Virus Detection

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Chemical Probes for Drug-Resistance Assessment by Binding Competition (RABC): Oseltamivir Susceptibility Evaluation**

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For the treatment of influenza infections, neuraminidase (NA) inhibitors oseltamivir (Tamiflu) and zanamivir (Relenza) are the most useful therapies.[1–3] Oseltamivir is an oral prodrug that is converted by an endogenous esterase to oseltamivir carboxylate (OS, **1**, Figure 1). Upon the binding of OS to NA, an induced fit of NA to reorient the Glu276 residue toward Arg224 creates a larger hydrophobic pocket for the side chain of OS.[4,5] NAs can evolve resistance to OS with mutations such as H274Y to alter the hydrophobic pocket and thus, result in decreased binding with OS by several hundred fold. In contrast, zanamivir is effective for the H274Y mutant[5] and most influenza viruses because it is structurally similar to the natural substrate and its binding does not require a conformational change of the enzyme. The worldwide surge of drug-resistant influenza viruses has prompted the need for the development of quick tests for OS susceptibility. We propose a new method, namely a resistance assessment by binding competition (RABC) assay, using labeled zanamivir as the chemical probe to identify the influenza subtypes that are resistant to OS but still sensitive to zanamivir. As illustrated in Figure 1, in the presence of OS, zanamivir-based probes can bind the OS-resistant viruses but not the OS-sensitive viruses because of competitive binding of OS. This detection method can be further developed into a quick membrane-based test to enable visual detection, or combined with sugar-array analysis for simultaneous determination of drug resistance and virus serotypes. Recently, many activity-based chemical probes have been reported.[6–8] The RABC assay strategy is not limited to OS resistance detection; it can also be applied to assess other pathogens by using some of the activity-based probes and inhibitors that interact with the same target.

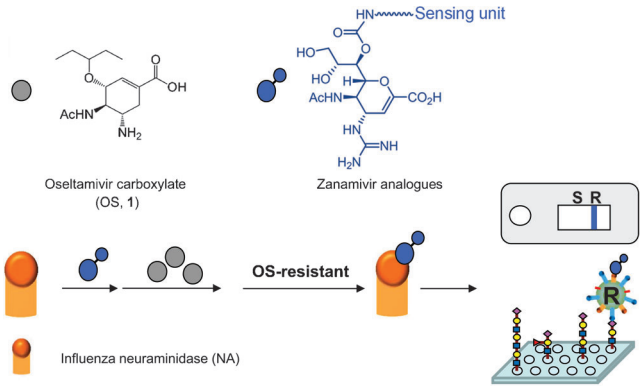

Figure 1. Principle of resistance assessment by binding competition (RABC) assay using influenza neuraminidase as an example. Zanamivir can bind both OS-sensitive and OS-resistant NA in the absence of OS; however, it can only bind the OS-resistant NA when OS is present for binding competition. This method was developed into a membrane-based visual assay or combined with sugar array analysis for simultaneous determination of drug resistance and virus serotypes. S = OS-sensitive viruses; R = OS-resistant viruses.

As a demonstration, the zanamivir-biotin (ZB) conjugate **2** was used in this study, but other zanamivir derivatives with appropriate reporter moieties (Supporting Information, Figure S1) could also be used for the RABC assay. The biotin modification was performed at the 7-OH position of zanamivir (Scheme 1), which is known to have little effect on NA inhibition.[9,10] Indeed, ZB inhibits the NA activity of A/WSN/1933 (H1N1) virus with an IC_{50} value of 7.7 nM, similar to zanamivir (2.1 nM).

Differential ZB binding to OS-sensitive and OS-resistant NAs was demonstrated using NA-transfected cells (Figure S2), virus-infected cells (Figure S3,S4), and the viruses alone (Figure 2). We first used virus-infected cells to identify the optimal conditions to differentiate OS-sensitive from OS-resistant viruses by the RABC assay. The resistance factor (r), defined as the fraction of the binding signal obtained with ZB + OS versus those with ZB alone, was significantly different ($p < 0.001$) between the cells infected with OS-sensitive and OS-resistant viruses (Figure S5). OS/ZB ratios

