Research Article

Journal of Molecular Recognition

Received: 28 February 2014, Revised: 2 July 2014, Accepted: 10 August 2014, Published online in Wiley Online Library

(wileyonlinelibrary.com) DOI: 10.1002/jmr.2417

Development of a surface plasmon resonance assay to measure the binding affinity of wild-type influenza neuraminidase and its H274Y mutant to the antiviral drug zanamivir

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Influenza is one of the most common infections of the upper respiratory tract. Antiviral drugs that are currently used to treat influenza, such as oseltamivir and zanamivir, are neuraminidase (NA) inhibitors. However, the virus may develop resistance through single-point mutations of NA. Antiviral resistance is currently monitored by a labelled enzymatic assay, which can be inconsistent because of the short half-life of the labelled product and variations in the assay conditions. In this paper, we describe a label-free surface plasmon resonance (SPR) assay for measuring the binding affinity of NA-drug interactions. Wild-type (WT) NA and a histidine 274 tyrosine (H274Y) mutant were expressed in High Five™ (*Trichoplusia ni*) insect cells. A spacer molecule (1,6-hexanediamine) was site-specifically conjugated to the 7-hydroxyl group of zanamivir, which is not involved in binding to NA, and the construct was immobilized onto a SPR sensor Chip to obtain a final immobilization response of 431 response units. Binding responses obtained for WT and H274Y mutant NAs were fitted to a simple Langmuir 1:1 model with drift to obtain the association (k_a) and dissociation (k_d) rate constants. The ratio between the binding affinities for the two isoforms was comparable to literature values obtained using labelled enzyme assays. Significant potential exists for an extension of this approach to test for drug resistance of further NA mutants against zanamivir and other antiviral drugs, perhaps paving the way for a reliable SPR biosensor assay that may replace labelled enzymatic assays.

Keywords: surface plasmon resonance; influenza neuraminidase; binding kinetics; antiviral drugs; zanamivir; drug resistance

INTRODUCTION

Influenza results in the deaths of millions of people annually (Maines *et al*., 2005; Yuen and Wong, 2005). In the early 20th century, new strains of the influenza virus emerged, killing up to between 50 and 100 million people. In April 2009, a new influenza strain, 'swine flu', that combined human, pig and bird influenza genes emerged (Trifonov *et al*., 2009), and on June 11 that year, the World Health Organisation declared the outbreak of swine flu to be a pandemic. The ability of this influenza strain to spread rapidly made it a severe threat to public health. Several antiviral drugs are now used to control symptoms and to slow the spread of influenza, but it is currently not possible to know whether a particular influenza strain will respond to a particular drug. Current methods for testing for viral resistance to a particular antiviral drug are time-consuming and can give variable results. A rapid and reliable method for testing the likely binding affinity between a new strain of influenza and an antiviral drug is needed so that the most efficacious drug can be prescribed early in a pandemic outbreak.

Influenza viruses belong to the family *Orthomyxoviridae* (Amano and Cheng, 2005; Shtyrya *et al*., 2009) and are classified as influenza A, B and C (Wagner *et al*., 2002; von Itzstein, 2007). Influenza A is the most commonly occurring of these and is further classified based on its surface glycoproteins hemagglutinin (HA) and neuraminidase (NA), e.g. H1N1, H5N1, which refer to viral strains that contain identical NA but different HA isoforms on their surface (Oxford *et al*., 2002). The severity of each strain of the virus depends on the type of HA and NA it carries (Fouchier *et al*., 2004). Currently, there are 16 subtypes of HA and 9 subtypes of NA (Liu *et al*., 1995; von Itzstein, 2007; Colman, 2009), classified by their interaction with antibodies. All variants within a given subtype will be neutralized by a similar set of antibodies (Boonsoongnern *et al*., 2005). These surface glycoproteins recognize sialic acid [*N*-acetylneuraminic acid (NANA)] in humans (von Itzstein, 2007), and both proteins play a significant role in viral infection. The mechanism of virus infection has been

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studied extensively (Palese *et al*., 1974; Liu *et al*., 1995; Oakley *et al*., 2010; von Itzstein, 2007; Matrosovich & Klenk 2003; Nelson *et al*., 1993; Sauter *et al*., 1992; Suzuki *et al*. 2000; Watowich *et al*. 1994), and once a clear understanding was developed, both HA and NA were proposed as potential anti-influenza drug discovery targets (Anderson *et al*., 1948; Colman, 2002; von Itzstein, 2007; Colman, 2009). Current antiviral drugs such as oseltamivir (Roche's Tamiflu™) and zanamivir (GlaxoSmithKline's Relenza™) are NA inhibitors (NIs) that bind more tightly to NA than its natural substrate, sialic acid blocking the action of NA and inhibiting the release of the virus from the host cell (Kim *et al*., 1997). For this reason, NIs have become the first line of defence against seasonal influenza attack. However, the virus can acquire resistance to NIs by developing single-point mutations [such as histidine 274 tyrosine (H274Y)] in the target protein NA (Colman, 2002; Ferraris and Lina, 2008; Colman, 2009). The ability of the virus to develop resistance against NIs varies with the mutation, and so it is important to monitor the sensitivity of currently circulating strains to NI drugs.

