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# The Val430Ile neuraminidase (NA) substitution, identified in influenza B virus isolates, impacts the catalytic 116Arg residue causing reduced susceptibility to NA inhibitors

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ABSTRACT

As part of a 2015–2018 clinical trial of peramivir treatment for acute influenza infections in the elderly, an influenza B/Yamagata/16/1988-like isolate harbouring a Val430Ile neuraminidase (NA) substitution was recovered from a single patient. This substitution was detected in respiratory samples collected before and during peramivir treatment. In NA inhibition assays, oseltamivir, zanamivir and peramivir IC50s of the Val430Ile isolate were 4-, 15- and 16-fold higher compared to a wild-type (WT) strain. In reverse genetics experiments, the Ile430Val reversion restored the drug susceptible phenotype. The Val430Ile mutant and the WT strain had comparable replication kinetics in ST6GalI-MDCK cells and the NA mutation was stable after four passages in that cell line. Molecular dynamics simulations suggested that Val430Ile impacts the NA binding through a mechanism involving the catalytic Arg116 residue. The potential of some NA mutations not part of the active site to alter the susceptibility to NA inhibitors highlights the need to develop novel antiviral strategies against influenza B infections.

## 1. Introduction

Influenza B viruses from B/Yamagata/16/1988 and B/Victoria/2/1987 antigenic lineages are co-circulating since the 1980s (Rota et al., 1990). Influenza B infections generally cause 20–30% of total seasonal influenza but this rate can increase to ≥50% during some seasons (Paul Glezen et al., 2013). For example, during the 2017–18 influenza epidemic, influenza B viruses from the B/Yamagata/16/1988 antigenic lineage accounted for 60% and 50% of all influenza infections in Europe (Adlhoch et al., 2018) and Canada (Skowronski et al., 2018), respectively.

Neuraminidase inhibitors (NAIs), including oseltamivir, zanamivir and peramivir constitute the main class of antivirals currently recommended for the prophylaxis and treatment of influenza A and B infections (Samson et al., 2013). Influenza B isolates have higher $IC_{50}$ values against NAIs (in particular against oseltamivir) compared to influenza A viruses (Boivin and Goyette, 2002). Accordingly, oseltamivir was found to be less effective against influenza B than against influenza A infections with regard to duration of fever and virus persistence (Kawai et al., 2006). Therefore, management of clinical influenza B infections could be more complicated in the advent of influenza B variants with NA substitutions conferring incremental reduced susceptibility to NAIs.

Previous in vitro studies and clinical reports showed that reduced susceptibility to NAIs generally results from amino acid (a.a.) substitutions within the framework or catalytic region of the NA enzyme (McKimm-Breschkin, 2013). For instance, framework (Glu117Ala, Ile221Thr, His273Tyr and Asp197Glu/I; B numbering here and throughout the text) and catalytic (R150K and Arg374Lys) NA substitutions have been shown to confer reduced inhibition (RI, between 5- and 50-fold increases in the $IC_{50}$ value over the WT) or highly reduced inhibition (HRI, > 50-fold increases in the $IC_{50}$ value) for one or more NAIs in influenza B variants (WHO, 2011). Additionally, drug-resistant variants with mutations outside the NA catalytic site have been reported (Abed et al., 2019; Hatakeyama et al., 2007).

Herein, we report on the detection and characterization of a clinical influenza B/Yamagata/16/88 variant containing a new NA substitution (Val430Ile), responsible for reduced susceptibilities to NAIs. This

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**Table 1**
Susceptibility profiles of influenza B isolates determined by NA assays using the fluorescent (MUNANA) substrate.

