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Solid phase assay for comparing reactivation rates of neuraminidases of influenza wild type and resistant mutants after inhibitor removal

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ABSTRACT

The influenza virus neuraminidase inhibitors are normally slow binding inhibitors, but many mutations leading to resistance, also result in the loss of the slow binding phenotype. Mutations can also affect the rate of dissociation of the inhibitors from the neuraminidase, but the assays to measure this require large amounts of virus and are time consuming. To more fully understand the impacts of mutations on the binding and dissociation of the neuraminidase inhibitors we have developed a solid phase reactivation assay, which can use small amounts of crude virus sample bound to an ELISA plate. Multiple viruses can be assayed simultaneously against multiple inhibitors. Using this assay we have demonstrated differences in the relative rates of dissociation of the inhibitors and reactivation of enzyme activity among different influenza A and B viruses for zanamivir, oseltamivir and peramivir. In general oseltamivir dissociated the fastest, and dissociation of peramivir was much slower than both the other inhibitors. Viruses with H274Y, E119V and E119G mutations demonstrated faster dissociation of the inhibitor to which they were resistant. Dissociation of zanamivir and oseltamivir were faster from the D197E mutant, but not of peramivir.

1. Introduction

The influenza virus neuraminidase inhibitors (NAIs) are described as being time dependent slow binding inhibitors (Barrett et al., 2011; Baum et al., 2003; Blick et al., 1995; Kati et al., 1998; Pegg and von Itzstein, 1994; Varghese et al., 1998; Wang et al., 2002). This means that in the enzyme assay used to measure drug sensitivity, in order to achieve optimal inhibition, virus and the NAI must be preincubated, prior to the addition of substrate. Many mutations which lead to NAI resistance, also lead to loss of slow binding of the NAI (Barrett et al., 2011; Baum et al., 2003; Blick et al., 1995; McKimm-Breschkin et al., 1998; Oakley et al., 2010). We have recently developed a simple phenotypic assay which allows the easy identification of slow and fast binding of NAIs and multiple viruses without requiring purified virus or NA, or a detailed knowledge of enzyme kinetics (Barrett et al., 2011; McKimm-Breschkin et al., 2013a; McKimm-Breschkin et al., 2012; McKimm-Breschkin et al., 2013b; Oakley et al., 2010). The analysis uses two assays, where we follow the changes in IC_{50}, each 10 min for 60 min after addition of substrate. In one assay we preincubate virus and the NAI prior to the addition of substrate. In the second assay we simultaneously add virus, NAI and substrate. With the simultaneous addition of all reagents we see a gradual decrease in IC_{50} as the NAI occupies the active site if it is slow binding. For slow binding NAIs pre-incubation enhances binding, leading to lower IC_{50}s compared to the no preincubation reaction. While the NAIs bind slowly to wild type viruses, we saw a loss of slow binding with viruses with NA mutations conferring reduced susceptibility (Barrett et al., 2011; McKimm-Breschkin et al., 2013a; McKimm-Breschkin et al., 2013b; Oakley et al., 2010).

However, we also observed that in the 60 min following addition of substrate in the preincubation reaction, the IC_{50}s generally increased. This suggested some dissociation of the inhibitors despite their continued presence. The rate varied with both virus and NAI. Dissociation of oseltamivir appeared to be faster than zanamivir and peramivir was the slowest. We wanted to understand if this observation truly represented differences in the dissociation rates of the different inhibitors. Analysis of the dissociation or reactivation of NAIs is currently carried out in solution with the

Abbreviations: NA, neuraminidase; NAI, neuraminidase inhibitor; IC_{50}, drug concentration which reduces enzyme activity by 50% compared to untreated enzyme.

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virus/NA reacted with excess inhibitor, and then unbound inhibitor is removed by column chromatography (Bantia et al., 2006; Bantia et al., 2011; Kim et al., 2013; Kiso et al., 2010; Watts et al., 2006). However this is labor intensive, requires large amounts of virus, and only a few samples can be handled, limiting the number of replicates and drugs which can be studied. Thus in addition to our IC_{50} kinetics assay for studying whether the NAIs were slow or fast binding, our aim was to develop a higher throughput 96 well based assay to evaluate the impacts of mutations on dissociation of the NAIs.

