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Short Communication

Neuraminidase mutations conferring resistance to laninamivir lead to faster drug binding and dissociation

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ABSTRACT

The neuraminidase (NA) inhibitors oseltamivir and zanamivir are administered twice daily for 5 days for treatment of influenza. Laninamivir is a 7-methoxy derivative of zanamivir, but a single dose is effective when taken as the laninamivir octanoate prodrug. We show here in IC_{50} kinetics assays and a solid phase reactivation assay that compared to zanamivir laninamivir also demonstrates slow binding to but slower dissociation from multiple wild type NAs. A D197E mutation in an influenza B and an E119G in an N9 neuraminidase which confer 15- and 150-fold resistance to laninamivir result in faster binding and dissociation. Despite similar IC_{50}s our assays demonstrate more rapid dissociation of laninamivir from clade 1 compared to 2 H5N1 NAs.

Currently two types of influenza neuraminidase inhibitors (NAI) are licensed globally for the treatment and prevention of influenza. Zanamivir (Relenza) is taken twice daily for 5 days by inhalation and oseltamivir (Tamiflu) is taken orally twice daily for 5 days. Two newer drugs peramivir (Rapiacta) which is administered intravenously and a long acting derivative of zanamivir, laninamivir octanoate (prodrug lnavir, active compound laninamivir) delivered by a single inhaled dose, have been available in Japan since 2010. Laninamivir, generated from laninamivir octanoate, not only resides in the lungs of mice for many days (Kakuta et al., 2013; Kubo et al., 2010), but its dissociation from seasonal and pandemic N1, N2 and B wild type neuraminidases is slower than for other NAIs (Kiso et al., 2010; Yamashita et al., 2011). Some studies have been carried out on the susceptibility to laninamivir of strains primarily resistant to oseltamivir and more recently zanamivir (Fujisaki et al., 2012; Leang et al., 2014; Samson et al., 2014; Thabet et al., 2010). As for zanamivir, laninamivir retains activity against H1N1 H274Y, N294S and H3N2 E119V oseltamivir resistant mutants, but E119G and E119A confer cross-resistance to zanamivir and laninamivir.

The NAIs are defined as being time dependent or slow binding inhibitors (Hart and Bethell, 1995; Kati et al., 1998; Pegg and von Itzstein, 1994). This means that preincubation with inhibitor is necessary to achieve maximum occupancy of the NA enzyme active site and thus maximum inhibition. Many mutations which affect drug sensitivity also lead to loss of slow binding, hence preincubation does not lead to enhanced inhibitor binding and IC_{50}s are similar with or without preincubation (Barrett et al., 2011; Baum et al., 2003; Fujisaki et al., 2012; McKimm-Breschkin et al., 2013; Oakley et al., 2010). However, mutations can also impact on the rate of dissociation of the NAIs. Thus higher IC_{50}s can be as a result of loss of slow binding and/or faster dissociation.

We have developed a simple 96-well based phenotypic IC_{50} kinetics assay to evaluate whether drugs are fast or slow binding, based on changes in IC_{50} over time, (Barrett et al., 2011; McKimm-Breschkin et al., 2012; Oakley et al., 2010). We have also recently developed a 96-well based solid phase assay for monitoring dissociation of drug, by following reactivation of enzyme activity. (Barrett and McKimm-Breschkin, 2014). This assay can handle many more virus-drug combinations than the classic method of using individual tubes and spin columns for each sample (Bantia et al., 2011; Kim et al., 2013; Kiso et al., 2010).These assays have provided a greater insight into the impacts of mutations on drug binding and dissociation than single IC_{50} values. We have shown a strong correlation between loss of slow binding of zanamivir, oseltamivir and peramivir and faster dissociation for drugs to which the mutant is resistant. Here we have further explored the impacts of mutations on the kinetics of laninamivir binding and dissociation.

We had a series of matched wild type and mutant virus pairs, which allowed us to directly compare the impacts of mutations

Abbreviations: NA, neuraminidase; IC_{50}, amount of drug to reduce enzyme activity by 50%; ELISA, enzyme linked immunosorbent assay.