| Isolates | Patient#/Peramivir status[a] | Peramivir $IC_{50}$ (nM)[b] | Fold[c] [phenotype][d] | Oseltamivir $IC_{50}$(nM)[b] | Fold[c] [phenotype][d] | Zanamivir $IC_{50}$(nM)[b] | Fold[c] [phenotype][d] |
|---|---|---|---|---|---|---|---|
| 1 | 008-605/Day 1 | 0.31 ± 0.03 | 0.79 [NI] | 7.12 ± 0.05 | 0.56 [NI] | 0.70 ± 0.03 | 0.83 [NI] |
| 2 | 006-606/Day 1 | 0.30 ± 0.07 | 0.76 [NI] | 9.81 ± 0.81 | 0.77 [NI] | 0.74 ± 0.11 | 0.88 [NI] |
| 3 | 011-607/Day 1 | 0.47 ± 0.04 | 1.20 [NI] | 16.99 ± 1.81 | 1.34 [NI] | 1.23 ± 0.22 | 1.46 [NI] |
| 4 | 011-607/Day 3 | 0.37 ± 0.05 | 0.94 [NI] | 14.78 ± 0.32 | 1.16 [NI] | 1.01 ± 0.10 | 1.20 [NI] |
| 5 | 011-608/Day 3 | 0.40 ± 0.07 | 1.02 [NI] | 9.82 ± 2.4 | 0.77 [NI] | 0.76 ± 0.04 | 0.90 [NI] |
| 6 | 011-609/Day 1 | 0.53 ± 0.05 | 1.35 [NI] | 12.48 ± 7.21 | 0.98 [NI] | 0.82 ± 0.14 | 0.97 [NI] |
| 7 | 011-609/Day 3 | 0.49 ± 0.01 | 1.25 [NI] | 12.57 ± 0.63 | 0.99 [NI] | 0.87 ± 0.17 | 1.03 [NI] |
| 8 | 020-607/Day 1 | 6.06 ± 0.87 | 15.53 [RI] | 50.28 ± 5.19 | 3.96 [NI] | 13.68 ± 0.11 | 16.28 [RI] |
| 9 | 020-607/Day 3 | 7.17 ± 1.53 | 18.38 [RI] | 42.98 ± 9.82 | 3.39 [NI] | 12.41 ± 0.97 | 14.77 [RI] |
| B/Phuket/3073/13 | Na | 0.39 ± 0.02 | 1 [NI] | 12.67 ± 0.71 | 1 [NI] | 0.84 ± 0.06 | 1 [NI] |

Na, not applicable.
[a] Day 1 is the pre-therapy sample; Day 3 is a sample collected after 3 days of peramivir therapy.
[b] Mean $IC_{50}$ values ± SD were obtained from two independent experiments in duplicate.
[c] Fold changes as compared to the B/Phuket/3073/13 $IC_{50}$.
[d] Resistance phenotype in accordance with WHO criteria: Normal inhibition (NI, ≤ 5-fold increase in $IC_{50}$ value over the WT); RI, Reduced Inhibition (between 5- and 50-fold increase); HRI, Highly Reduced Inhibition (> 50-fold increase).

isolate was recovered on Day 1 (pre-therapy) and Day 3 of peramivir part of a clinical trial of peramivir treatment for acute influenza infections (BioCryst, BCX1812-306). This was a phase 3, US multicenter, single-arm, open label study to evaluate the safety, pharmacokinetics and effectiveness of intravenous peramivir in elderly subjects with acute uncomplicated influenza infection and in subjects at higher risk for influenza complications (NCT 02635724; 2015–2018).

Molecular characterization of these viruses was done by specific RT-PCR amplifications of the hemagglutinin (HA) and neuraminidase (NA) genes and Sanger sequencing (ABI Prism 377 DNA sequencer, Applied Biosystems, Foster City, CA). Nasopharyngeal samples (NPS) were also inoculated into ST6GalI-MDCK cells for phenotypic characterization.

As shown in Table 1, a total of 9 influenza B isolates from 6 patients were characterized. Positive cultures could be obtained with both Day 1 and Day 3 NPSs from patients 011–607, 011–609 and 020–607 whereas only Day 1 or Day 3 sample grew viruses for the remaining patients. No viral growth was obtained with any NPS collected on Day 7. HA/NA sequence analysis of both NPS and their respective ST6GalI-MDCK grown isolates revealed that these B viruses were related to the B/Phuket/3073/2013 vaccine strain which is part of the B/Yamagata/16/88 antigenic lineage.

In NA inhibition assays using the methylumbelliferyl-N-acetylneuraminic acid substrate (MUNANA) (Samson et al., 2014), $IC_{50}$ values obtained for isolates 1–7 were similar to those of the WT control (B/Phuket/3073/2013, kindly provided by the NIBSC), ranging between 0.30 nM and 0.53 nM, 7.12 nM and 16.99 nM, and 0.70 nM and 1.23 nM for peramivir, oseltamivir and zanamivir, respectively. By contrast, isolates 8 and 9 (Days 1 and 3 samples, respectively, from patient 020–607) exhibited RI phenotypes against peramivir and zanamivir and had an oseltamivir $IC_{50}$ that increased by ≈ 4-fold compared to the WT. The NA and HA genes from patient 020–607 were identical for days 1 and 3 and shared 99.5% and 98.9% amino acid identities, respectively, with B/Phuket/3073/2013.