For initial screening studies, the plaque reduction assay (PRA) is usually performed to detect a broad range of resistant influenza phenotypes. NIs prevent the release of virus from infected cells, leading to the formation of smaller plaques in a PRA, and this can be used to evaluate drug sensitivity. However, the PRA has not been used successfully to determine the sensitivity of NIs, and the main limitation of this assay is that many clinical isolates do not form plaques well, and so this assay is considered unreliable for determining whether a particular influenza strain is resistant or sensitive to a specific NI (Tisdale, 2000; Wetherall *et al*., 2003). This unreliability led to the development of biochemical inhibition assays (Gubareva *et al*., 1998; Gubareva *et al*., 2000), which have been extended to determine viral sensitivity to NI drugs. The most commonly used substrate for detecting enzyme activity and inhibition is the fluorogenic 2′-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid known as MUNANA (Wetherall *et al*., 2003), the use of which was initially described by Potier *et al*. (1979). Cleavage of MUNANA by NA in a sample releases a fluorescent substance, methylumbelliferone, and the measured fluorescence is directly proportional to the amount of enzyme activity. Currently, several fluorometric enzyme assays are used in practise, using various MUNANA concentrations and buffers (Wetherall *et al*., 2003). In most experiments, IC_{50} values of the NI drugs are determined, where IC_{50} is the concentration of inhibitor required to inhibit 50% of the enzyme reaction. Time-dependent enzyme assays have also been performed to determine the inhibition constant (k_i) to evaluate the effectiveness of the inhibitor. Collins *et al*. (2008) have used the time-dependent fluorometric enzyme assay to measure the binding affinity of NI drug interactions with NA. Binding affinity data was used to determine which of the two commonly used NI drugs could serve as potential inhibitors to treat a particular mutant strain.

The inconsistencies in the MUNANA assay (Gubareva *et al*., 2002) led to the development of a more sensitive chemiluminescent substrate (a 1,2-dioxetane derivative of sialic acid, NA-STAR) with up to 67-fold higher sensitivity for NA detection than the former fluorometric enzyme assays. However, the chemiluminescent substrate is a flash emitter with a half-life of 5 min, and the signal intensity must therefore be measured immediately and consistently with regard to time (Wetherall *et al*., 2003). This calls for a high level of technical competence to perform the assay, which otherwise might give false positive or false negative results, and for this reason, this substrate is not currently used to monitor drug resistance. Hence, there is a need for a simple, reliable and label-free assay to monitor resistance of influenza to antiviral drugs.

Surface plasmon resonance (SPR) is an optical, label-free, real-time biosensor technique first reported in 1983 (Liedberg *et al*., 1995) and now used for measuring various biomolecular interactions such as protein-protein, antigen-antibody and receptor-ligand binding (Navratilova and Myszka, 2006; Lee *et al*., 2008). An SPR response is proportional to the molecular weight ratio of the solution phase antigen and the surface-tethered ligand, so if applied to NA-antiviral drug interactions, the highest signal amplitude will result from tethering the low molecular weight antiviral drug (≈300 Da) to the SPR sensor chip as the ligand and following the binding of NA (240 kDa) as the analyte in solution. In this paper, we describe the synthesis of a zanamivir-spacer conjugate, immobilization of the conjugate to the sensor chip, and the development of a simple, label-free, real-time SPR assay to measure the affinity of zanamivir and NA (WT and H274Y mutant) interactions. We show that the results from this new assay are equivalent to values reported in the literature using other assay methods.

MATERIAL AND METHODS

Materials

The pBac 1 vector (Novagene) was purchased from EMD Millipore (Billerica, Massachusetts, USA). A QuikChange® multi-site-directed mutagenesis (SDM) kit was purchased from Stratagene (San Diego, California, USA). The following primers were purchased from Gene Works Pty Ltd (Adelaide, Australia):

pBac-1-NA forward primer
(5′AATAAAAAAACCTATAAATATAGGATCCA
TGAACCCG AACCAGAAA ATT 3′)

pBac-1-NA reverse primer
(5′AGTGGTGGTGGTGGTGCTCGAGTTATT
TATCAATGGTAAACGGCAGTTCCG 3′)

SDM primer
("5′-G AAC GCG CCG AAC AGC TAT TAT GAA GAATGC AG-′3")

Insect cell lines (Sf9 and High Five Trademark) were gifted by the Protein Expression Facility, University of Queensland. Sf-900II serum-free medium (SFM), 0.1% trypan blue, Cellfectin reagent, Grace's insect medium, unsupplemented, antibiotics and antimycotics, 100X, were purchased from Life Technologies Corporation (Carlsbad, California, USA). FlashBAC DNA was purchased from Oxford Expression Technologies (Oxford, UK). Influenza A H1N1 (Swine Flu 2009) NA antibody and Mouse IgG secondary antibody horseradish peroxidase conjugate were purchased from Sino Biologicals (Beijing, China). A Resource™ Q GL prepacked ion exchange column and Superdex 200 10/300 GL prepacked gel filtration column were purchased from GE Healthcare Life Sciences (Uppsala, Sweden). All purification experiments were conducted using an AKTAexplorer 10 chromatography system (GE Healthcare Life Sciences).

