavidin, for instance in ELISA-type assays or in Biacore systems. To give an example, treatment of readily available streptavidin-coated microtiter plates with a solution of 3d (or an analog with optimised spacer length) should result in a phospha-oseltamivir-coated plate with a strong affinity for influenza NA. Fluorescein is one of a family of fluorophores widely used in biochemistry and virology (e.g. the Alexafluor® group). This will aid direct fluorimetric detection and quantification of influenza viruses or influenza NA independent of antibodies and the standard sialidase assay which is based on the unselective substrate 2'-(4-methylumbelliferyl)-α-D-N-acetylneuraminic acid

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Fig. 1 Influenza neuraminidase inhibitors and target compounds 3a-d.

(MUNANA). The cross-reactivity of oseltamivir with other sialidases is known to be low,¹⁵ *i.e.* an assay employing **3d** or **3e** might even allow the presence of mixtures of sialidase activities in test samples. Moreover, we hope that such compounds will help to identify irregular distributions of neuraminidase ('neuraminidase patches') on the viral surface, again without involvement of antibodies or the influenza HA. It is known that the functional balance between NA and HA is an important factor for the replicative fitness of the virus.¹⁹

The synthesis of target conjugates 3a-e has been achieved through a strategy introduced by us for inhibitors 2, all containing the phospha-oseltamivir motif and inhibiting influenza NA in the subnanomolar range.^{13,16} Protected monoester 4 is readily available *via* our Hunsdiecker–Barton approach^{13,20} from the 'acetamido-azide', the precursor of oseltamivir in the industrial synthesis (Scheme 1).²¹ ω -Azido-hexyltriflate **9** was synthesised from commercially available hexanediol through standard procedures. In brief, mono-silylation of the diol gave **5** which was tosylated to give **6**. Substitution of the tosylate using sodium azide gave **7** which was deprotected to yield azido alcohol **8**²² Both **8** and commercially available 1-hexyne-6-ol were activated with triflic anhydride and lutidine to give triflates **9** and **10**, respectively.

Monomethyl phosphonate 4 was alkylated with the ω azidohexyl- and ω -ethynylbutyltriflates 9 and 10, respectively, to give the corresponding mixed diesters 11 and 12 in good yield. Mixtures of diastereoisomers were typically obtained which were used as such. Cleavage of the methyl esters 11 and 12 followed by deprotection of the amine under acidic conditions resulted in 'clickable' inhibitors 3a and 3b.



Scheme 1 Synthesis of 'clickable' inhibitors 3a and 3b.

To demonstrate the suitability of the approach for the production of phospha-oseltamivir multimers, we 'clicked' azide **11** with 1,4-diethynylbenzene under Sharpless/Huisgen-conditions to give protected dimer **13** which was readily deprotected to give dimeric inhibitor **3c** (Scheme 2).

For (+)-biotin conjugate **3d**, azide **11** was reduced with trimethyl phosphine followed by hydrolysis of the aza-ylide. The resulting amine was then coupled to (+)-biotin using benzotriazol-1-yl-oxypyrrolidinephosphonium hexafluorophosphate (PyBOP) as condensing agent. Protected conjugate **14** was deprotected with sodium iodide and then triffic acid to yield **3d** in 64% yield over 2 steps.

The same set of coupling and deprotection reactions was applied for the fluorescein conjugate **3e** (*via* protected **15**) which was obtained in a comparable yield of 75% (Scheme 3).

To confirm binding, compounds **3a–e** were tested for inhibition of the neuraminidase isolated from the influenza virus X31 (H3N2)²³ and compared to benchmark inhibitors oseltamivir carboxylate **1** and zanamivir.²⁴ As expected, all 5 compounds are potent inhibitors of the hydrolysis of 2'-(4-methylumbelliferryl)- α -D-neuraminic acid (MUNANA) by this neuraminidase (Table 1). This confirms that the 'clickable' derivatives **3a** and **3b** are attractive candidates for the generation of multivalent phospha-



Scheme 2 Synthesis of a dimeric inhibitor via 'click'-chemistry.



Scheme 3 Synthesis of conjugates of phospha-oseltamivir and reporter groups biotin and fluorescein.

oseltamivir-based inhibitors and phospha-oseltamivir-coated surfaces. Interestingly, dimeric inhibitor **3c** exhibits are a marked improvement compared to the monomer and certainly belongs to the most powerful influenza NA inhibitors ever synthesized. Whether this effect can be attributed to statistical rebinding leading, for instance, to a lower k_{off} -rate or other effects remains to be investigated but it will be interesting to see its performance in plaque reduction assays.