2. Materials and methods

2.1. Viruses and inhibitors

Stocks of the following viruses were grown in Madin Darby Canine Kidney Cells, (MDCK): A/Mississippi/03/01 H1N1 wild type and oseltamivir resistant H274Y mutant, (Monto et al., 2006), A/Fukui/45/04 H3N2 wild type and E119V oseltamivir resistant mutant (Tashiro et al., 2009), B/Perth/211/01 influenza B wild type and D197E mutant with decreased susceptibility to all NAIs (Hurt et al., 2006), NWS/G70C H1N9 wild type and E119G mutant with decreased susceptibility to zanamivir and peramivir (Blick et al., 1995; Smith et al., 2002). H5N1 clade 1 A/Chicken/Vietnam/08/2004 and clade 2 A/Chicken/Bangli/BBVD-563/2007, the latter with decreased sensitivity to oseltamivir, (McKimm-Breschkin et al., 2013a) were grown in eggs at CSIRO AAHL and the allantoic fluid was gamma irradiated as previously described (McKimm-Breschkin et al., 2013a).

Zanamivir and peramivir were provided by GlaxoSmithKline (Stevenage, UK) and oseltamivir carboxylate was provided by Dr. Keith Watson (Walter and Eliza Hall Institute, Australia). The fluorescent substrate 4-Methylumbelliferyl N-acetyl-α-D-neuraminic acid (MUNANA) was obtained from Carbosynth (Berkshire, UK) and was diluted to a final concentration of 100 μM in 50 mM sodium acetate pH 5.5 and 5 mM $CaCl_2$.

We used a BMG FLUOstar Optima reader and the kinetics function for real time monitoring of the fluorescent signals. Fluorescence was read using excitation and emission filters 355 and 460 nm respectively. Black ELISA plates (Greiner Fluorotrack-600 high binding plates) were used for all assays.

2.2. Optimization of virus coating dilution

Virus lysates were prepared by freezing and thawing infected MDCK cultures. Samples thus contained cell free virus and NA bound to cell membrane fragments. Immediately prior to use samples were sonicated for at least 3 sequential 1 min cycles in a Soniclean 30 A+ bath, (Transtek systems Australia) sitting on ice between each cycle for 1 min. 50 μl of each virus was added to wells in a 96-well black ELISA plate and serial twofold dilutions were carried out in PBS. Virus was bound at 4 °C overnight. Unbound virus was removed and plates were washed with PBS. 50 μL each of PBS and MUNANA mix were added to each well and the plates were incubated in the fluorimeter at 37 °C. The signal was monitored each 10 min for two hours. A virus dilution was selected for reactivation experiments which still had enzyme activity with a linear rate of reaction and was less than 30% of the threshold linear value for the machine, at the end of the two hours, so that activity would remain linear for at least four hours.

2.3. Inhibition and reactivation

After binding 50 μL of the selected dilution of virus overnight, unbound virus was removed and plates were blocked with PBS-Tween 20 (0.05%). Each inhibitor, at a concentration of approximately 50 times the IC_{50} (Barrett et al., 2011; McKimm-Breschkin et al., 2013a) was diluted in PBS and was added to three or four virus coated wells. PBS was added to two or three wells for the untreated virus controls. The plates were incubated for 30 min at room temperature to allow inhibitor to bind. To minimize time from removal of inhibitor to addition of substrate plates were then rapidly washed four times by submerging in a small container with 0.05% Tween-20 in PBS. The same initial concentration of inhibitor was added back to one well for each virus-inhibitor set of replicates to confirm inhibition, and 50 μl of PBS was added to the remaining wells. MUNANA, 50 μl was added to all wells and the plate was placed in the fluorimeter at 37 °C. Fluorescent units (FU) were recorded and graphed for each well every 10 min for up to 4 h. For each virus inhibitor combinations two or three replicates were done in a minimum of two independent assays (4–9 replicates total). For the H5N1 a single assay in triplicate was carried out. The individual rates of enzyme activity were calculated for each 10 min interval from 0 to 30 then each 30 min to 240 min. Rates were then plotted as a percentage of the maximum rate of the uninhibited control. T½ was calculated as the time taken to reach half the maximum rate of the uninhibited control. The means and standard deviations were then calculated from all replicates. The T½ values were compared between each wild type and mutant pair for each drug by analysis of variance (ANOVA) using Sigmaplot. A probability value of 0.05 was chosen to indicate the values were significantly different.