Amine coupling reagents 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide-HCl (EDAC, 0.4 M), *N*-hydroxysuccinimide (NHS,

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0.1 M), ethanolamine-HCl (1.0 M, pH 8.5) and GLC biosensor chips were purchased from Bio-Rad Laboratories (Hercules, CA, USA). Regeneration buffer (10 mM glycine-HCl, pH 3.0), immobilization buffer (20 mM sodium phosphate, pH 8.0) and running buffer (20 mM sodium phosphate, 300 mM sodium chloride, 0.1% Triton-100, pH 6.0) were prepared in the laboratory using analytical grade chemicals purchased from Sigma Aldrich (St. Louis, Missouri, USA). Zanamivir and zanamivir intermediates were gifted by GlaxoSmithKline Ltd (GSK, Stevenage, UK).

A ProteOn XPR36 protein interaction system (Bio-Rad Laboratories) was used for SPR assays. Instrument control and data analysis were carried out using ProteOn Manager Software Version 3.0.

Recombinant NA expression and purification

Influenza NA [Influenza A virus (A/Puerto Rico/8/1934(H1N1))] Gene ID: 956530 (GOI) was cloned into pBac 1 vector through the homologous cloning method. An SDM primer was used to obtain the desired mutation of H274Y by following the QuikChange® multi-SDM kit protocol. To optimize the protein expression, Sf9 (*Spodoptera frugiperda*) and High Five (*Trichopulsia ni*) insect cells were infected with baculoviruses encoding for WT NA and the H274Y mutant. Western blots were performed to confirm the presence of influenza NA in High Five and Sf9 samples collected at different time intervals (24, 48 and 72 h post-infection for High Five cells and 72 and 96 h post-infection for Sf9 cells). Cell lysis was performed using the method of Dalakouras *et al.* (2006), with minor modifications. Recombinant insect cells were harvested and suspended in 4 ml of lysis buffer (20 mM sodium phosphate, 5.7% TritonX-100, pH 6.0). Protease inhibitors were added, and the cells were immediately sonicated on ice to release intracellular proteins. The final cell lysate was centrifuged at 16,500*g* for 15 min at 4°C, and the supernatant containing the soluble proteins was collected and used as a source of NA. The recombinantly generated NAs were purified by anion exchange and size exclusion chromatography. A 1 ml Resource™ Q anion exchange column was equilibrated with buffer A (20 mM sodium phosphate, 0.1% TritonX-100, pH 6.0). The sample was injected onto the column through an injection loop, and the column was then washed with buffer A for five column volumes (CV) to remove loosely or non-bound materials. Bound proteins were eluted by a linear gradient over 20 CV to 100% buffer B (20 mM sodium phosphate, 0.1% TritonX-100, 1 M NaCl, pH 6.0). The elution fractions with positive western blot signal were combined and further purified by size exclusion chromatography using a Superdex S200 10/300 GL column.

Zanamivir-spacer conjugate synthesis

4-Dimethylaminopyridine (DMAP) (152 mg, 2.5 eq.) and 4-nitrophenyl chloroformate (121 mg, 1.2 eq.) were added to a solution of **1** (Figure 1) (286 mg, 0.5 mmol) in pyridine (1.5 ml) under N_2, and the mixture was stirred at room temperature. After 3 h, *N*-Boc-1,6-hexanediamine (0.13 ml, 1 eq.) was added, and the resulting mixture was then left to stir overnight. After 16 h, the mixture was diluted with ethyl acetate (EtOAc, 20 ml) and extracted with aqueous HCl (2 M, 40 ml). The aqueous phase was then extracted with EtOAc (2 × 20 ml), and the combined organic extracts were then dried by adding excess magnesium sulphate ($MgSO_4$), filtered, and concentrated under vacuum. The structure of the product 2 was confirmed by high-resolution mass spectroscopy (HRMS) using electrospray ionization/time of flight (ESI/TOF) (Bruker MaXis 3G, Bruker Daltronics, Bremen, Germany) [HRMS (ESI-TOF): calculated for $C_{36}H_{58}N_6O_{15}H^+$: 815.4033, measured: 815.4040 (MH^+)].

The crude compound 2 (Figure 1) was dissolved in trifluoroacetic acid (TFA, 5 ml) and was stirred for 1 h at room temperature under N_2. After this time which the reaction was concentrated under vacuum. The residue was dissolved in 50% (*v*/*v*) aqueous methanol (40 ml), triethylamine (10 ml) was added, and the mixture was stirred at room temperature. After 6 h, the reaction mixture was concentrated under vacuum, and the residue was then freeze dried to yield a yellow viscous gel-like substance (compound **3**). The structure of the product (1,6-hexanediamine-zanamivir conjugate, HDA-zanamivir) was