Clustal alignments of the NA amino acid sequences were performed to identify a molecular marker of NAI resistance. As shown in Fig. 1A, the 8 functional and 11 framework residues (Colman et al., 1993) were conserved in the B/Phuket/3073/2013 and 020–607 NA protein sequences whereas they differed at 5 positions elsewhere (Gly17Glu, Ala67Val, Asp34Asn, Lys373Gln and Val430Ile). Except for the Val430Ile substitution, the other four changes were detected in isolates with unaltered NAI susceptibilities and these substitutions were common among recent influenza B strains as revealed by blast analysis of public sequence database (not shown). Of interest, Ile430 (Ile427 in N2 numbering) is a highly conserved residue among influenza A subtypes (Fig. 1B). We have recently shown that the Ile430Thr substitution conferred a cross-RI phenotype against all tested NAIs in recombinant influenza A(H1N1)pdm09 virus (Tu et al., 2017). Contrasting with influenza A viruses, the influenza B NA protein contains valine at position 430 and this residue is also conserved among both B/Yamagata/16/1988 (accession number CY018767) and B/Victoria/2/1987 (AB036870) viruses and their current representative vaccine strains [i.e., B/Phuket/3073/2013 (EPI544263) and B/Colorado/06/2017 (ASK81297)], respectively (Fig. 1B).

To confirm that the Val430Ile change was responsible for the altered NAI susceptibility observed for 020–607 isolates, the NA gene from patient 020–607 was cloned into the bidirectional pBZ plasmid and the reversion mutation (Ile430Val) was inserted by PCR-directed mutatgenesis. The resulting pBZ-$NA_{430Ile}$ or pBZ-$NA_{Ile430Val}$ plasmid was co-transfected with pBZ plasmids containing the remaining seven B/Phuket/3073/2013 segments into 293T-MDCK co-cultures for the rescue of 7:1 reassortants by reverse genetics (Fage et al., 2018). NA inhibition assays performed with 7:1 reassortants confirmed RI/HRI phenotypes for the reassortant containing the NA from patient 020–607 (430Ile), with mean peramivir, oseltamivir and zanamivir $IC_{50}$ values of 44.54 (114.1-fold over the WT 7:1 reassortant), 215 (16.9-fold) and 125 (148.6-fold), respectively; and demonstrated that the Ile430Val reversion was associated with a susceptible phenotype with mean $IC_{50}$ values of 0.68 nM (1.07-fold over the 7:1 WT reassortant), 15.03 nM (1.06-fold) and 0.99 nM (1-fold) for peramivir, oseltamivir and zanamivir, respectively.

When assessed by replication kinetics experiments using ST6GalI-MDCK cells infected at a multiplicity of infection (M.O.I.) of 0.0001 PFUs/cell (Abed et al., 2019), the WT and Val430Ile isolates showed comparable titers at all time-points (Fig. 2A). In the NA enzyme kinetics experiments using the equivalent of $10^{10}$ copies/mL of viruses, based on quantitative RT-PCR targeting the NS1 gene (Selvaraju and Selvarangan, 2010), the WT and Val430Ile NA enzymes displayed comparable mean Km (39.45 vs 25.84 μM) and Vmax (19.97 vs 15.12 U/sec) values (Fig. 2B). After 4 passages in ST6GalI-MDCK cells and A549 cells, we found that Val430Ile mutation was conserved with no additional HA/NA sequence changes, suggesting the genetic stability of this NA mutant.

In order to decipher the molecular basis of the observed reduced inhibition to NAIs, we performed molecular dynamics (MD) simulations and trajectory analysis as detailed in the supplementary text (Tu et al., 2017). As summarized in Fig. 3, some interactions of the Val430 residue with His439, Leu132 and Arg116 neighboring a.a. are significantly increased in the mutant (due to the additional methyl in isoleucine) compared to the WT (valine). In the WT system (Fig. 3A–C), the interactions Val430-Arg116, Val430-His439 and Arg116-His439 were

**A**

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                10        20        30        40        50        60
V430I   MLPSTIQTLTLFLTSGEVLLSLYVSASLSYLLYSDILLKFSRTEVTAPIMPLDCANASNV
        ::::::::::::::: ::::::::::::::::::::::::::::::::::::::::::
Phuket  MLPSTIQTLTLFLTSGGVLLSLYVSASLSYLLYSDILLKFSRTEVTAPIMPLDCANASNV
                10        20        30        40        50        60

