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Phylogenetically based establishment of a dengue virus panel,

representing all available genotypes, as a tool in dengue drug

discovery

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- 10
- 11 Abstract

12 Dengue fever is the most widespread of the human arbovirus diseases, with approximately one third of the world's population at risk of infection. Dengue viruses are members of the 13 14 genus *Flavivirus* (family *Flaviviridae*) and, antigenically, they separate as four closely related 15 serotypes (1-4) that share 60 to 75 % amino acid homology. This genetic diversity complicates the process of antiviral drug discovery. Thus, currently no approved dengue-16 17 specific therapeutic treatments are available. With the aim of providing an efficient tool for 18 dengue virus drug discovery, a collection of nineteen dengue viruses, representing the 19 genotypic diversity within the four serotypes, was developed. After phylogenetic analysis of 20 the full-length genomes, we selected relevant strains from the EVAg collection at Aix-21 Marseille University and completed the virus collection, using a reverse genetic system based 22 on the infectious sub-genomic amplicons technique. Finally, we evaluated this dengue virus 23 collection against three published dengue inhibitory compounds. NITD008, which targets the

highly conserved active site of the viral NS5 polymerase enzyme, exhibited similar antiviral 24 25 potencies against each of the different dengue genotypes in the panel. Compounds targeting less conserved protein subdomains, such as the capsid inhibitor ST-148, or SDM25N, a ∂ 26 27 opioid receptor antagonist which indirectly targets NS4B, exhibited larger differences in 28 potency against the various genotypes of dengue viruses. These results illustrate the 29 importance of a phylogenetically based dengue virus reference panel for dengue antiviral 30 research. The collection developed in this study, which includes such representative dengue 31 viruses, has been made available to the scientific community through the European Virus 32 Archive to evaluate novel DENV antiviral candidates.

33 Keywords

34 Dengue virus; phylogenetic analysis; reverse genetic; drug discovery; virus panel; dengue inhibitors

35 Short communication

36 Dengue virus (DENV) is a major threat to human health, with approximately one third of the 37 world's population at risk of being infected. DENV is the causative agent of dengue fever, as 38 well as the more severe dengue haemorrhagic fever (DHF)(Messina et al., 2014) and dengue 39 shock syndrome (DSS). It belongs to the genus Flavivirus (Flaviviridae family), which 40 comprises other clinically important human pathogens, such as yellow fever virus, West Nile virus and the recently emerging Zika virus(Vasilakis and Weaver, 2017). DENV is an 41 42 arthropod borne virus transmitted through the bite of infected mosquitoes from the genus Aedes (Stegomyia). Epidemiological transmission of DENV is confined to urban and peri-43 44 urban cycles for which Aedes aegypti and Ae albopictus mosquitoes, respectively, are the 45 primary transmission vectors(Chen and Vasilakis, 2011). Dengue is a positive-sense single 46 stranded RNA virus with a 10.7 kb genome encoding a single polyprotein which is post-47 translationally processed into three structural proteins, viz., capsid (C), pre-membrane (prM), envelope (E) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and
NS5)(Gebhard et al., 2011). Four antigenically closely related serotypes of DENV (1-4)
which share 60 to 75 % amino acid homology, have been identified(Guzman and Harris,
2015). Within this serotype demarcation, the DENV are also grouped into genotypes, with
varying terminology between authors(Carrillo-Valenzo et al., 2010; Weaver and Vasilakis,
2009) (hereunder we refer to the grouping proposed by Weaver and Vasilakis(Weaver and
Vasilakis, 2009)).

Hence, many of the DENV diagnostic tools do not readily distinguish between DENV 55 56 serotypes. Moreover, co-circulation of different serotypes during DENV epidemics(Vilela et 57 al., 2016) increases the complexity of virus identification. Added to these factors, antibody 58 dependent enhancement of the disease i.e., when patients contract a heterotypic secondary 59 DENV infection(Katzelnick et al., 2017) is a potential additional complication for effective 60 treatment of patients. Consequently, scientists are faced with the challenge of developing 61 Directly Active Antivirals (DAA) that can inhibit the entire spectrum of genetically diverse 62 serotypes and/or genotypes of DENV. However, despite the tireless efforts to provide an 63 antiviral therapy(Canard, 2012; Coutard et al., 2008; van Cleef et al., 2013; Yin et al., 2009), 64 there are still no approved drugs on the market to treat dengue infections. At present, the treatments available are merely supportive(Kaptein and Neyts, 2016). 65