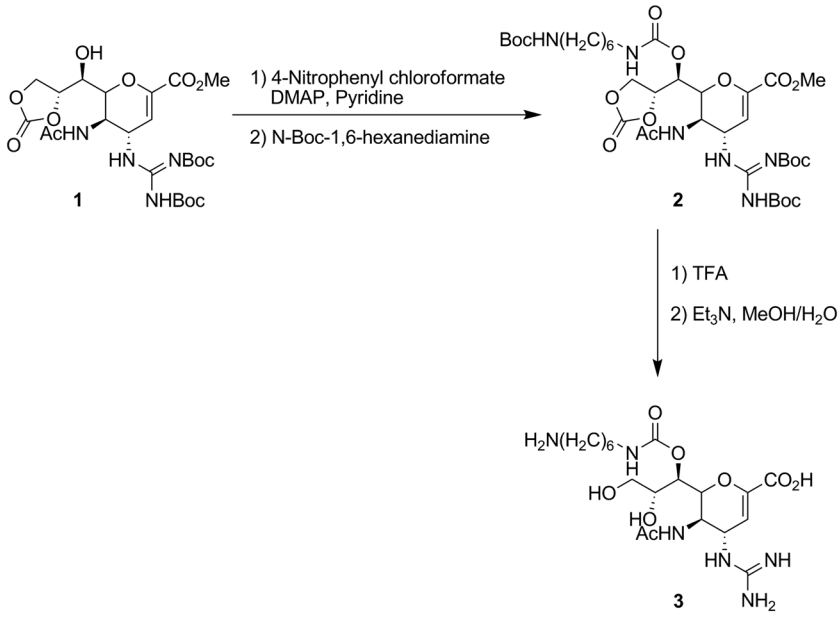

Figure 1. Synthesis of HDA-zanamivir.

confirmed by mass spectrometry [HRMS (ESI-TOF): calculated for $C_{19}H_{34}N_6O_8H^+$: 475.2509, measured: 475.2511 (MH^+)].

Ligand preparation

The synthesised ligand (50 mg) was dissolved in 1 ml of immobilization buffer (20 mM sodium phosphate, pH 8.0). The solution was centrifuged at 16,500*g* for 20 min at 25°C. The supernatant was further diluted with immobilization buffer (1:1) and again centrifuged at 16,500*g* for 20 min at 25°C. The final supernatant (300 μl) was used for ligand immobilization as described in the succeeding texts.

Biosensor surface preparation

Immobilization was carried out at 35°C using a standard amine coupling technique following the manufacturer's instructions. The ProteOn XPR36 system uses a 6 × 6 flow channel system, in which six parallel channels (ligand, L1–L6) are injected simultaneously, with the flow through the second set of six parallel channels (analyte, A1–A6) oriented normal to the first set. Thus, 36 intersection points are formed, each of which is a sensor spot. Channels L1 and L2 were activated simultaneously with a mixture of EDAC and NHS (1:1 *v/v*, 30 μl/min, 5 min) to form ester groups for the amine coupling, followed by running buffer in L1 and HDA-zanamivir in L2. Finally, residual unreacted surface ester groups in both channels were deactivated with ethanolamine-HCl (1 M, pH 8.5, 30 μl/min, 5 min). Channel L1 was used as the reference channel to subtract SPR responses caused by buffer refractive index changes from those caused by analyte-ligand interactions.

Analyte sample preparation

A dilution series of NA samples in SPR running buffer were used as analytes. The starting enzyme concentrations used in the assay were 5.2 and 6.1 nM for the WT and H274Y mutant, respectively.

Surface plasmon resonance biosensor assay

Six analyte samples (WT or H274Y NA) were injected simultaneously through channels A1 to A6 for 300 s (25 μl/min) at 35°C. The binding responses were acquired during the 300 s association phase (analytes injection) and 600 s dissociation (running buffer injection). The sensor surface was regenerated between experiments by two quick injections of regeneration buffer (18 s, 100 μl/min) to remove NA bound to zanamivir. The SPR signal after reference channel (L1) response subtraction corresponded to the binding of the analyte to the immobilized ligand. The SPR curves were fitted to a simple Langmuir 1:1 model and a Langmuir 1:1 model with drift to obtain kinetic parameters. The goodness of the fit was determined from the residuals and χ^2 values (the average of the squared differences between the measured data points and the fit).

RESULTS AND DISCUSSION

Expression optimization

Glycosylation is important for obtaining an active form of NA (Deroo *et al*., 1996), so a eukaryotic expression system is required to express active NA. Although some research groups (Martinet *et al*., 1997; Yongkiettrakul *et al*., 2009) have used a yeast expression system to express NA, more commonly, insect cells are used (Mather *et al*., 1992; Deroo *et al*., 1996; Dalakouras *et al*., 2006; Oakley *et al*., 2010). Hence, an insect cell expression system was employed in this work. Interestingly, Deroo *et al*. (1996) obtained soluble, active NA secreted into the cell culture media, whilst Dalakouras *et al*. (2006) had to lyse the cells, using detergent, to obtain soluble NA, although both groups reportedly used the same expression system.

Several groups have reported that affinity tags may interfere with the activity of NA (Castrucci *et al*., 1992; Yano *et al*., 2008; Schmidt *et al*., 2011; Xu *et al*., 2008), so affinity tags were not used in NA expression, even though this may have made purification simpler.

Analysis by Western blot (Figure 2) confirmed the presence of NA in all samples (both WT and H274Y mutant). The molecular weight of the active NA tetramer is 240 kDa, and a predominant band was seen at about 55 kDa, corresponding to the NA monomer molecular weight for both cell lines in sodium dodecyl sulphate (SDS)-PAGE gels run under reducing conditions. In the absence of a reducing agent, NA migrated to the equivalent of a 110 kDa band on SDS-PAGE gel (data not shown). This is consistent with the view that the protein is internally linked by disulfide bridges to form a dimer, which further associates by non-covalent interactions to form a tetrameric active form of the protein (Deroo *et al*., 1996). Similar SDS-PAGE results under both reducing and non-reducing conditions have been reported by Wu *et al*. (2009).