                70        80        90       100       110       120
V430I   QAVNRSVTKGVTPLLPEPEWTYPRLSCPGSTFQKALLISPHRFGETKGNSAPLIIREPFI
        ::::::.::::::::::::::::::::::::::::::::::::::::::::::::::::
Phuket  QAVNRSATKGVTPLLPEPEWTYPRLSCPGSTFQKALLISPHRFGETKGNSAPLIIREPFI
                70        80        90       100       110       120

               130       140       150       160       170       180
V430I   ACGPKECKHFALTHYAAQPGGYYNGTREDRNKLRHLISVKLGKIPTVENSIFHMAAWSGS
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Phuket  ACGPKECKHFALTHYAAQPGGYYNGTREDRNKLRHLISVKLGKIPTVENSIFHMAAWSGS
               130       140       150       160       170       180

               190       200       210       220       230       240
V430I   ACHDGREWTYIGVDGPDSNALLKIKYGEAYTDTYHSYAKNILRTQESACNCIGGDCYLMI
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Phuket  ACHDGREWTYIGVDGPDSNALLKIKYGEAYTDTYHSYAKNILRTQESACNCIGGDCYLMI
               190       200       210       220       230       240

               250       260       270       280       290       300
V430I   TDGPASGISECRFLKIREGRIIKEIFPTGRVKHTEECTCGFASNKTIECACRDNSYTAKR
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Phuket  TDGPASGISECRFLKIREGRIIKEIFPTGRVKHTEECTCGFASNKTIECACRDNSYTAKR
               250       260       270       280       290       300

               310       320       330       340       350       360
V430I   PFVKLNVETDTAEIRLMCTKTYLDTPRPNDGSITGPCESDGNEGSGGIKGGFVHQRMASK
        :::::::::::::::::::::::::::::::::::::.::::::::::::::::::::::
Phuket  PFVKLNVETDTAEIRLMCTKTYLDTPRPNDGSITGPCESDGDEGSGGIKGGFVHQRMASK
               310       320       330       340       350       360

               370       380       390       400       410       420
V430I   IGRWYSRTMSKTQRMGMGLYVKYDGDPWTDSEALALSGVMVSMEEPGWYSFGFEIKDKKC
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Phuket  IGRWYSRTMSKTKRMGMGLYVKYDGDPWTDSEALALSGVMVSMEEPGWYSFGFEIKDKKC
               370       380       390       400       410       420

               430       440       450       460
V430I   DVPCIGIEMIHDGGKTTWHSAATAIYCLMGSGQLLWDTVTGVNMTL
        ::::::::::::::::::::::::::::::::::::::::::::::
Phuket  DVPCIGIEMVHDGGKTTWHSAATAIYCLMGSGQLLWDTVTGVNMTL
               430       440       450       460
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**B**