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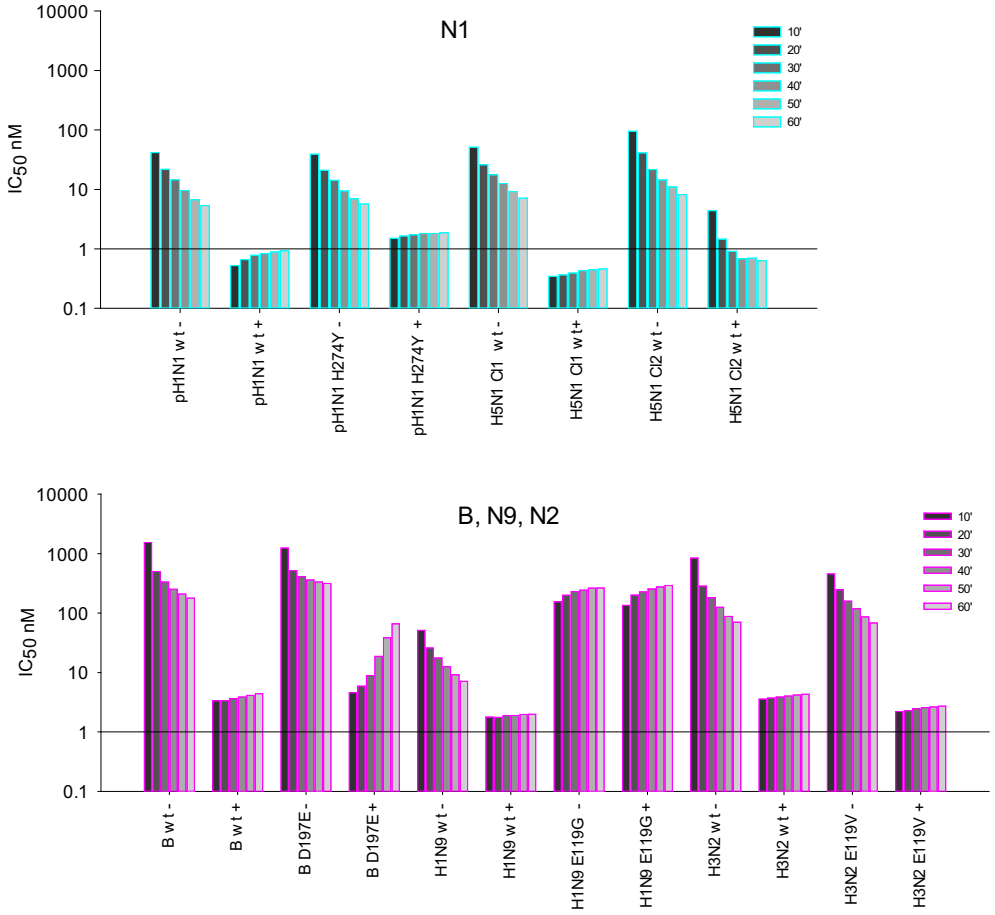

Fig. 1. IC_{50} kinetics of inhibition by laninamivir. Wild type and mutant viruses were preincubated for 30 min with laninamivir (+) before addition of the MUNANA substrate, or virus, laninamivir and MUNANA substrate were co-incubated, with no preincubation (−) (Barrett et al., 2011). Slow binding is shown by higher IC_{50}s without preincubation compared to with preincubation. (a) Laninamivir bound slowly to all N1 NAs, but appeared to bind even slower to the clade 2 H5N1 NA, since the IC_{50} continued to decrease even after preincubation. (b) The IC_{50}s for the B virus with the D197E mutation increase faster compared to the wild type in the preincubation reaction. Complete loss of slow binding was seen for the E119G mutant, as values are similar with or without preincubation. Results are means of duplicate assays. Virus abbreviations, wt = wild type, mutants have specific mutation defined. pH1N1 = A/Perth/265/09, pH1N1 H274Y = A/Perth/261/09, H5N1 cl1 = A/Chicken/Vietnam/08/2004 clade 1, H5N1 cl2 = A/Chicken/Bangli/BBVD-563/2007 clade 2, B = B/Perth/211/01, H1N9 = A//NWS/Tern Australia/G70C, H3N2 = A/Fukui/45/04.

Table 1
Binding and dissociation kinetics of laninamivir.

Virus	IC_{50} nM[a]			IC_{50} nM		Reactivation
	−pre 60′[b]	+pre 60′[b]	60′ −pre/+pre IC_{50}s[c]	+pre 10′	IC_{50} 60′/IC_{50} 10′[d]	*T½* min (SD)[e]
pH1N1 wt	6.7	0.9	7.4	0.5	1.8	⩾240
H274Y	7.0	1.9	3.7	1.5	1.2	⩾240
H5N1 clade 1	7.1	0.5	14.2	0.3	1.3	59.1 (16.1)
Clade 2	8.2	0.6	13.7	4.4	0.1	⩾240*
H3N2 wt	70.1	4.3	16.3	3.6	1.2	>240
E119V	68.4	2.7	25.3	2.2	1.2	219.3 (37.8)**
H1N9 wt	7.1	2.0	3.6	1.8	1.1	118.8 (13.1)
E119G	264	290	0.9	134	2.2	⩽10*
B wt	179.1	4.4	40.7	3.3	1.3	120.9 (11.9)
D197E	315.4	66	4.8	4.6	14.4	23.8 (2.8)*

[a] IC_{50}s are calculated from duplicate assays.
[b] −pre = no preincubation, virus, laninamivir and substrate all coincubated, +pre = virus + laninamivir preincubated for 30 min prior to addition of substrate.
[c] −pre/+pre gives an indication of fast or slow binding. The higher the ratio, the slower the binding. Fast binding inhibitors have ratios around 1.0 as preincubation makes no difference.
[d] IC_{50} 60′/IC_{50} 10′ indicates the relative rate of inhibitor dissociation in the IC_{50} kinetics assay.
[e] *T½* values are calculated from the solid phase reactivation assay, with 2–3 replicates in 2–3 assays (6–9 replicates).
* Difference between pairs significant by one way ANOVA ($p < 0.005$).
** As *T½* ≫ 240, using a wild type value of 300 min for ANOVA gave $p < 0.005$.

on both the IC_{50} kinetics of laninamivir binding as well as the relative rates of dissociation. Stocks of the following viruses were grown in Madin Darby Canine Kidney Cells, (MDCK), A/Perth/265/09 pandemic H1N1 wild type and A/Perth/261/09 oseltamivir resistant H274Y mutant (ISIRV Antiviral Group) A/Fukui/45/04 H3N2 wild type and E119V oseltamivir resistant mutant (Tashiro