3. Results and discussion

In order to further understand the impacts of mutations on NAI binding and dissociation, we developed a solid phase reactivation assay which allowed the simultaneous comparison of dissociation of several NAIs and reactivation of enzyme activity from multiple wild type and mutant viruses. We were able to use crude extracts of cell culture grown viruses or crude allantoic fluid, and only 50 μl of diluted virus was needed to coat each well in an ELISA plate based assay. We used a panel of four wild type and mutant virus pairs, as well as a clade 1 and clade 2 H5N1 virus, (McKimm-Breschkin et al., 2013a; McKimm-Breschkin et al., 2007) to evaluate the impacts of the mutations on the dissociation rate of the NAIs. Virus could be bound to ELISA plates, maintaining the NA activity, even when gamma irradiated as for the H5N1 viruses. Mean NA activity was plotted as fluorescent units versus time (FU/min, Figs. 1 and 2) to compare the relative rates of reactivation.

For all the influenza A wild type viruses the dissociation of oseltamivir was generally the most rapid, with little variation in the T½ values from 20 to 30 min (Table 1, Figs. 1 and 2). For the H1N1 H274Y, H3N2 E119V, and clade 2 H5N1 viruses with reduced oseltamivir sensitivity, dissociation was even faster, from 12 to 16 min ($p < 0.001$ compared to each wild type pair). Interestingly T½ values were similar for the mutants, regardless of how high the IC_{50} was (H274Y 2440 nM, E119V 260 nM and clade 2 H5N1 19.6 nM, compared to wild type values of H1N1 3.1 nM, H3N2 5.3 nM and clade 1 H5N1 of 0.6 nM)(Barrett et al., 2011; McKimm-Breschkin et al., 2013a).

Dissociation of zanamivir from wild type viruses was generally slower than of oseltamivir, and with a greater range of T½ from 28 to 109 min. Interestingly while we previously showed that the clade 2 IC_{50} was only slightly lower than the clade 1 IC_{50} (clade 1 = 2.1 nM and clade 2 = 1.3 nM)(McKimm-Breschkin et al., 2013a) between the H5N1 clade 1 and 2 viruses the T½ varied from 28 to 73 min ($p < 0.001$). This result is consistent with our previous IC_{50} kinetics analysis where we showed that from 10 to 60 min after addition of substrate in the preincubation reaction, there was a greater increase in IC_{50} for a clade 1 (4.5-fold) compared to