The expression of NA in High Five cells was at its peak 72 h post-infection, and there was a significant drop in cell viability after 72 h post-infection (Figure 2). The level of expression in Sf9 cells was relatively low, reaching a maximum 96 h post-infection. The cell viability dropped below 80% only after 96 h post-infection for Sf9 cells. Hence, the western blot signal was strongest after 96 h post-infection. The results show that High Five cells were superior to Sf9 cells for NA expression and that 72 h was the optimum time for harvesting the cells post-infection.

Ligand immobilization

Immobilizing (tethering) a ligand *via* a primary amine group to an activated SPR chip surface is convenient, rapid and simple. However, the steric proximity of the chip may have a significant effect on ligand binding by an analyte (Fee, 2013). To avoid the

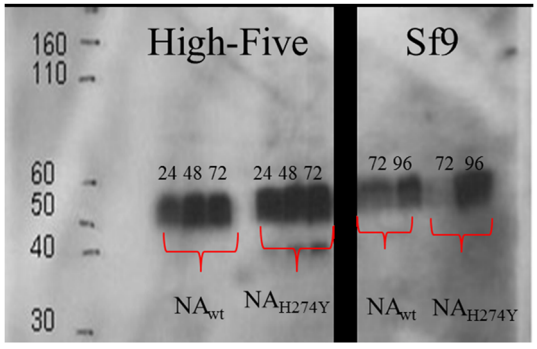

Figure 2. Western blot analysis of the time course of expression of influenza NA in Sf9 and High Five insect cells, detected with the monoclonal influenza A H1N1 (swine flu 2009) NA/neuraminidase antibody.

chip surface interfering with NA-zanamivir binding, zanamivir was first conjugated to a spacer molecule that could present a single primary amine suitable for amine coupling. The resulting HDA-zanamivir was then immobilized on the SPR chip surface. It is possible that altering the chemical structure of zanamivir through conjugation with a spacer could interfere with analyte binding, but McKimm-Breschkin *et al*. (2003) identified 1,6-hexanediamine as an appropriate spacer molecule when tethering zanamivir (*via* its 7-hydroxyl group) to microspheres, reporting that the antiviral effect was unaltered. Hence, 1,6-hexanediamine was chosen as a spacer in this work for immobilizing zanamivir to the SPR sensor chip. We did not specifically test for the effect of the spacer on drug activity or binding but believe this was unnecessary because we were interested only in the relative binding between WT and H274Y NA and not the absolute values of binding constants.

From preliminary experiments (data not shown), it was found that HDA-zanamivir was difficult to solubilize, and that removal of insoluble material by centrifugation was required prior to immobilization. HDA-zanamivir was immobilized on channel L2 of GLC sensor chips, using L1 as a reference channel. After the ligand coupling and deactivation steps, the final ligand immobilization responses (ΔRU) was 431 RU (Figure 3).

SPR interaction analysis

Preliminary SPR experiments with NA cell lysates showed a positive binding response with the immobilized HDA-zanamivir (data not shown). Moreover, the cell lysates from High Five control (cells that were not infected with recombinant baculovirus) did not show any sign of binding to the immobilized HDA-zanamivir. Identical binding responses were observed when NA cell lysates were injected at different flow rates, suggesting that the interactions were not mass transfer limited.

As shown by the SPR responses in Figures 4 (WT) and 5 (H274Y), immobilized HDA-zanamivir showed specific binding to NA, with responses increasing with analyte concentration. At the end of analyte injection, a very slow dissociation phase was observed in both cases, although neither analyte dissociated completely from the ligand, indicating a strong binding affinity with the immobilized ligand. The sensor surface was regenerated twice with a regeneration buffer in between analyte-binding runs. The drop in pH during regeneration was expected to change the conformation of bound NA, thereby releasing NA from the NA-zanamivir complex formed on the chip surface. The regeneration step was useful to obtain a stable baseline for subsequent analyte injections, with ligand activity maintained for at least 25 cycles (data not shown).

To study the association (k_a) and dissociation (k_d) rate constants between WT and H274Y NA, sensorgrams were obtained over a range of analyte concentrations. Experiments were repeated five times in each case and all curves used to determine the rate constants. To avoid systematic errors, analyte samples were assigned to the six analyte channels in a random order and were again randomized between injections. Figures 4 and 5 show that the responses at each concentration were consistent, with all five repeated runs overlapping at each analyte concentration. The binding curves were fitted to the 1:1 Langmuir binding model (data not shown) and the 1:1 Langmuir binding model with drift (Figures 4 and 5) using ProteOn Manager™ Software tools. The 1:1 Langmuir model with drift uses the same kinetic equation as 1:1 Langmuir model but calculates drift as a linear drift with respect to time (D^*t, where D is the slope of the drift). Both models assume that analyte and the ligand are homogenous and the binding events are independent. Initial visual inspection showed that the lines of the resulting fit passed through the experimental data for both models. However, when the results were analysed further with the residuals, the following observations were made. For the highest two concentrations, the fitted line passed slightly below the experimental curves for the mutant NA, whilst they passed through the middle of the experimental curves for the WT NA. The fitted line for the third concentration passed above the curves in the dissociation phase for both proteins. The three lowest concentrations fitted very well for both proteins. The goodness of the fit was then examined by a χ^2 test (Table 1). For a good fit, χ^2 is expected to be less than 10% of R_{max}. Global fitting of the data using the 1:1 model resulted in a good fit for the H274Y mutant, yielding a χ^2 value of $<$10% of R_{max}. However, this value was found to be slightly $>$10% for the WT protein, indicating that the fit was not as good as that for the mutant, which could be because of slight baseline drift observed in the dissociation phase, which can be taken into

