Extended dengue virus panel: application to antiviral compounds studies.

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Abstract

Dengue is the most widespread arboviral disease with approximately 1/3 of the world population at risk. Dengue viruses belong to the genus *Flavivirus* (*Flaviviradea* family) and are divided into four closely related serotypes (1-4) that share 60 to 75 % identity at the amino acid level. This viral diversity complicates the development of antivirals and currently they are still no approved treatment. With the aim of providing an efficient tool for dengue virus research particularly to evaluated compounds efficacy, we developed a panel of 19 dengue viruses covering nearly all genotypes in the four serotypes. After a phylogenetic analysis we selected relevant strains from our collection and design reverse genetic system based on the ISA technic to generate the missing ones. Finally, we evaluated our dengue panel against three, already published, compounds. We demonstrated that NITD008, which targeted the very conserved active site of the polymerase, exhibited very similar EC50s regardless of the dengue genotypes. But compounds targeting either directly or indirectly less conserved proteins such as the capsid (ST-148) or NS4B (SDM25N) exhibited large differences of activity toward the various genotypes of dengue viruses. These data reinforce our conviction that there is a real need to evaluate compounds activity against a large panel of dengue viruses.

Article

Dengue virus is a major threat to human health with approximately 1/3 of the world population at risk of being infected. Dengue virus is the causative agent of the dengue fever and the more severe Dengue Hemorrhagic Fever (DHF) [1] and Dengue Shock syndrome (DSS). It belongs to the genus *Flavivirus* (*Flaviviridae* family) which comprises other clinically important human pathogens such as Yellow fever virus, West Nile Virus and the recently emerging Zika Virus [2]. Dengue is an arthropod born virus transmitted by the bites of mosquitoes from the genus *Aedes* (Stegomya). This virus has a transmission cycle that alternates between a sylvatic enzootic cycle using arboreal mosquitoes as vectors and an urban cycle which include humans using *Aedes aegypti* and *albopictus* mosquitoes [3]. Dengue

is a positive single strand RNA virus with a 10.7 kb genome coding for a single polyprotein which is processed in three structural proteins (C, PrM and ENV), and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) [4]. Dengue is divided into four closely relative serotypes (1-4) that share 60 to 75 % identity at the amino acid level [5]. Inside this serotype classification, Dengue virus are also classified as genotypes with a variable terminology between authors[6,7].

For researchers studying dengue virus, it can be really challenging to develop Direct Active Antivirals (DAA) against such a large viral diversity. Despite the huge effort to provide an antiviral therapy [8–11], they are still no approved drugs. At this time treatments are only supportive [12].

With the aim of providing an efficient tool for dengue virus research particularly to evaluated compounds efficacy, we developed a panel of dengue viruses covering near all genotypes in the four serotypes (figure 1). Whenever possible, we chosen to select strains that exhibited low passages in cell cultures and were issued from recent outbreaks. The *ad. hoc*. strains were selected from the European Virus Archive (EVA) collection [13], the French National Reference center for arbovirus (CNR) and using our in house reverse genetic technic that allow us to generate infectious viruses from published full length genome sequences [14,15].