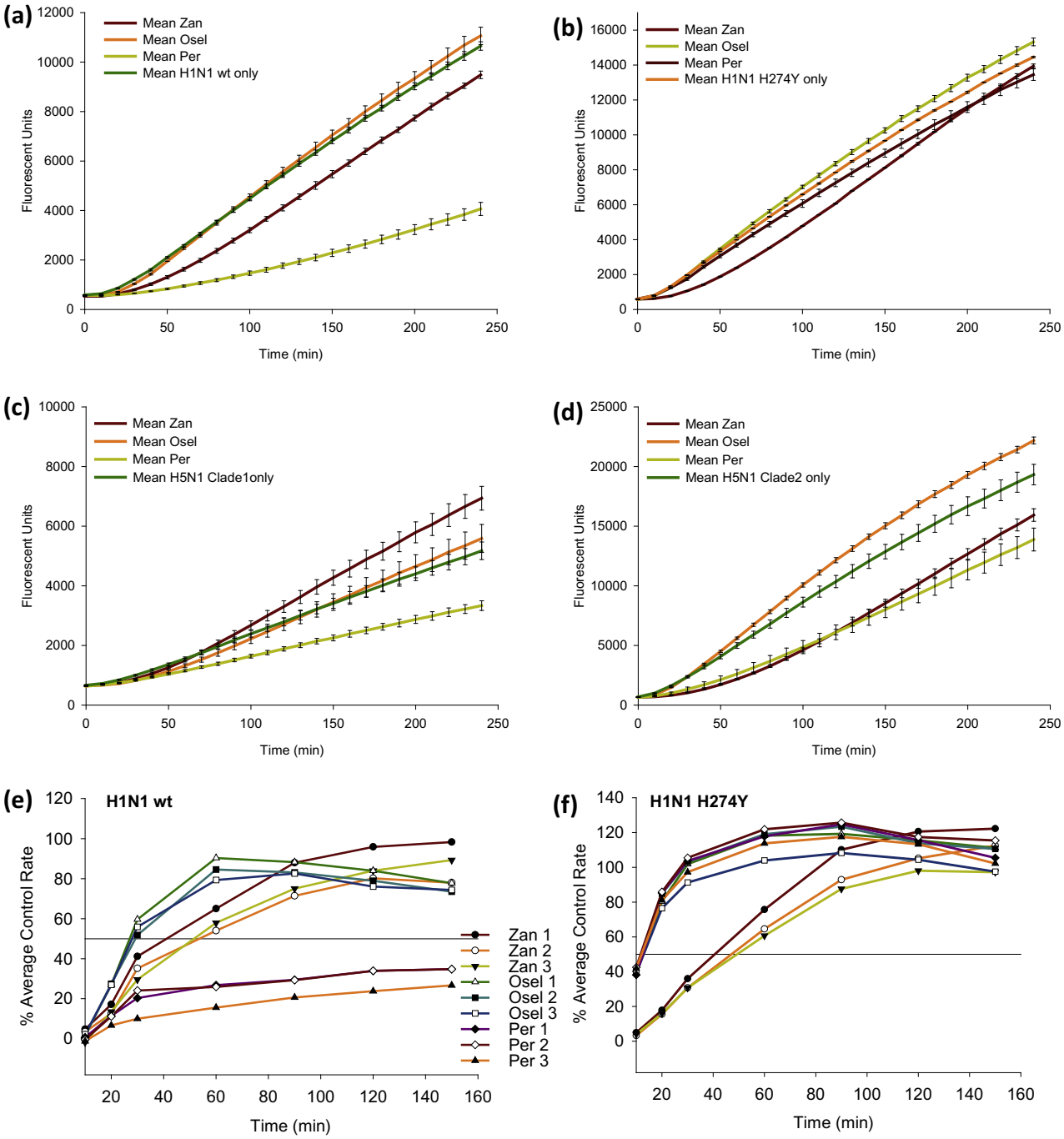

Fig. 1. Reactivation kinetics for N1 viruses. Time dependent recovery of enzyme activity after removal of inhibitors and addition of MUNANA substrate. (a) A/Mississippi/3/01 H1N1 wild type, (b) A/Mississippi/3/01H1N1 H274Y oseltamivir and peramivir resistant mutant, (c) A/Chicken/Vietnam/08/2004 clade 1 H5N1 wild type, (d) A/Chicken/Bangli/BBVD-563/2007 clade 2 H5N1 with decreased oseltamivir sensitivity. The three drugs were all assayed simultaneously against the same virus on the same plate. Each curve represents the mean of two or three replicates from one experiment, and standard error bars are shown. Individual plots of rate of enzyme activity against time used to calculate T½ values for (e) A/Mississippi/3/01 H1N1 wild type (f) A/Mississippi/3/01 H1N1 H274Y mutant. The H274Y mutation results in very rapid dissociation of both oseltamivir and peramivir, correlating with resistance to both.

a clade 2 (2.2-fold) for zanamivir, suggesting a faster dissociation (Barrett et al., 2011). For the E119G virus with high zanamivir resistance, dissociation was so rapid, <10 min, we could not determine any difference from the uninhibited virus control. Although the E119V mutation does not affect the IC_{50} for zanamivir, there was faster reactivation compared to the wild type virus (70 min compared to 109 min, p value < 0.005). This surprising observation is also consistent with our previous IC_{50} kinetics results comparing the changes in IC_{50} from 10 to 60 min with zanamivir in the preincubation reaction 1.9-fold compared to 1.3-fold for the wild type (Barrett et al., 2011). Thus this novel assay demonstrates that there are subtle impacts on drug binding both with the H5N1 and E119V mutant not readily seen by just determining just a single IC_{50}.

Enzyme reactivation after peramivir treatment was slower for all wild type influenza A viruses than for the other two NAIs; many had a T½ longer than the 240 min reaction time. Mutations confer-