A major barrier to evaluating the activity spectrum of potential DENV-inhibitory molecules arises from the non-availability of a well-defined panel of viruses that specifically represents the genetic variability of all characterised DENV isolates. With the aim of providing a tool for DENV research, with which to assess the antiviral activity of potential inhibitory molecules, we have developed a collection of DENV with sequences that include representative genotypes from within the four DENV serotypes (figure 1). Wherever possible, we selected clinical strains with a limited number of passages in cell culture. Strains were selected from either the European Virus Archive (EVA) collection(Romette et al., 2018), the French
National Reference Centre for arboviruses (CNR), or the World Reference Center for
Emerging Viruses and Arboviruses (WRCEVA). Viruses that could not be obtained but for
which full length genome sequences were available, were re-created using the versatile
infectious sub-genomic amplicons (ISA) reverse genetics technology(Aubry et al., 2015,
2014).

79 In order to select representative genotypes, we collected dengue full-length genome sequences 80 from the NCBI database and complemented this database with those of our, still unpublished, 81 "in house" and CNR strains. We performed phylogenetic reconstructions with the maximum 82 likelihood method to assign all available genome sequences to a genotype in a serotype 83 (supplementary material Fig 1, 2, 3 and 4). Within each genotype, we focused on strains that 84 were not subjected to extensive cell passage and were either available as biological isolates in 85 virus collections or as full-length sequences in GenBank. Six dengue genotypes were 86 available only as complete genome sequences in the NCBI database without any biological 87 strain counterparts in referenced collections (DENV-1 genotype III, DENV-2 genotype sylvatic and Asian II, DENV-3 genotype V and DENV-4 genotype III and sylvatic). Two 88 89 genotypes were not available at all because of incomplete genome sequence (DENV-1 90 genotype II and DENV-3 genotype IV). To obtain the biological viruses from the completely 91 sequenced strains, we designed reverse genetics systems based on the ISA technique(Atieh et 92 al., 2016; Aubry et al., 2015, 2014) and generated synthetic overlapping DNA fragments that 93 covered each of the entire genome, bordered by a CMV promoter on the 5' end and a 94 Ribozyme and poly-adenylation signal on the 3' end. The overlapping fragments were co-95 transfected into a mix of human and hamster embryonic kidney cell lines (HEK 293 and 96 BHK-21 purchased from the American Cell Culture Collection). This enabled us to recover 97 the missing biological strains to complete the collection. The initial viral stocks were

amplified in Vero E6 cells and fully sequenced. All the DENV strains used, have been made
available through the EVAg collection (https://www.european-virus-archive.com/).

100 Various specific dengue inhibitors that target several viral proteins involved in different 101 replication steps, have been discovered. ST-148, an inhibitor targeting the capsid structural 102 protein, has been reported to inhibit all DENV serotypes in cell culture, although with varying 103 efficiency. This inhibitor also appears promising in the AG-129 mouse model when infected 104 with a strain of DENV-2(Byrd et al., 2013). NITD008, an adenosine analogue inhibitor that 105 targets the RNA-dependent RNA polymerase activity, was shown to be inhibitory against all 106 dengue serotypes as well as other flaviviruses, including West Nile virus, yellow fever virus 107 and tick-borne Powassan virus(Yin et al., 2009). SDM25N, a ∂ opioid receptor antagonist, has 108 been reported to target the NS4B protein, probably indirectly through a cellular factor. Thus 109 far, it has only been shown to be active against a DENV-2 strain(van Cleef et al., 2013). 110 Based on the different mechanisms of action of the 3 compounds, their respective target and 111 its associated sequence variability across the different genotypes, we hypothesize that the 112 antiviral activity of the compounds might differ between all of the genotypes of DENV. 113 Therefore, the antiviral activity of these three compounds was assessed using a single 114 common protocol based on a viral RNA yield reduction assay(Delang et al., 2016). The assay did not depend on the cytopathogenic potential of the strain, thus allowing for the inclusion of 115 116 any dengue strain in the panel tested. Because all these strains differed in their replication 117 kinetics, prior to the assay, all DENV MOI and times of readout of the assay were calibrated 118 so that the replication growth were still in the log growth curve at time of the collection of the 119 supernatant. Although the maximum reduction of virus yield may depend of the specific strain 120 and assay conditions, the half inhibitory doses (IC50s) are not expected to be affected in these 121 settings and will depend only on the inhibitor efficiency. The compounds were assayed from 122 10 to 0.004µM, with 3-fold step-dilution in triplicate. The amount of viral RNA in the

supernatant medium, sampled at pre-determined time in the growth cycle, was quantified by
qRT-PCR to determine the 50% maximal effective concentration (EC₅₀) (Table 1).