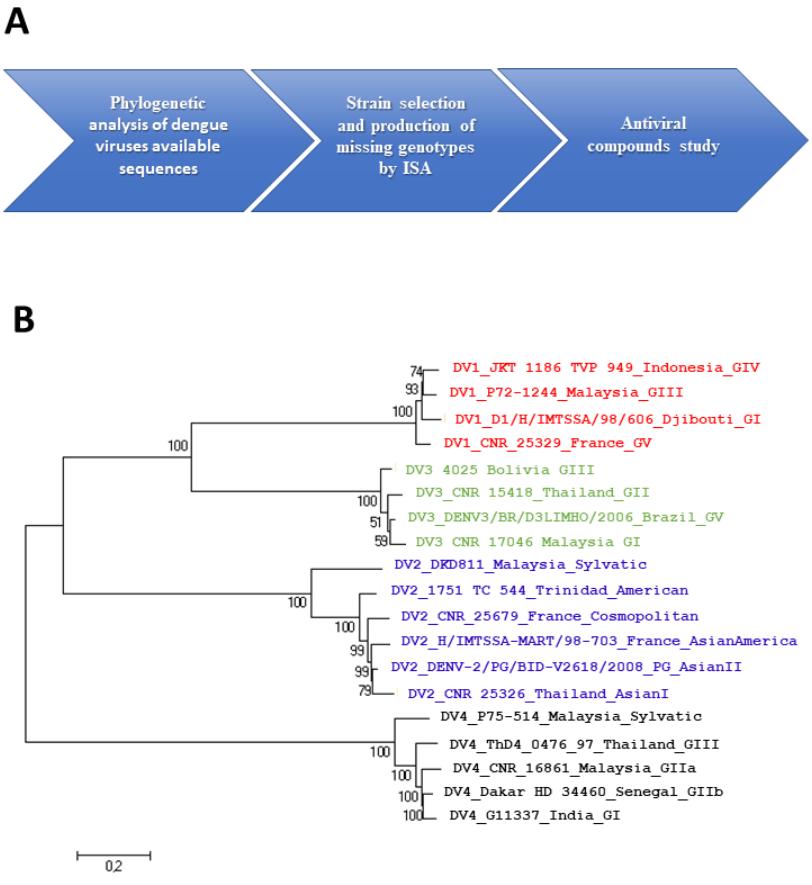

Figure 1 : Dengue panel construction A: Structure of the workflow used for the panel construction. B: phylogenetic tree of the panel using full nucleic acid coding sequence and the maximum likelihood method

(GTR+G+I model) with 500 bootstraps. Strains information: DENV-1: Genotype I: (Djibouti, AF298808,); Genotype III (Malaysia, EF457905.1); Genotype IV (Indonesia, EUO7031); Genotype V (France, MF004384); DENV-2: Genotype AsianAmerica (France Martinique, AF208496); Genotype American (Trinidad, EU073981.1); Genotype Cosmopolitan (France, GenBank MF004385,); Genotype Asian1 (Thailand, MH888331); Genotype Asian 2 (Papua New Guinea, FJ906959.1); Genotype Sylvatic (Malaysia, FJ467493.1); DENV-3: Genotype I (Malaysia, MF004386, Genotype II (Thailand, MH888332); Genotype III (Bolivia, MH888333); Genotype V (Brazil, JN697379.1); DENV-4 : Genotype IIb (Senegal, MF004387,); Genotype IIa (Malaysia, MH888334); Genotype III (Thailand, AY618988.1); Genotype Sylvatic (Malaysia, JF262779.1); Genotype I (INDIA, JF262783.1). Complete information relative to the strains of the panel are more fully detailed in the supplemental material.

For this purpose, we collected dengue complete genome sequences from the NCBI database and complemented this large database with those of our still unpublished “in house” and CNR strains. We performed phylogenetic reconstructions with the maximum likelihood method to assign all available genome sequences to a genotype in a serotype (reduced versions of phylogenetic trees are provided in Supplemental material fig 1,2,3 and 4). From this phylogenetic analysis, we noticed that six dengue genotypes were available only as complete genome sequences in the NCBI database but without biological strain counterparts (DENV-1 genotype III, DENV-2 genotype sylvatic and Asian II, DENV-3 genotype V and DENV-4 genotype III and sylvatic). To obtain these strains, we designed reverse genetics systems based on ISA technic and generated synthetics overlapping DNA fragments that cover each of the whole genome, bordered by a CMV promoter on the 5’ end and a Ribozyme and poly-adenylation signal on the 3’ end. The overlapping fragments were co-transfected in permissible cell lines and allowed us to recover the missing biological strains to complete our panel. The viral stocks were grown on Vero E6 cells and were fully sequenced. All these dengue strains have been made available through the EVA collection.