Biotin-conjugate **3d** and fluorescein-conjugate **3e** are equally strong inhibitors. The strong binding of **3d** prompted us to look at the impact of binding on the fluorescence of **3d** (Fig. 2).

The binding of NA to the fluorescein conjugate **3e** quenches the fluorescence and shifts the emission maximum to a longer wavelength (Fig. 2a and 2b). The time course for the quenching is consistent with an association rate constant of 2×10^6 M⁻¹ s⁻¹

| Table 1 | Inhibition | constants (| K | [nM] |) for | target | phos | phonat | es |
|---------|------------|-------------|-----|------|-------|--------|------|--------|-----|
| Table 1 | minontion | constants (| 111 | լույ | , 101 | unger | phos | phonat | C,C |

| 3a | 3b | 3c | 3d | 3e | Oseltamivir | Zanamivir |
|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|
| 0.234 ± 0.036 | 0.224 ± 0.037 | 0.040 ± 0.010 | 0.241 ± 0.052 | 0.162 ± 0.029 | 0.130 ± 0.032 | 0.268 ± 0.039 |

^{*a*} Inhibition of MUNANA hydrolysis catalysed by the neuraminidase from influenza virus X31 (H3N2); inhibition constants were determined as described in the Supporting Information; $K_{\rm M}$ for MUNANA = 20.2 ± 3.3 μ M.



Fig. 2 (a) Decrease in fluorescence intensity (510 nm) of **3e** over time upon binding to influenza virus NA. The binding experiment was carried out at 5 °C and concentrations of **3e** and NA were comparable (9 nM and 12 nM, respectively). (b) Red-shift of fluorescence maximum of **3e** upon binding to influenza NA. Top line: unbound **3e**. Bottom line: **3e** bound to NA. (c) Fluorescence titration of **3e** with influenza virus NA, resulting in a dissociation constant K_d of 0.23 ± 0.05 nM.

measured (at 37 °C) using the methods described in Collins *et al.*²⁵ Fluorescence titration of **3d** with influenza NA resulted in a dissociation constant K_d of 0.23 ± 0.05 nM which is in good agreement with the value determined by MUNANA hydrolysis (Fig. 2c and Table 1). The changes in fluorescence are rather large and more than adequate for binding studies, given that fluorescein is not generally considered an environmentally sensitive probe. **3e** can therefore be used for the determination of dissociation constants by fluorescence-based displacement titration. In addition, these effects suggest new opportunities for direct quantification of influenza neuraminidases that are independent of calorimetry or hydrolysis of substrates such as MUNANA.

We conclude that this project has resulted in conjugatable compounds based on the oseltamivir motif which display selective, high-affinity binding to influenza neuraminidase. The potential of the approach has been demonstrated through the synthesis of dimeric inhibitor **3c** and 2 diagnostic tools containing reporter groups widely used in biological research. The binding of one of them, fluorescein conjugate **3e**, to influenza NA has been investigated in a quantitative fashion and an excellent correlation between the fluorescence titration and the MUNANA-based assay has been found. It can safely be expected that both compounds will find various applications in influenza research for instance quantification of NA, virus counting or detection of NA clusters on the virus surface to name only a few.