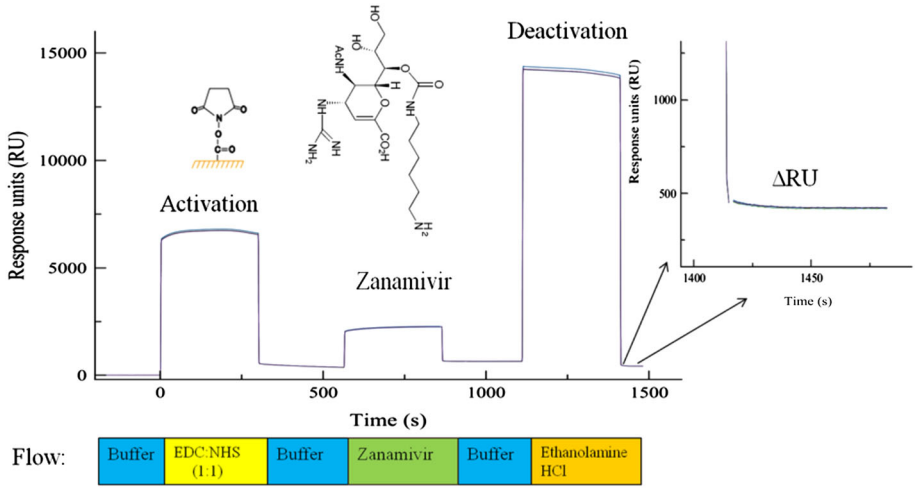

Figure 3. Immobilization of HDA-zanamivir to SPR GLC sensor chip. The figure shows activation of the chip surface with a mixture of EDAC and NHS, followed HDA-zanamivir and capping of un-reacted surface ester groups with ethanolamine-HCl.

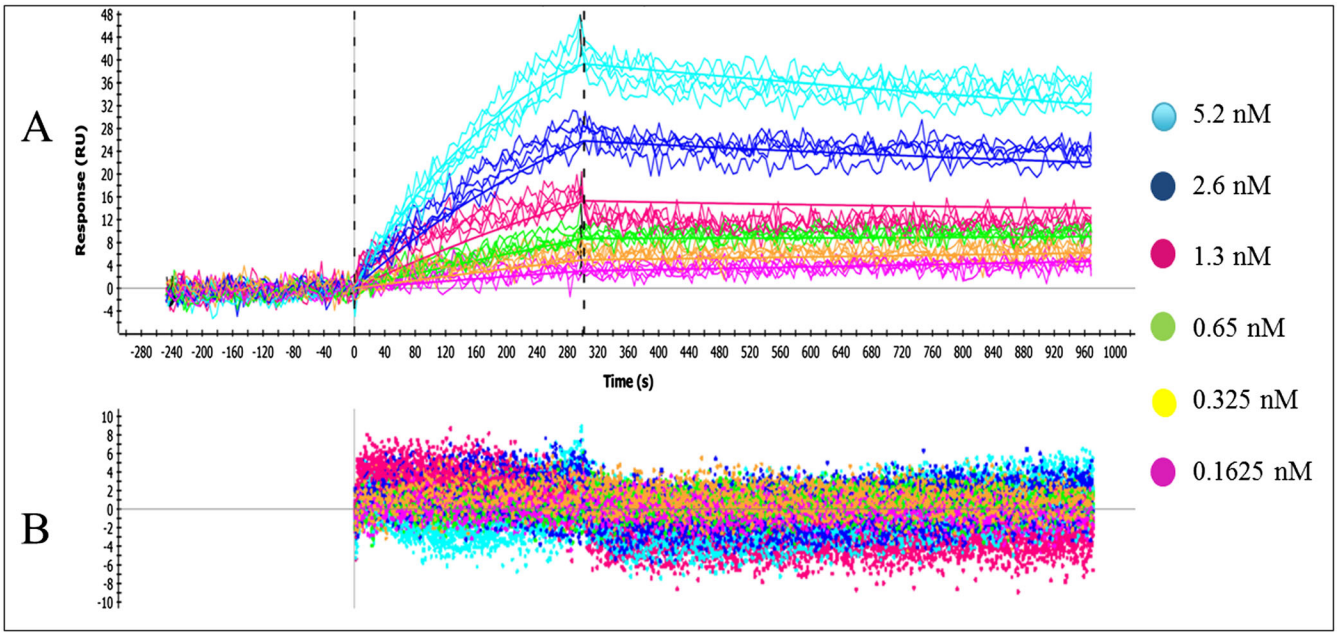

Figure 4. WT NA SPR binding curve fitting using Langmuir 1:1 model with drift. (A) The data presented here are of five independent experiments for six concentrations yielding identical results. The fitted lines (solid lines) pass through the experimental curves. (B) The residuals, showing the goodness of the fit with the original experimental data.