Various specific dengue inhibitors that targeted several viral proteins involved in different replication steps have been developed. ST-148, an inhibitor targeting the capsid structural protein, has been reported to inhibit all dengue serotypes in cultures, although with varying efficiency, and seems promising in AG-129 mouse model infected with a Dengue 2 strain [16]. NITD008, a adenosine analog inhibitor that target the RNA-dependent RNA polymerase activity was shown to be efficient against all dengue serotypes as well as other flaviviruses [11]. SDM25N a ∂ opioid receptor antagonist has been reported to target, probably indirectly, the NS4 B protein. So far it has only been shown to be active against a dengue 2 strain [10]. Depending on the target and its sequence variability and the various mechanisms of action of these compounds their efficiency is likely to differ a lot regarding all the genotypes of dengue viruses. To evaluate our reference dengue panel, we assess the antiviral activity of these three compounds using a single common protocol based on a viral RNA yield reduction assay that do not depend on the cytopathogenic potential of the strain and allow the inclusion of any dengue strain in the panel. The compounds were assayed from 10 to 0.005µM with 3-fold step-dilution in triplicates and the amounts of viral RNA in the supernatant during the log-growth curve was quantified by qRT-PCR so as to determine the EC50 (table1).

Table 1: Dengue panel susceptibility to three antiviral compounds assessed by yield reduction assay. Anti-capsid ST-148, nucleoside analog NITD008 and anti-NS4B SDM25N were tested against the dengue panel 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**	**I**	**>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**	**A**	**1** ±0,7	**0,3**±0,06	**2,9**±0,01
	CNR_25679 **France**	**C**	**1,1** ±0,3	**0,2**±0,07	**1,9**±0,03
	CNR 25326 **Thailand**	**AsianI**	**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**	**I**	**>10**	**2,8**±0,3	**>10**
	CNR 15418 **Thailand**	**II**	**>10**	**1,2**±0,3	**>10**
Dengue 4	G11337 **India**	**I**	**>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**

For NITD008 we measured EC50 ranging from 0.2µM to 2.8µM in accordance to previously described activity of the compound against different serotypes [17]. All dengue strains in the panel were somewhat similarly sensitive to NITD008, which is fully consistent with the flaviviral broad spectrum activity of this nucleoside analog.

Concerning the capsid inhibitor ST-148, all genotypes of dengue 2 strains were sensitive with EC50 ranging from 0.25 to 1.1µM. However, we found only one sensitive genotype for DENV-1 (DENV-1 GIII at 0.5µM) as the EC50 of the other dengue 1 genotypes were all above 10µM. Similarly, only one DENV-4 genotype was sensitive to the compound: (DENV-4 GIII at 0.3µM), the EC50s of the others were above 10µM. Lastly, all DENV3 genotypes were resistant to the capsid inhibitor, with EC50 > 10µM. Although Byrd and co-workers found that DENV-2 serotype was the most sensible serotype to this capsid inhibitor and

showed some variability in the inhibition, they could not fully assess the poor and highly variable susceptibility of strains from other serotypes.

For SDM25N, most of the dengue 2 genotypes and half of dengue 1 genotypes were moderately sensitive to the compound with EC50 ranging from around 2 to 8 µM and all dengue 3 and 4 genotypes were less sensitive with EC50 above 10 µM.

Overall, these results showed that compounds, like NITD008, that targeted very conserved sites such as the active site of the polymerase exhibited very similar EC50s regardless of the dengue genotypes but compounds targeting either directly or indirectly less conserved proteins such as the capsid or NS4B exhibited large differences of activity toward the various genotypes of dengue viruses. These data reinforce our conviction that there is a real need to evaluate compounds activity against a larger panel of clinically relevant dengue viruses such as our reference panel of near all available dengue genotypes.

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Contributions

FT, XDL and GQ conceived the experiments. XDL allowed the funding of this study. FT, CB, and GQ performed the experiments. FT and GQ analyzed the results. FT and GQ wrote the paper. FT, CB, GQ and XDL reviewed and edited the paper.

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