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B/Colorado      FSGVMVSMKEPGW--YSFG----FEIKDKKCDVPCIGIEMVHDGGKE----TWHSAATAI 445
B/Phuket        LSGVMVSMEEPGW--YSFG----FEIKDKKCDVPCIGIEMVHDGGKT----TWHSAATAI 445
B/Yamagata      PSGVMVSMKEPGW--YSFG----FEIKDKKCDVPCIGIEMVHDGGKK----TWHSAATAI 445
B/Victoria      PSGVMVSMKEPGW--YSFG----FEIKDKKCDVPCIGIEMVHDGGKK----TWHSAATAI 445
N5              -SKKVEVLNNKNWSGYSGAFTIPTAMTSKNCIVPCFWLEMIRGKPEERT-SIWTSSSSTV 442
N8              -IKRQVVVDNLKWSGYSGSFTLPVELTKRNCLVPCFWVEMIRGKPEEK--TIWTSSSSIV 444
N1              -SIKQDIVGINEWSGYSGSFVQHPELTGLDCIRPCFWVELIRGRPEEN--TIWTSGSSIS 444
N4              -NGVQDIIDNDNWSGYSGSFSIRGETTGRNCTVPCFWVEMIRGQPKEK--TIWTSGSSIA 445
N7              -KERQEIVGNDNWSGYSGSFIDYWN-DNSECYNPCFYVELIRGRPEEAKYVEWTSNSLIA 446
N6              -VAHQVIVNNQNWSGYSGAFIDYWA-D-RECFNPCFYVELIRGRPKESS-VLWTSNSIVA 447
N9              -TQGQTIVLNTDWSGYSGSFMDYWA-E-GECYRACFYVELIRGRPKEDK-VWWTSNSIVS 447
N2              QINRQVIVDRGDRSGYSGIFSV----EGKSCINRCFYVELIRGRKEE-TEVLWTSNSIVV 445
N3              KSVTQTLVSNNDWSGYSGSFIV----KTKDCFQPCFYVELIRGRPNKNDDVSWTSNSIVT 445
                              **            *   *   *           * *
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**Fig. 1. A-** Comparison of the neuraminidase (NA) amino acid sequences between the clinical Val430Ile variant and B/Phuket/2073/2013 vaccine strain. The eight functional (shaded in gray) and 11 framework residues (in boxes) are highlighted. **B-** Multiple ClustalW alignment of NA amino acid sequences of influenza A and B viruses. Only residues 391–445 are shown. The alignment includes B/Yamagata/16/1988 (GeneBank accession number EPI544263), B/Victoria/2/1987 (BAB32609), and their current B/Phuket/2073/2013 (EPI544263) and B/Colorado/06/2017 (ASK81297) representatives, respectively. Also, are included N1–N9 subtypes among influenza A type viruses: A/Michigan/45/2015 (H1N1, KU933495), A/Indiana/17/2016 (H3N2; KY116080), A/mallard/Alberta/58/2017 (H1N3; MH411954), A/gray-teal/Australia/2/79 (H4N4; AY207533), A/shearwater/Australia/1/1972 (H6N5; EU429794), A/sanderling/Delaware/1258/86 (H6N6; AY207557), A/swine/England/191973/92 (H1N7; AAC57417), A/turkey/Minnesota/42782–1/2013 (H6N8; KM244070) and A/tern/Australia/G70C/1975 (H11N9; M17813).
The 430 residue in B sequences (427 in A sequences) is highlighted in gra
y.
Asterisks denote identical residues among all sequences.

observed in 70%, 60% and 70% of the trajectory frames, respectively. By contrast, in the Ile430 system (Fig. 3B–C), these occupancies increased to 90%, 93% and 97%, respectively. On the other side, the interactions between Arg116 and Leu132 are reduced from 99% for the WT to 68% for Val430Ile. Consequently, Arg116 is positioned farther from Leu132 but closer to residue 430 in the mutant than in the WT. Thus, this displacement puts Arg116 farther from the ligand binding site in the mutant which may destabilise the critical interaction with NAIs.

Finally, we investigated whether Val430Ile NA variants are

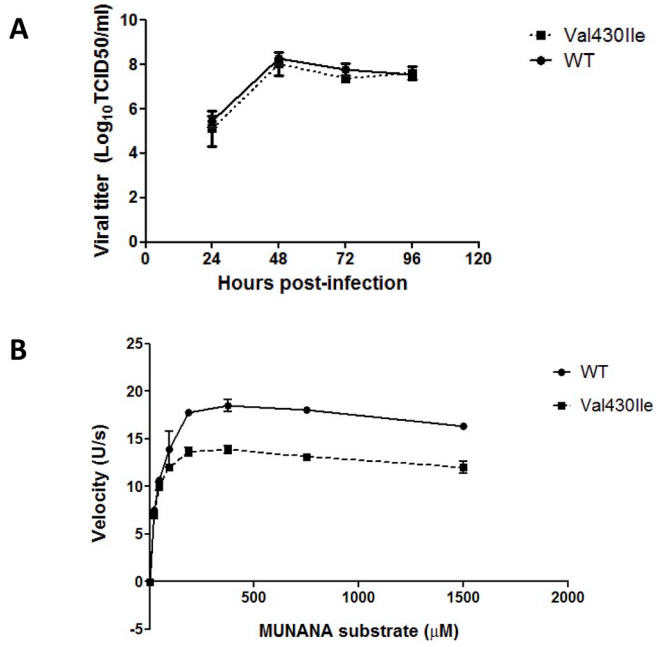


**Fig. 2. A).** ***In vitro*** **replicative capacities of clinical influenza B viruses.** Viral titers were determined at the indicated time points from supernatants of ST6GalI-expressing MDCK cells infected with the B/Phuket/2073/2013 susceptible virus (430Val) and the drug-resistant (430Ile) isolate at a multiplicity of infection (MOI) of 0.0001. Mean viral titers ± SD from triplicate experiments were determined by TCID50 assays. **B).** Determination of Km and Vmax values for influenza B viruses. NA enzyme kinetics experiments were performed by using $10^{10}$ copies/mL of viruses as determined by quantitative RT-PCR assay.