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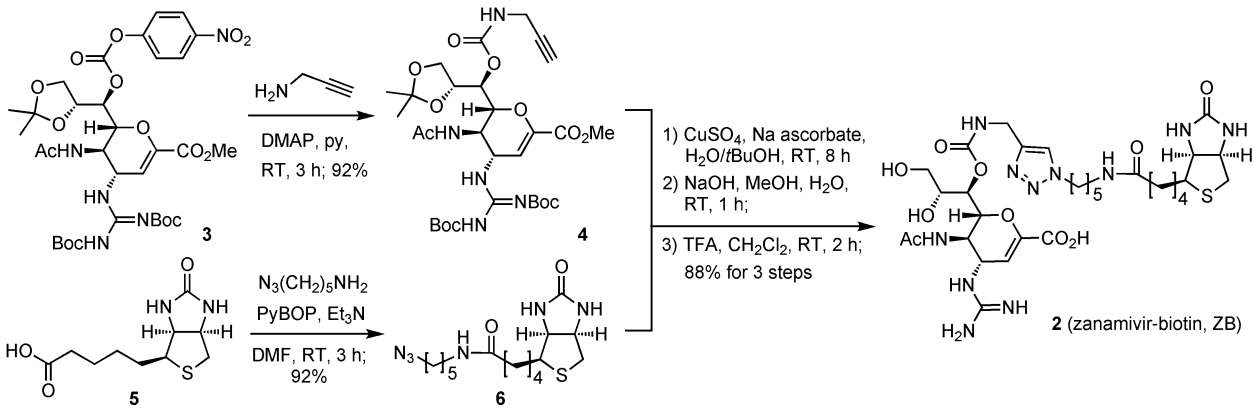

Scheme 1. Synthesis of zanamivir-biotin (ZB) conjugate **2**. Boc = *tert*-butoxycarbonyl, DMAP = 4-dimethylaminopyridine, DMF = *N*,*N*-dimethylformamide, py = pyridine, PyBOP = benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate, TFA = trifluoroacetic acid.

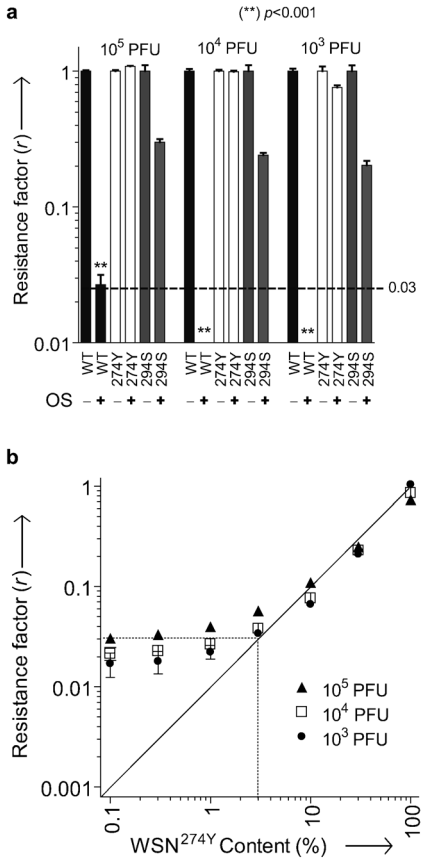

Figure 2. a) The virus samples were immobilized in anti-HA coated microplate wells at 10^5, 10^4, or 10^3 PFU per well. The immobilized viral samples in triplicate wells were incubated with 30 nM ZB or with 30 nM ZB + OS at 150 nM followed by incubation with alkaline phosphatase (AP) conjugated streptavidin. ** = $p < 0.001$. b) Mixed WSN^{wt} and WSN^{274Y} samples at eight different ratios were immobilized in triplicate each in anti-HA coated microwells at the indicated titers and detected as described above.

in the range of 0.5–10 can be used to differentiate the OS-resistant, such as H274Y and N294S, NA mutants (Figure S5). The RABC assay was also used to determine the OS susceptibility of influenza viruses. We used anti-influenza hemagglutinin (HA) coated microtiter wells to capture influenza viruses. After incubation with ZB in the presence or absence of OS, the *r* factor was determined. For the OS-sensitive WSN at 10^3–10^4 plaque forming units (PFU), the measured *r* values are less than 0.01 (Figure 2a). A higher *r* factor of 0.03 was determined, because of increased background values, for higher titer samples at 10^5 PFU (Figure 2b). These results suggested that a threshold for the *r* factor of 0.05 can be applied to define the OS resistance of immobilized influenza samples at the range of 10^3–10^5 PFU. We also determined the detection limit in terms of the percentage of resistant viruses in a given virus pool. Eight 10^5 PFU/mL viral samples containing mixtures of WSN^{wt} and WSN^{274Y} viruses with 0, 0.1, 0.3, 1, 3, 10, 30, and 100% WSN^{274Y} were prepared and processed to determine the *r* factor. Figure 2b shows that the determined *r* factor correlated well with the percentage of OS-resistant viruses for samples with 3% or higher WSN^{274Y} viruses, suggesting that the *r* factor can be used to determine the percentage of the resistant viruses in a given virus sample. For samples with lower OS-resistance contents, the estimation is much inflated.