125 The DENV strains of the collection showed similar sensitivity towards the nucleoside 126 analogue inhibitor NITD008 with EC_{50} 's ranging from 0.2μ M to 2.8μ M, which is in 127 accordance with previously published results (Xie et al., 2015).

128 The capsid inhibitor ST-148 inhibited all DENV-2 genotypes with EC₅₀'s ranging from 0.25 129 to 1.1µM. However, only one genotype of DENV-1 (DENV-1 GIII at 0.5µM), and one of 130 DENV-4 (DENV-4 GIII at 0.3µM), were inhibited by this compound. Finally, no activity was 131 observed against our DENV-3 genotypes, with all EC_{50} 's > 10µM. Although Byrd and co-132 workers(Byrd et al., 2013) found that the DENV-2 serotype was the most sensitive serotype to 133 this capsid inhibitor and showed up to two log of variability in the inhibition against other 134 serotypes, they did not fully evaluate the variation in susceptibility to other serotypes 135 sufficiently comprehensively to draw conclusions. In their study, they associated ST-148 136 resistance to a Leucine at position 34 instead of a Serine in DENV-2. However, looking at 137 capsid amino-acid alignment of all our DENV panel and their study's strains, and regardless 138 of their sensibility to ST-148, all strains exhibited a Serine at position 34 except DENV-2 139 from Trinidad (1751 TC 544), which presented a Proline at this position, as the Modoc virus 140 that they reported to be sensitive to ST-148 (Byrd et al., 2013). Thus, if resistance for ST-148 141 can arise from S34L mutations in some DENV-2 strains it is clear that it cannot be 142 unequivocally associated to a Leucine in position 34 in other serotypes and genotypes. This 143 suggests that other residues or domains in the capsid protein may be involved in the 144 interaction.

SDM25N showed moderate efficacy, with EC_{50} 's ranging from 1.7 – 7.7 μ M against a large proportion of the DENV-2 genotype strains, and half of the DENV-1 genotypes. However, no 147 activity was observed against any of the DENV-3 and 4 genotypes, as EC_{50} were all above 10 148 μ M. This result suggests that the binding affinity of NS4B to the hypothetical cellular factor 149 targeted by SDM25N varies greatly among various DENV genotypes and/or that this cellular 150 factor might be dispensable for efficient replication of some DENV genotypes.

Overall, the results demonstrate that compounds targeting highly conserved sites, exemplified by nucleoside analogue inhibitor NITD008 (targeting the active site of the polymerase), had a broader pan-serotypic activity, with similar EC_{50} 's regardless of the DENV genotype. In contrast, compounds targeting less conserved proteins or protein subdomains, either directly (*e.g.* the capsid) or indirectly through an interaction with a host factor of the cell (*e.g.* SDM25N), exhibited larger differences in activity towards the various genotypes of DENV.

157 Importantly, these data illustrate the fact that a sound in cellulo evaluation of anti-dengue 158 candidate molecules requires the use of a complete reference virus panel that enables 159 estimates of the antiviral activity against each of the identified DENV genotypes to be 160 obtained. Modern reverse genetics techniques have enabled us to develop such a 161 representative collection, and it has been made available to the scientific community through 162 the European Virus Archive collection (EVA). We believe that the availability of this new 163 tool will enable the independent assessment of pan-serotypic activity of anti-dengue 164 candidates in the future, fulfilling a critical requirement for a successful dengue antiviral 165 small molecule.

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171 **Contributions**

- 172 OG, MVL, GQ and XDL generated the idea of the panel. FT, XDL and GQ conceived the
- 173 experiments. XDL proposed the study design. FT, CB, and GQ performed the experiments.
- 174 FT and GQ analysed the results. FT and GQ wrote the paper. FT, CB, GQ, OG, MVL and
- 175 XDL reviewed and edited the paper.