prevalent among currently circulating influenza B clades. For that purpose, ≥ 2800 influenza B NA sequences submitted to GISAID (https://www.gisaid.org) between 2016 and 2019 were downloaded and analysed with Fluserver (https://fluserver.bii.a-star.edu.sg). Among 2875 NA sequences from the Yamagata-lineage, only one Val430Ile variant (B/Idaho/25/2016) could be recorded (0.03%); however Val430Ala substitutions were found in 4 sequences (0.13%). No Val430Ile substitution could be detected among 6856 NA sequences from the Victoria-lineage (0%) whereas 4 Val430Ala sequences (0.05%) were detected in that lineage.

To our knowledge, this is the first report on the detection of a Val430Ile substitution mediating cross-RI phenotypes in influenza B isolates. Our MD simulation experiments showed that the impact of the Val430Ile substitution on RI phenotypes involved the catalytic Arg116 residue which is highly conserved in influenza A and B viruses. Interestingly, this variant was identified before NAI treatment, was found to be genetically stable and did not demonstrate signs of viral attenuation in vitro. These features suggest that this substitution may not alter viral replicative properties. Therefore, such variants should be taken into account during surveillance programs for NAI-resistance monitoring. Finally, due to a lower efficacy of some NAIs against influenza B viruses and the potential for emergence of variants with RI phenotypes, there is an urgent need to develop novel anti-influenza alternatives.

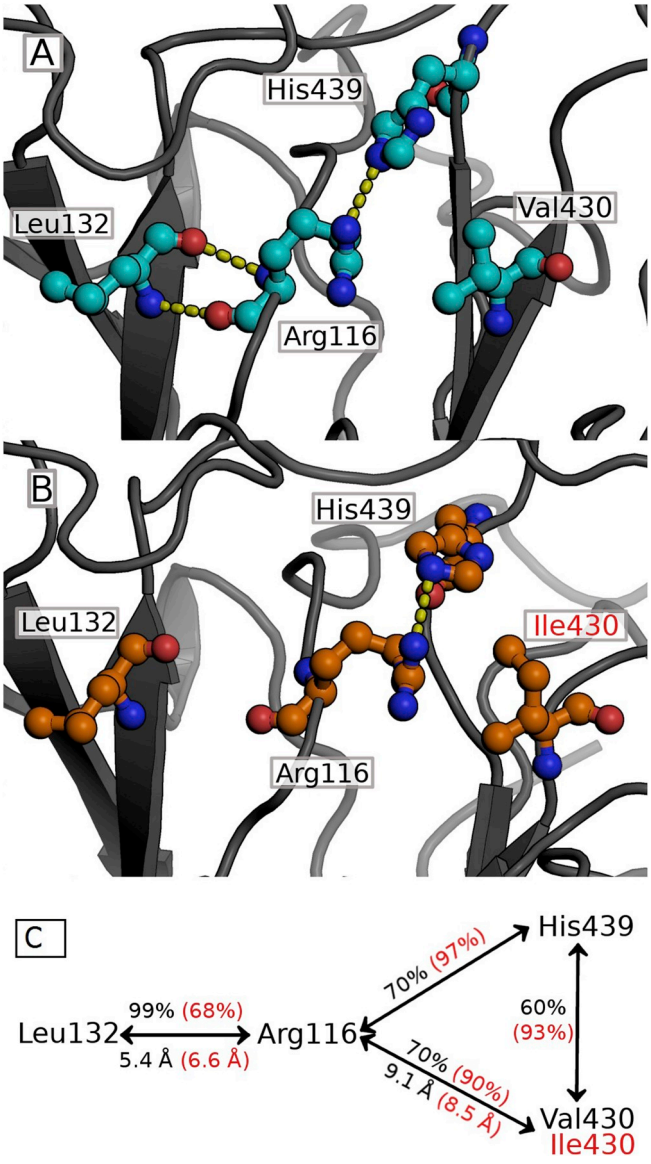


**Fig. 3. Molecular dynamics simulations of the Val430Ile variant.** Typical structures of the interaction network of Arg116 for the WT (A) and Val430Ile variant (B). The trajectories show protein in gray cartoon and the residues involved in the network in spheres. Polar interactions are identified with yellow dash lines. (C) Diagram of the interaction network for the WT and the mutant shows black arrows for the interactions, with the occupancy value expressed as percentage. The values in black are for the WT, and those in red are for the Val430Ile mutant.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.antiviral.2019.104561.

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