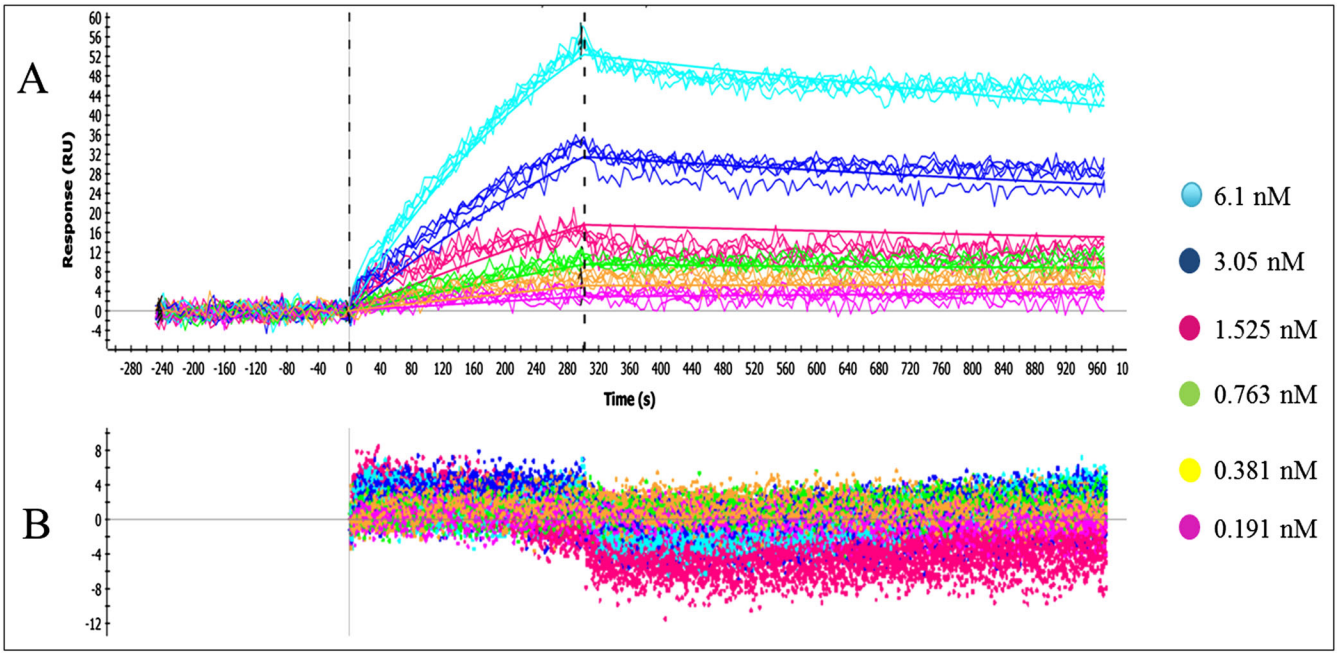

Figure 5. H274Y NA SPR binding curve fitting using Langmuir 1:1 model with drift. (A) The data presented here are of five independent experiments for six concentrations yielding identical results. The fitted lines (solid lines) pass through the experimental curves. (B) The residuals showing the goodness of the fit with the original experimental data.

account using a 1:1 Langmuir binding with drift model. The 1:1 model with drift resulted in χ^2 values $<$10% in both cases, indicating a better fit than using the 1:1 model without drift. Moreover, we are interested in relative binding values between NA isoforms and zanamivir. Hence, the kinetic parameters obtained from 1:1 model with drift, which fit the experimental data best, are used for subsequent discussion. The k_a value of the WT protein was observed to be twice that of H274Y mutant, indicating that the WT NA protein had a slightly stronger affinity to the immobilized ligand than that of the mutant NA. However, there was little difference between the k_d values for WT and H274Y NA. This suggests that after binding to the ligand, both proteins interacted in a similar manner and that the conformational change brought about by the H274Y mutant did not have

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Table 1. SPR kinetic parameters obtained using ProteOn Manager™ Software tools

Curve fitting model	Protein	k_a (1/Ms)	k_a error (1/Ms)	k_d (1/s)	k_d error (1/s)	K_D (nM)	R_{max} (RU)	R_{max} error (RU)	χ^2 (RU)
Langmuir 1:1 without drift	WT	1.05×10^{6}	4.86×10^{3}	2.15×10^{-4}	3.56×10^{-6}	0.205	48.16	1.5×10^{-1}	5.88
	H274Y	5.32×10^{5}	3.17×10^{3}	2.92×10^{-4}	3.11×10^{-6}	0.55	85.60	3.84×10^{-1}	6.86
Langmuir 1:1 with drift	WT	8.01×10^{5}	5.63×10^{3}	4.13×10^{-4}	4.58×10^{-6}	0.516	56.28	3.28×10^{-1}	5.01
	H274Y	4.36×10^{5}	3.91×10^{3}	3.87×10^{-4}	3.91×10^{-6}	0.88	98.62	6.78×10^{-1}	6.50

The kinetic parameters were determined from five independent measurements. k_a and k_d are the association and dissociation rate constants, respectively. The goodness of the fit was then examined by χ^2 value (the average of the squared differences between the measured data point and the fit).

a major impact with zanamivir, in agreement with the results of (Hurt *et al*., 2009). Table 1 summarizes the dissociation constants measured for both proteins.