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Notes and References

- 1 WHO, Global Alert and Resistance (GAR)/Influenza, http://www. who.int/csr/disease/influenza/en/.
- 2 Health Protection Agency, HPA National Influenza Report, http://www.hpa.org.uk.
- 3 M. von Itzstein, Nat. Rev. Drug Discovery, 2007, 6, 967– 974.
- 4 E. De Clerq, Nat. Rev. Drug Discovery, 2006, 5, 1015-1025.
- 5 J. Rich, Design and Synthesis of Sialidase Inhibitors for Influenza Virus Infections, Elsevier, Amsterdam, 2007.
- 6 C. U. Kim, W. Lew, M. A. Williams, H. Liu, L. Zhang, S. Swaminathan, N. Bischofberger, M. S. Chen, D. B. Mendel, C. Y. Tai, W. G. Laver and R. C. Stevens, J. Am. Chem. Soc., 1997, 119, 681–690.
- 7 M. von Itzstein, W.-Y. Wu, G. B. Kok, M. S. Pegg, J. C. Dyason, B. Jin, T. V. Phan, M. L. Smythe, H. F. White, S. W. Oliver, P. M. Colman, J. N. Varghese, D. M. Ryan, J. M. Woods, R. C. Bethell, V. J. Hotham, J. M. Cameron and C. R. Penn, *Nature*, 1993, **363**, 418–423.
- 8 P. Chand, P. L. Kotian, A. Dehghani, Y. El-Kattan, T.-H. Lin, T. L. Hutchison, Y. S. Babu, S. Bantia, A. J. Elliott and J. A. Montgomery, J. Med. Chem., 2001, 44, 4379–4392.
- 9 W. P. Burmeister, B. Henrissat, C. Bosso, S. Cusack and R. W. H. Ruigrok, *Structure*, 1993, **1**, 19–26.
- 10 J. N. Varghese, W. G. Laver and P. M. Colman, *Nature*, 1983, 303, 35–40.
- 11 J. N. Varghese, J. L. McKimm-Breschkin, J. B. Caldwell, A. A. Kortt and P. M. Colman, *Proteins: Struct., Funct., Genet.*, 1992, 14, 327– 332.
- 12 P. Bossart-Whitaker, M. Carson, Y. S. Babu, C. D. Smith, W. G. Laver and G. M. Air, *J. Mol. Biol.*, 1993, **232**, 1069–1083.
- 13 B. Carbain, P. J. Collins, L. Callum, S. R. Martin, A. J. Hay, J. McCauley and H. Streicher, *ChemMedChem*, 2009, 4, 335–337.
- 14 H. Busse and H. Streicher, *Bioorg. Med. Chem.*, 2006, **14**, 1047–1057.
- 15 K. Hata, K. Koseki, K. Yamaguchi, S. Moriya, Y. Suzuki, S. Yingsakmongkon, G. Hirai, M. Sodeoka, M. von Itzstein and T. Miyagi, *Antimicrob. Agents Chemother.*, 2008, **52**, 3484–3491.

- 16 B. Carbain, S. R. Martin, P. J. Collins, P. B. Hitchcock and H. Streicher, Org. Biomol. Chem., 2009, 7, 2570–2575.
- 17 H. C. Kolb, M. G. Finn and K. B. Sharpless, Angew. Chem., Int. Ed., 2001, 40, 2004–2021.
- 18 S. J. F. Macdonald, R. Cameron, D. A. Demaine, R. J. Fenton, G. Foster, D. Gower, J. N. Hamblin, S. Hamilton, G. J. Hart, A. P. Hill, G. G. A. Inglis, B. Jin, H. T. Jones, D. B. McConnell, J. McKimm-Breschkin, G. Mills, V. Nguyen, I. J. Owens, N. Parry, S. E. Shanahan, D. Smith, K. G. Watson, W.-Y. Wu and S. P. Tucker, J. Med. Chem., 2005, 48, 2964–2971.
- 19 R. Wagner, M. Matrosovich and H.-D. Klenk, *Rev. Med. Virol.*, 2002, 12, 159–166.
- 20 B. Carbain, P. B. Hitchcock and H. Streicher, *Tetrahedron Lett.*, 2010, 51, 2717–2719.
- 21 S. Abrecht, M. C. Federspiel, H. Estermann, R. Fischer, M. Karpf, H.-J. Mair, T. Oberhauser, G. Rimmler, R. Trussardi and U. Zutter, *Chimia*, 2007, **61**, 93– 99.
- 22 G. Mantovani, V. Ladmiral, L. Tao and D. M. Haddleton, *Chem. Commun.*, 2005, 2089–2091.
- 23 The strain is a standard in influenza research, http://www. uniprot.org/taxonomy/132504.
- 24 M. A. Rameix-Welti, F. Agou, P. Buchy, S. Mardy, J. T. Aubin, M. Veron, S. van der Werf and N. Naffakh, *Antimicrob. Agents Chemother.*, 2006, 50, 3809–3815.
- 25 P. J. Collins, L. F. Haire, Y. P. Lin, J. Liu, R. J. Russel, P. A. Walker, J. J. Skehel, S. R. Martin, A. J. Hay and S. J. Gamblin, *Nature*, 2008, **453**, 1258–1262.