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Proximity Ligation-Based Fluorogenic Imaging Agents for Neuraminidases

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Abstract

Reagents to visualize and localize neuraminidase activity would be valuable probes to study the role of neuraminidases in normal cellular processes as well as during viral infections or cancer development. Here we describe a new class of neuraminidase-imaging probes that function as proximity ligation reagents by releasing a highly reactive fluorophore that tags nearby cellular material. We further demonstrate that it is possible to create an influenza virus-specific reagent, and show that it can specifically detect influenza virus infections in mammalian cells. These reagents have potential use as specific histological probes independent of viral antigenicity, and therefore offer some advantages over commonly used anti-neuraminidase antibodies.

COMMUNICATION

A histological imaging agent was developed for the influenza neuraminidase. The reagent reacts with the neuraminidases to release a highly reactive fluorophore that tags nearby cellular materials, thereby functioning as a proximity ligation agent for neuraminidases and locally visualizing neuraminidase activity.

Keywords

influenza virus; neuraminidase; histological imaging; fluorogenic probes

Influenza virus has two important surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA), which play key roles in cellular entry and release. The main function of NA is to cleave sialoside glycosidic linkages present on the surface of infected host cells, thus releasing newly formed virus particles to infect new cells and individuals. There is also some evidence to suggest that NA activity may be involved in the initiation stage of virus

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infection, possibly during the endocytosis steps^[1–4]. To investigate the precise role NA plays during virus infection, an imaging agent would be useful to localize its activity. While X-Neu5Ac (5-bromo-4-chloroindol-3-yl α -N-acetylneuraminic acid) can function as an imaging reagent for sialidases by releasing a chromophore that becomes water-insoluble upon dimerization, its sensitivity is very low, limiting its application. The Suzuki group recently developed a more sensitive fluorogenic and precipitating imaging reagent, BTP-Neu5Ac, that has been used to visualize mammalian tissue sialidases^[5], influenza virus neuraminidase^[6], and sialidase activity in several other viruses^[7,8]. Concurrently, the Wong group developed a fluorescent difluorosialic acid (DFSA)-based, cell-permeable probe to label and visualize a variety of human and influenza neuraminidases^[9]. However, this reagent only labels stoichiometrically, limiting its sensitivity. Although these types of probes are versatile reagents for study of various sialidases, their generally low sensitivity and lack of specificity hinders useful application.

We have a long-term interest in the use of fluorogenic quinone methide precursors (QMs) as specific labeling reagents to locally visualize enzyme activities within living cells via proximity-based ligation. Glycosyl-QMs, in particular, function as superior probes for imaging, since substrate hydrolysis liberates the fluorescent and electrophilic QM leaving group, which immediately reacts with surrounding nucleophiles, thereby minimizing diffusion of the fluorophore. Importantly these reagents release multiple fluorophores per enzyme, and thus are inherently more sensitive than stoichiometric probes. We originally developed fluorogenic QM-based histological imaging agents for use as α -glucuronidase activity markers in an *Arabidopsis thaliana* plant line^[10]. These reagents allowed high-quality, imaging of enzymatic activity that was much more stable and discrete than that with the commonly used X-GlcA^[10]. This concept has since been picked up by others and expanded to probe the biological activities of phosphatases^[11], sulfatases^[12], β -lactamases^[13] and β -galactosidases^[14]. In this paper, we describe our development of proximity ligation-based imaging agents for influenza virus neuraminidase in which a fluorogenic CF₂Mu group, difluoromethyl coumarin, is attached to the anomeric position of sialic acid, CF₂Mu-Neu5Ac, **1**. These proximity ligation-based reagents covalently “tag” the surrounding environment after release by the sialidase/neuraminidase (Figure 1), allowing the localization of sialidase activity.

The synthesis of CF₂Mu-Neu5Ac started with conversion of sialic acid methyl ester to per-*O*-acetylated sialyl chloride (see Scheme 1 and SI Methods). After coupling of the 6-CHO-coumarin the aldehyde was converted to a difluoromethyl group, yielding intermediate **4**. Finally, sugar acetate and methyl ester groups were deprotected to give the desired imaging reagent **1**.

With **1** in hand, we first compared its kinetic behavior with that of the common sialidase substrate, CF₃Mu-Neu5Ac, using a bacterial sialidase NedA from *Micromonospora viridifaciens*. As expected, these two substrates have very similar K_M values of 4.0 M for CF₃Mu-Neu5Ac and 2.4 M for CF₂Mu-Neu5Ac. To test whether the latent QM can tag biomolecules, we incubated CF₂Mu-Neu5Ac with *Escherichia coli* (*E. coli*) cells for 1 h, in the presence of exogenous NedA sialidase. The excess reagent was then washed away and the NedA-treated cells, shown to be fluorescent, suggesting that the fluorophore was

covalently attached to the cell surface (Fig 2 A). In another experiment, we incubated *E. coli* transformed with NedA-expressing plasmids with **1** for 3 h and these too were shown to be fluorescent, even after extensive washing. These results demonstrate that cleavage of **1** by a sialidase can indeed fluorescently label surrounding environments.