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- 271

Table 1 Dengue virus collection-susceptibility to three antiviral compounds assessed by yield reduction assay. Anti-capsid ST-148, nucleoside analogue NITD008 and ∂ opioid receptor antagonist SDM25N were independently tested twice, with 3 replicates per experiment, against the dengue collection from 10µM to 0.005µM. AA: Asian American, A: American, C: cosmopolitan.

			ST-148	NITD008	SDM25N
	Virus	Genotype	EC50 (µM)	EC50 (µM)	EC50 (µM)
Dengue 1	D1/H/IMTSSA/98/606 Djibouti	Ι	>10	0,9 ±0,1	>10
	JKT 1186 TVP 949 Indonesia	IV	>10	0,3 ±0,03	5,5± 3,67
	CNR_25329 France	V	>10	2,7± 4	7,4 ±0,04
	P72-1244 Malaysia	III	3 ±0,5	0,9± 0,2	>10
Dengue 2	H/IMTSSA-MART/98-703 France	AA	0,8 ±0,5	0,9± 0,3	2,9 ±0,95
	_1751 TC 544 Trinidad	Α	1 ±0,7	0,3 ±0,06	2,9 ±0,01
	CNR_25679 France	С	1,1 ± 0,3	0,2 ±0,07	1,9± 0,03
	CNR 25326 Thailand	Asian I	0,1 ±0,03	0,9± 0,2	7,7 ±0,04
	DENV-2/PG/BID-V2618/2008 Papua New Guinea	Asian II	0,2 ±0,16	0,3 ±0,5	4,1 ±0,02
	DKD811 Malaysia	Sylvatic	0,4 ±0,18	0,4 ±0,1	>10
Dengue 3	DENV3/BR/D3LIMHO/2006 Brazil	V	>10	1±0,09	>10
	4025 Bolivia	III	>10	1± 0,05	>10
	CNR 17046 Malaysia	Ι	>10	2,8± 0,3	>10
	CNR 15418 Thailand	II	>10	1,2± 0,3	>10
Dengue 4	G11337 India	Ι	>10	1,2 ±0,03	>10
	Dakar HD 34460 Senegal	IIb	>10	0,9± 0,3	>10
	CNR_16861 Malaysia	IIa	>10	0,4 ±0,01	>10
	ThD4_0476_97 Thailand	III	0,3 ±0,08	0,2 ±0,08	>10
	P75-514 Malaysia	Sylvatic	>10	1±0,05	>10

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Phylogenetic analysis of dengue available sequences

Strain selection and production of missing genotypes by ISA

Antiviral compounds study

B



0,2

Figure 1 : Global serotype-representative DENV collection A: Pipeline of the workflow employed for the virus collection. B: Maximum likelihood phylogenetic tree (GTR+G+I model with 500 bootstraps), based on the complete nucleic acid sequences of the virus collection. Strain information: DENV-1: Genotype I: (Djibouti, 1998, AF298808); Genotype III (Malaysia,1972, EF457905.1); Genotype IV (Indonesia, 1977, EUO74031); Genotype V (France, 2014, MF004384); DENV-2: Genotype Asian-America (France Martinique, 1998, AF208496); Genotype American (Trinidad, 1953, EU073981.1); Genotype Cosmopolitan (France, 2014, MF004385); Genotype Asian 1 (Thailand, 2014, MH888331); Genotype Asian 2 (Papua New Guinea, 2008, FJ906959.1); Genotype Sylvatic (Malaysia, 2008, FJ467493.1); DENV-3: Genotype I (Malaysia, 2012, MF004386); Genotype II (Thailand, 2012 MH888332); Genotype III (Bolivia,2011, MH888333); Genotype V (Brazil,2006, JN697379.1); DENV-4 : Genotype IIb (Senegal, 1981, MF004387); Genotype IIa (Malaysia, 2013, MH888334); Genotype III (Thailand, 1997, AY618988.1); Genotype Sylvatic (Malaysia, 1975, JF262779.1); Genotype I (INDIA, 1961, JF262783.1). Complete information relevant to the strains of the collection are more fully detailed in the supplemental material.