The RABC assay was then used to query a total of 137 Taiwan seasonal H1N1 clinical isolates collected in the years 2005–2009 for OS-susceptibility determination in a blind test. The results suggested that all of the tested seasonal H1N1 isolates collected before 2008 (Figure 3a) or early in 2008 (Figure 3b) were OS susceptible. Not until mid 2008 was OS-resistant H1N1 isolated in Taiwan (Figure 3b). Similarly, 48 out of 50 seasonal H1N1 samples collected in 2009 were scored as OS resistant (Figure 3c). We also examined the OS susceptibility of the 2009 pandemic H1N1 isolates collected in Taiwan. Figure 3d showed that isolates 1–4 were OS-susceptible and isolates 5–7 were OS-resistant viruses. To evaluate the susceptibility prediction using the RABC assay, we randomly picked (by lab staff not involved in this study) 60 isolates of the seasonal H1N1 samples and the seven pandemic H1N1 strains from Figure 3 to analyze their NA sequences. Consistent with the RABC results, all samples predicted as OS sensitive by the RABC assay have histidine, while those predicted to be OS resistant have tyrosine at residue 274 of the NA (Table S1).

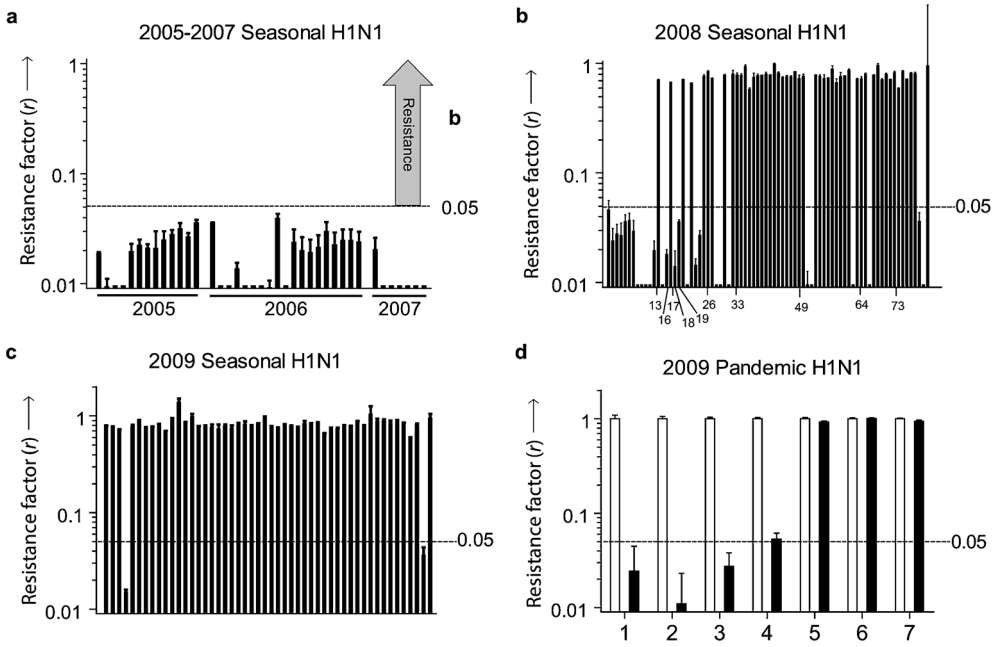

Figure 3. Application of the RABC assay for monitoring the emergence of resistant virus isolates in Taiwan. Seasonal H1N1 clinical isolates collected in years 2005–2007 (a), 2008 (b), and 2009 (c) were used for an OS-susceptibility study. The dashed lines at the *r* factor of 0.05 was the threshold for the susceptibility status of the tested viruses. d) The OS susceptibility of seven 2009 pandemic H1N1 isolates was measured in an identical fashion. Both total (white) and OS-resistant ZB binding (black) are shown.

A quick test on a membrane for visual assessment of the OS susceptibility of influenza viruses was also devised. As a proof of principle, viruses incubated with ZB or ZB + OS were spotted on a poly(vinylidene) fluoride (PVDF) membrane and processed for color development. The staining of the OS-susceptible WSN^{wt} by ZB was blocked by OS competition while the staining of the OS-resistant WSN^{274Y} was resistant to the same competition over 10^4–10^6 virus titers (Figure 4a). In addition to the most-prevalent 274Y mutation, the RABC assay can be applied to the less-resistant 294S mutation using a lower OS concentration for competition (Figure 4b). RABC on a membrane was further shown useful for monitoring the OS susceptibility of several seasonal (Figure 4c), pandemic H1N1 isolates (Figure 4d), and other influenza viruses including H1N1, H3N2, H5N1, and type B influenza viruses (Figure 4e).