To-date, four human sialidases (Neu1, Neu2, Neu3, and Neu4) that serve important roles in modifying glycoconjugates within different subcellular compartments have been characterized^[15], with comparable homologs identified in other mammalian species. Since reagent **1** is a non-specific imaging reagent, capable of being hydrolyzed by a wide variety of sialidases, we initially utilized this probe to visualize endogenous mammalian sialidase activity within Madin-Darby canine kidney (MDCK) cells, a widely employed host cell line for growth of influenza viruses. **1** (200 μ M, final) was incubated with native MDCK cells grown on glass cover slips for 90 min, after which cells were washed, fixed and permeabilized, probed for common cellular markers, and imaged by confocal microscopy.

Cells were stained with Wheat-Germ Agglutinin (WGA), a generic glycoprotein marker typically used to visualize the cell surface/cell membrane; phalloidin, a fungal toxin specific for F-actin used to label the cytoskeleton; (optionally) an anti-Perth/2009 NA antibody; and QM proximity-labelling reagents. Incubation with **1** resulted in fluorescent labelling of cells, with all cells visualized showing a strong signal above background in the blue (405 nm) channel (see Fig. 3A, far left panel), consistent with the spectral profile of coumarin-derived leaving groups (ex./em. 360/470 nm). To confirm that fluorescent labeling is due to endogenous MDCK sialidase activity, cells were treated with 300 M (final) *N*-Acetyl-2,3-dehydro-2-deoxyneuraminic acid (DANA; a broadly active sialidase inhibitor), leading to a near-complete reduction in the observed signal (Fig. 3B).

Typically sialic acids are more cell-permeable if they are acetylated: cellular esterases then remove the acetate moieties once the probe has entered^[9]. We therefore carried out similar fluorescent imaging analysis using our protected intermediate **4** as the probe. However, while treatment of native MDCK cells with **4** did result in some sialidase-dependent cell labelling (Fig. 3C & 3D), the fluorescent signal is substantially lower than for **1**. These results contrast with earlier findings using the stoichiometric DFSA-based probes, where the acetylated probes were superior^[9]. These differences may be due to the presence of the F₂Mu moiety in **1**, which may improve membrane permeability; and also to the longer incubation times (approximately 15 h) used for protected DFSA-based probes^[9], suggesting that the kinetics of absorption, deprotection by cellular esterases, and subsequent hydrolysis by endogenous sialidases within both of these experimental settings is particularly slow.

As validation of our proximity-ligation strategy, we conducted imaging analysis of MDCK cells treated with commercial Mu-Neu5Ac (Sigma) as a negative control. Here, as expected in the absence of the difluoromethyl modification, no fluorescence signal was detected (Fig. 3E).

We next attempted to probe viral neuraminidase (NA) and endogenous sialidase activity in MDCK cells infected with influenza virus. MDCK cells were infected with a contemporary seasonal H3N2 virus, A/Perth/16/2009, at MOI = 1.0 (multiplicity of infection) for 24 hours.

Following infection, surviving cells were washed, incubated with QM reagents **1** or **4** for 90 min, and imaged as before for uninfected cells (Fig. 4). As expected, treatment with compound **1**, which functions as a non-specific sialidase substrate, resulted in extensive fluorescent-labeling of influenza-infected MDCK cells (Fig. 4A; although anti-NA staining is weaker than in panels B and C, both a positive signal and distortion of normal cellular morphology indicate successful infection). Treatment of identically infected cells at 24 HPI (hours post infection) with 50 nM (final) oseltamivir for 1 hour, prior to incubation with **1**, resulted in similar fluorescence signals (Fig. 4B). Oseltamivir is a potent and highly-specific inhibitor of viral NA, thus suggesting that while **1** may be a substrate for influenza NA, the majority of cell labelling observed here still appears to be mediated by endogenous sialidase activities. Treatment of infected cells with DANA, during labelling, resulted in broad inhibition of fluorescence signal (Fig. 4C), consistent with native MDCK cells.