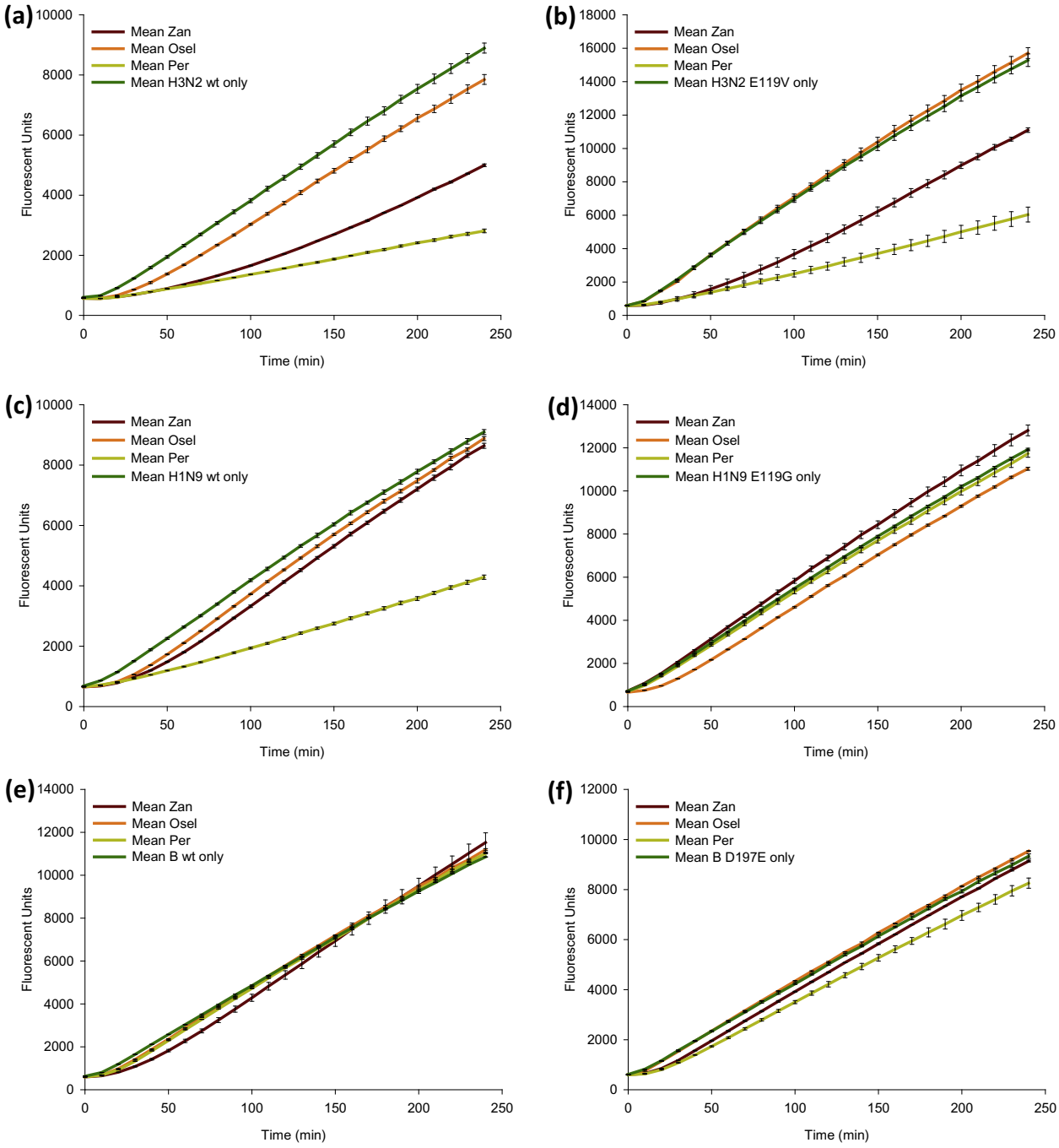

Fig. 2. Reactivation kinetics for N2, N9 and influenza B viruses. Time dependent recovery of enzyme activity after removal of inhibitors and addition of MUNANA substrate. (a) A/Fukui/45/04 H3N2 wild type, (b) A/Fukui/45/04 H3N2 E119 V oseltamivir resistant mutant, (c) NWS/G70C H1N9 wild type, (d) NWS/G70C H1N9 E119G zanamivir and peramivir resistant mutant, (e) B/Perth/211/01 wild type and (f) B/Perth/211/01 D197E multi resistant mutant. The three drugs were all assayed simultaneously against the same virus on the same plate. Each curve represents the mean of two or three replicates from one experiment, and standard error bars are shown. The E119G mutation results in very rapid dissociation of both zanamivir and peramivir, correlating with resistance to both, and all drugs dissociate rapidly from the influenza B virus.