Carbohydrate arrays have become a powerful method for identifying pathogens.[11–13] Because each influenza virus has its own characteristic receptor-binding fingerprint, features of the differential binding among influenza virus strains can be developed into a high-throughput method for the identification of influenza subtypes.[14–16] Such binding assays require the presence of NA inhibitors to prevent cleavage of the

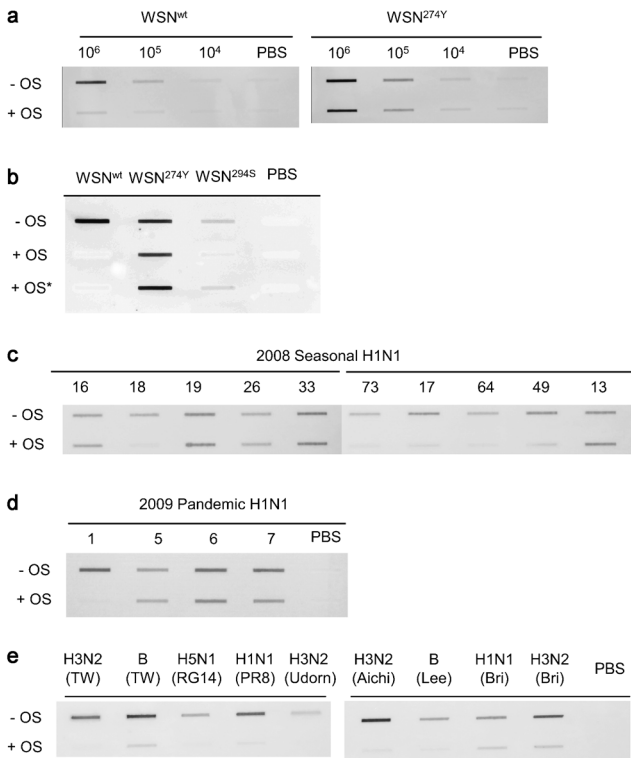

Figure 4. Development of the RABC visual assay and validation. Influenza viruses were incubated with either 30 nM ZB only (−OS) or 30 nM ZB and 150 nM OS (+OS). The treated samples were then absorbed onto a PVDF membrane that was previously immobilized with anti-HA antibodies. The membrane was then processed for color development using AP-conjugated SA. a) The WSN^{wt} or WSN^{274Y} mutant viruses at 10^4, 10^5, and 10^6 PFU. b) The WSN^{wt}, WSN^{274Y}, or WSN^{294S} viruses were also incubated with 30 nM ZB and 30 nM OS (+OS*) to probe the WSN^{294S} virus. c) Ten 2008 Taiwan seasonal H1N1 isolates from Figure 4b. d) Four pandemic H1N1 viral strains from Figure 4d. e) The treated virus samples indicated were directly immobilized onto a PVDF membrane and then processed in an identical fashion. PBS = no virus added to the PBS buffer.