The measured kinetic parameters suggest that the H274Y mutation did not affect the binding affinity of zanamivir. It is possible, although by no means evident, that minor quantities of one or more structurally similar ligands could have co-immobilised with HDA-zanamivir and contributed to the SPR response. Nevertheless, even in that case, the major ligand immobilised would have been HDA-zanamivir, so differences between the affinities of WT and H274Y for zanamivir would still be reflected in the SPR responses. Zanamivir possesses the same glycerol moiety as sialic acid, and hence, a mutation interfering with the binding of glycerol side chain is less likely to result in resistance to the drug without also weakening binding to sialic acid (Collins *et al*., 2008). This means that H274Y mutant is sensitive to zanamivir, whereas mutation that affects the binding of the guanidine group is more likely to cause resistance to zanamivir, as observed for Q136K mutant (Hurt *et al*., 2009).

Comparison with experimental data from the literature

Monitoring antiviral drug resistance requires measurement of the affinity with an NI as accurately as possible. The MUNANA assay is currently successfully used to monitor antiviral drug resistance. However, the major drawback of this assay has been its low consistency in monitoring clinical isolates with low NA activity. The chemiluminescence assay developed to overcome this drawback exhibited up to 67-fold higher sensitivity for NA detection than the MUNANA assay. However, the substrate used in this assay has a very short half-life of 5 min; the signal intensity must therefore be measured immediately (Wetherall *et al*., 2003), and variability is introduced by time delays prior to measurement. Although these assays are currently extensively used for antiviral drug monitoring, their inherent problems may have a significant impact on treatment guidance and drug development. The SPR method described here is a label-free technique that overcomes the challenges exhibited by the current assays. The SPR assay allows sensitive, real-time monitoring of NA-antiviral drug interactions, without sensitivity to assay time. The kinetic parameters obtained for NA-zanamivir interaction using the SPR assay were compared with inhibition kinetic data reported by (Collins *et al*., 2008). The latter group reported that the experimental inhibition constant (K_i) in their work was effectively the dissociation constant for the antiviral drugs and it was therefore used for comparison with the dissociation constant obtained from our SPR assay (Table 2). However, direct quantitative comparisons between the absolute values of the association and dissociation constants should be treated with caution because the experimental conditions differed, with the most marked difference being that Collins *et al*. used zanamivir in solution, whereas zanamivir was immobilized to a sensor chip in the SPR assay described here. Hence, we compared only the relative K_i and K_D values (H274Y/WT). The relative binding value obtained from the 1:1 Langmuir binding model with drift was 1.7 (and without drift, it was 2.68). The reasonable agreement between this and the relative binding value in the literature (1.9) indicates that the current SPR assay can provide similar results to the existing labelled enzymatic assay but has the advantage that it is highly repeatable and does not depend critically on assay timing.

CONCLUSIONS

This paper describes the development of an SPR assay for accurate monitoring of influenza antiviral drug resistance. A spacer molecule (1,6-hexanediamine) was site-specifically conjugated to the 7-hydroxyl group of zanamivir and the conjugate immobilized onto an SPR sensor chip to obtain a final SPR response of 431 RU. Preliminary SPR experiments showed that this immobilized zanamivir retained its ability to interact biospecifically with NA. The reference-subtracted binding responses obtained for WT and H274Y mutant NA were fitted to a simple Langmuir 1:1 model and a Langmuir 1:1 model with drift, to obtain kinetic parameters, with χ^2 values

Table 2. Comparison of kinetic parameters

Protein	Literature (Collins *et al*., 2008) K_i (nM)	SPR assay K_D (nM) (Langmuir 1:1 with drift)	SPR assay K_D (nM) (Langmuir without drift)
WT	0.1	0.52	0.205
H274Y	0.19	0.89	0.55
Relative binding (H274Y/WT)	1.9	1.7	2.68

<10% of the maximum response for the latter model showing the best fit. The repeatability of responses between runs and the similarity in relative binding values between the WT and H274 variant obtained from the literature and the current SPR assay (1.9 and 1.7, respectively) suggest that this SPR assay can provide equivalent results to the existing labelled enzymatic assay but with greater consistency and without assay time sensitivity.

The SPR assay developed in this work is the first step towards a general label-free biosensor assay for measuring the binding kinetics of NA-drug interactions. We expect that other antiviral drugs could be tethered to parallel ligand channels on the SPR sensor chip, to enable binding affinities of variants of NA with multiple drugs to be compared simultaneously. Thus, there is potential to extend our approach to rapidly screen for antiviral drug affinities as new strains of the influenza virus emerge and to replace low-consistency labelled enzymatic assays.

Acknowledgements

The authors thank the Biomolecular Interaction Centre, University of Canterbury and EBOS Group Ltd. for generously funding this project through a scholarship for BS. The gift of zanamivir intermediates by GlaxoSmithKline (UK) and the assistance of Dr. Robert Young are greatly appreciated.

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