ring peramivir resistance had the most dramatic effect on the dissociation rate of all NAIs, with reductions in T½ from >240 min to ⩽10 min for the E119G mutant and from >240 min to 14 min for the H274Y mutant. Others have also demonstrated that peramivir has a slower off rate compared to zanamivir, and this is slower than for oseltamivir for N1, N2, N9 and B wild type viruses (Bantia et al., 2006; Bantia et al., 2011; Kiso et al., 2010). While they used the classical method of spin column separation of reagents, neither group studied any mutants. Additionally one group (Kiso et al., 2010) used the time to reach a defined fluorescent units signal, and the relative reactivation was then compared to the time taken for the uninhibited control. However, the time to reach a predetermined signal is dependent on the amount of virus used and will vary for different amounts of virus. In contrast as we use rates of enzyme activity which are still in the linear range, the relative reactivation times can be compared across all viruses and drugs, independent of the absolute signal.

The NAIs show higher IC_{50}s for wild type influenza B viruses than for influenza A viruses (Barrett et al., 2011; McKimm-Breschkin, 2013) and here we show that the NAIs dissociated more

Table 1
Time (T½, min) for reaction rates to reach half the maximum rate of the uninhibited control.

Virus	Zanamivir		Oseltamivir		Peramivir	
	Mean	SD	Mean	SD	Mean	SD
H1N1 wt	41.3	7.7	25.4*	1.8	>240*	ND
H1N1 H274Y	43.2	9.2	14.5*	2.0	13.8*	1.9
H5N1 Clade 1	27.7*	2.7	31.2*	4.4	59.9	10.9
H5N1 Clade 2	72.8*	5.1	15.6*	0.6	84.4	0.3
H3N2 wt	108.7*	18.8	30.2*	3.2	>240	ND
E119V	70.1*	9.7	12.2*	0.5	>240	ND
H1N9 wt	32.1*	1.6	24.0	1.8	>240*	ND
E119G N9	<10*	ND	21.5	2.2	⩽10*	ND
B wt	24.6*	3.5	15.1*	1.3	18.9	0.9
B D197E	17.0*	1.9	10.9*	0.9	18.8	5.4

All drugs were assayed in duplicate or triplicate, in at least two independent assays. T½ values were calculated for each sample and means were calculated from all assays, (4–9 independent values), except for the H5N1 which were from triplicate wells in one assay.
* $P < 0.005$ for the comparison between wild type and mutant pair by one way ANOVA.

rapidly from the wild type B/Perth/211/01 virus compared to almost all the influenza A viruses., There was an especially dramatic difference for peramivir, of T½ values of >240 min for the human influenza A strains, compared to 19 min for the influenza B. However, although the D197E mutation reduces sensitivity to all three NAIs, (8.9 versus 258 nM for zanamivir, 144 versus 708 nM for oseltamivir and 2.8 versus 41.5 nM peramivir for wild type and mutant respectively) (Barrett et al., 2011) reactivation of the zanamivir and oseltamivir treated mutant viruses was faster compared to the zanamivir or oseltamivir treated wild type. In contrast, reactivation times of the peramivir-treated wild type and mutant virus were similar. This may be because dissociation of peramivir is already so much more rapid for the influenza B wild type virus compared to the other viruses that there is little further alteration in the rate for the mutant.

In our previous IC_{50} kinetics experiments (Barrett et al., 2011; McKimm-Breschkin et al., 2013a) all the influenza A mutants studied had lost slow binding to the specific inhibitor to which they were resistant, and also demonstrated faster apparent dissociation, despite the continued presence of the inhibitors. These results are consistent with our more direct assay presented here, where all the mutants, apart from the influenza B/Perth/211/01 D197E virus with peramivir, also showed faster dissociation compared to the wild type virus. Thus while not providing a quantitative comparison of the off rates, the IC_{50} kinetics graphs also provide a good qualitative indication of the relative dissociation rate for the wild type viruses, even though the NAIs remain in the assay. Either in the presence of the NAIs in the IC_{50} kinetics assays or our novel solid phase assay in the absence of the NAIs, we have demonstrated that in general dissociation of oseltamivir was faster than of zanamivir, with peramivir the slowest. The initial high IC_{50}s seen by the first 10 min in the IC_{50} kinetics preincubation reactions for many of the mutants correlate with the very rapid initial dissociation seen here.

Hence with our simple ELISA plate based reactivation assay and our IC_{50} kinetics assay we have now developed two high throughput assays which help provide an insight into the impacts of mutations on NAI binding and dissociation, and reveal subtle changes in binding/dissociation not previously observed.

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Conflicts of Interest

The authors have no conflicts of interest.

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