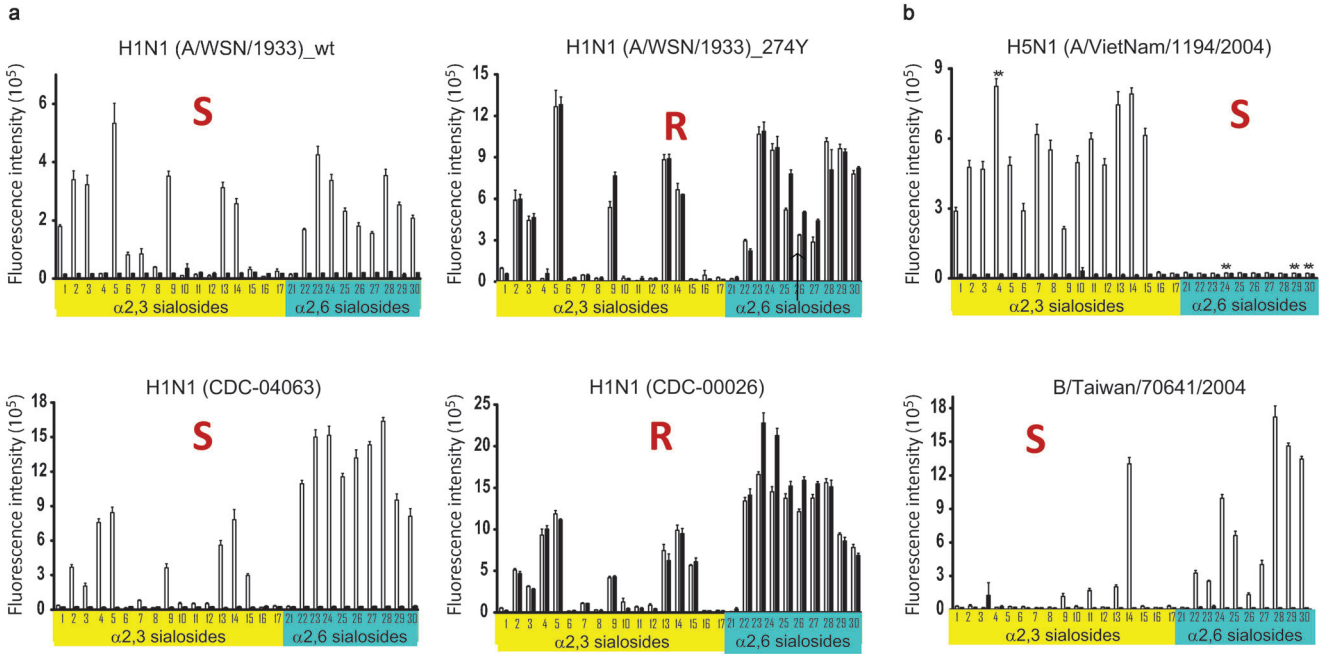

Figure 5. RABC assays on a sugar array allow simultaneous OS-susceptibility evaluation and virus serotyping. Binding profiles of OS-susceptible and OS-resistant H1N1 viruses (a) and other influenza viruses (b) on sialyl oligosaccharides were detected by staining using 100 nM ZB (white) and 100 nM ZB and 500 nM OS (black). S = OS-sensitive viruses; R = OS-resistant viruses. ** = $p < 0.01$ for differentiating the human versus avian influenza viruses.

terminal sialic acid of oligosaccharides in the array. We found that ZB can function as a potent NA inhibitor and can also replace HA antibodies as a universal detection reagent to monitor influenza-virus binding to sugar arrays. Herein, the RABC assay was evaluated on our previously reported sugar arrays containing α2,3- and α2,6-sialosides[16] (Figure S6) for simultaneous determination of OS resistance and the binding preferences of sialosides. As expected, OS susceptibilities of influenza viruses were clearly demonstrated in Figure 5a. The results were consistent with the RABC assays shown in Figure 2, Figure 3, and Figure 4. The sugar-binding patterns of other subtype viruses such as H5N1, or influenza B were also studied with ZB (Figure 5b). Thus, ZB can be used as a sensitive probe for simultaneous detection of HA subtypes and OS susceptibilities.

The existing methods for influenza surveillance are based mainly on immunological tests and sequence analysis. The immunological assay is rapid but limited by antibody specificities. The sequence analysis using reverse transcription polymerase chain reaction (RT-PCR) has high sensitivity in sequencing the defined regions or known mutation(s) of influenza virus genomes. Diagnosis using chemicals may provide an expedient alternative to the immunological assays. For example, biotinylated *S*-sialoside and zanamivir have been used to detect influenza viruses using the strong binding of HA and NA, respectively.[17,18] Our RABC method further showed that not only the most prevalent 274Y virus, but also other mutant viruses with reduced drug susceptibility can be monitored using the zanamivir-biotin conjugate. This is advantageous because our method can be generally applied for any NA mutant that has decreased binding to OS without prior knowledge of sequence changes, allowing surveillance of unprecedented drug-resistant mutations. The RABC assay is a binding-based assay that could be used to develop an instrument-free assay, such as the membrane assay described above, while most of the substrate-based assays, such as the one using an NA-Star kit, are difficult to perform without instruments.

Instead of counting the absolute particle numbers, we focused on using the RABC assay to determine the percentage of resistant viruses in a sample with a detection limit as low as 10^3 virus particles. As a prototype of quick test on a PVDF membrane, as few as 10^4 influenza viruses can be used for OS susceptibility assessment. Although the direct quantitative comparison of our RABC method with other previously reported diagnostic methods[19,20] is difficult because each method characterizes a specific property of the virus under various conditions, our present strategy appears to provide a unique opportunity for simultaneous detection of OS resistance and viral serotypes.

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