Slight increases in fluorescent-labeling of influenza-infected MDCK cells were observed upon identical incubation with compound **4** (Fig. S1). By 24 HPI, many cells are dead or severely infected with influenza leading to extreme stress and loss of cellular integrity, as evidenced by differences in staining of cellular revealed no fluorescent labelling of cells (Fig 5A & 5B), demonstrating that the 4,7-methylated QM substrates are not accessible to endogenous mammalian sialidases. Next, we carried out specific labeling of viral NA by incubation of **7** & **8** with infected MDCK cells at 24 HPI with Perth/09 H3N2 (MOI = 1.0). Significant fluorescent signal was observed after incubation with **8** for 90 min (Fig. 5C), while again, the protected reagent **7** gave rise to somewhat reduced labeling of NA (see Fig. S2A). Incubation of infected cells with both Oseltamivir and DANA resulted in significant reductions in the fluorescent signal for both compounds (Fig 5D & 5E; Fig. S2B & S2C). These results nicely confirm that reagent **8** is an influenza virus-specific NA probe with potential application as an antibody-independent reagent for viral detection and imaging. components by WGA and Phalloidin in Figs. 3 and 4 (uninfected cells show a “normal” morphology with defined absences corresponding to nuclei, whereas similar staining of infected MDCK cells appears diffuse and highly irregular). Under these conditions normal organization is disrupted, leading to loss of cytosolic components and likely permitting some deprotection of compound **4** in the extracellular environment. Nevertheless, compared with reagent **1**, treatment of infected cells with DANA resulted in a strong reduction of cell-labeling, while incubation with Oseltamivir appeared to have minimal effect (Fig. S1B & S1C). Clearly a modified reagent was needed that reacts only with the influenza NA and not the host enzymes.

Based on the report that introduction of methyl groups to the C4 and C7 positions of the sialoside moiety can greatly enhance the selectivity of substrate-based inhibitors and probes of influenza NA, with comparatively small effect on binding^[16,17] we sought to develop a virus-specific reagent for imaging influenza NA activities. Such a reagent could prove useful in specifically localizing viral NA activity, without interference from endogenous sialidase activities. To this end we synthesized CF₂Mu-4,7OMeNeu5Ac (**8**) as shown in Scheme 2. Following procedures^[17] described in the literature, a protected sialic acid derivative (**5**) was prepared in 8 steps and subsequently converted to the chloride by HCl gas. Subsequent steps were performed by analogy to the synthesis of **1**.

To confirm that methylation did not substantially affect affinity, kinetic parameters for the hydrolysis of reagent **8** by the NA activity of H3N2 virus (A/Hong Kong/1/68) were determined. Indeed, the K_M value of 159 M measured is comparable to that of the broad spectrum substrate CF₃Mu-Neu5Ac (56 M). As a further test of its specificity for the viral enzyme, we incubated **8**, and its protected version **7**, with native (uninfected) MDCK cells for 90 min, as described above. Confocal imaging analysis

In summary, we have developed fluorescent sialidase probes for the histological studies based on QM concepts. The nonspecific reagent **1** can image endogenous sialidases from MDCK cells, however, it cannot differentiate the types of sialidases. We further described the design and synthesis of an influenza NA-specific imaging reagent **8** incorporating methylation at the C4 and C7 hydroxyl groups and demonstrate its utility in cellular imaging.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Figure 1. Mechanism of action of neuraminidase imaging reagents. Influenza neuraminidase hydrolyzes the labeling substrate to generate reactive QM intermediates, which then react with surrounding nucleophiles to fluorescently tag the local environment, shown here as reaction with the cell surface.

Figure 2.

A). *E. coli* cell treated with **1**, with and without exogenous NedA. B). *E. coli* cell with and without NedA-expressing plasmid treated with **1**.

Figure 3.

MDCK cells shown stained with: QM imaging reagents, WGA-FITC (glycoproteins), Phalloidin-AF555 (F-actin), and as a merged image (far right panel). A) treatment with 0.2 mM **1**. B) cells treated with 0.3 mM DANA and 0.2 mM **1**. C) 0.2 mM **4**. D) treatment with 0.3 mM DANA and 0.2 mM **4**. E) 0.2 mM Mu-Neu5Ac.

Figure 4. Influenza-infected MDCK cells stained with: Imaging reagents, WGA-FITC (glycoproteins), Phalloidin-AF555 (F-actin), Anti-Perth/09 Neuraminidase-AF647, and shown as merged image (far right panel). A) incubation with 0.2 mM **1**. B) infected cells treated with 50 nM Oseltamivir and 0.2 mM **1**. C) treatment with 0.3 mM DANA and 0.2 mM **1**.

Figure 5.

Native MDCK (A and B) or virus-infected MDCK (C, D, and E) were stained with: Imaging reagents, WGA-FITC (glycoproteins), Phalloidin-AF555 (F-actin), Anti-Perth/09 Neuraminidase-AF647, and shown (far right) as a merged image. A) incubation with 0.2 mM **8**. B) incubation with 0.2 mM **7**. C) incubation with 0.2 mM **8**. D) treatment with 50 nM Oseltamivir and 0.2 mM **8**. E) treatment with 0.3 mM DANA and 0.2 mM **8**.

Scheme 2.
Synthesis of reagent 8.