Letter

Exploring the Implication of DDX3X in DENV Infection: Discovery of the First-in-Class DDX3X Fluorescent Inhibitor

Annalaura Brai,^V Adele Boccuto,^V Martina Monti, Serena Marchi, Ilaria Vicenti, Francesco Saladini, Claudia Immacolata Trivisani, Alessandro Pollutri, Claudia Maria Trombetta, Emanuele Montomoli, Valentina Riva, Anna Garbelli, Emanuele Maria Nola, Maurizio Zazzi, Giovanni Maga, Elena Dreassi,* and Maurizio Botta



attracting attention since it allows identification of broad-spectrum drugs endowed with high genetic barrier. In the last ten years our research group identified several small molecules DDX3X inhibitors and proved their efficacy against different viruses including novel emerging ones. Herein, starting from a screening of our compounds, we designed and synthesized novel derivatives with potent activity and high selectivity. Finally, we synthesized a fluorescent inhibitor that allowed us to study DDX3X cellular



localization during DENV infection in vitro. Immunofluorescence analysis showed that our inhibitor colocalized with DDX3X, promoting the reduction of infected cells and recovering the number of viable cells.

KEYWORDS: DDX3X, host factors, DENV, antiviral agents, resistance, immunofluorescence

engue Virus (DENV) is a positive sense, single stranded virus member of the Flaviviridae family. According to World Health Organization data, the global incidence of the five serotypes of DENV (DENV1-5) has increased in recent years, and about half of the world's population is now at risk of infection.¹ Nowadays DENV is transmitted in over 128 countries by Aedes aegypti and Aedes albopictus infected mosquitoes, with 390 million new cases per year.¹ The human migration flows, the inadequate vector control, and the global warming are the possible causes of the rapid spread of this virus. DENV infection is often asymptomatic or causes flulike symptoms, such as high fever, that usually terminate after 1 week. However, in a small number of cases DENV infection results in a potentially deadly illness named Dengue hemorrhagic fever (DHF) or in Dengue shock syndrome (DSS), characterized by high fever, bleeding, and circulatory failure which can cause shock and sometimes death.² No specific treatments are actually available, and vaccination is serotype-specific. In 2015 Sanofi Pasteur produced the first dengue vaccine, Dengvaxia, now approved for use in endemic areas.³ Nevertheless, its use is strictly recommended only to the patients that already contracted DENV infection, since the vaccination can increase the risk of contracting more severe DENV with respect to unvaccinated patients.³ In this context,

the search for novel effective antiviral drugs is an important challenge for the scientific community. Our research group already proved that the ATPase/RNA helicase X-linked DEAD-box polypeptide 3 (DDX3X) is an important target to develop antivirals with indirect acting mechanism of action.⁴⁻⁹ In 2016 we reported the first DDX3X inhibitor with a urea-based scaffold, endowed with broad spectrum antiviral activity against DENV, West Nile virus (WNV), Hepatitis C virus (HCV), and immunodeficiency virus type 1 (HIV-1).⁷ Furthermore, we discovered a sulfonamide series of DDX3X inhibitors with promising activities and improved in vitro pharmacokinetic properties, which were active against WNV infection.⁸ DDX3X inhibitors offer multiple advantages, in particular the possibility to fight different viruses including novel emerging ones with a unique molecule, and the reduced risk for drug resistance, since human genes coding for proteins

Special Issue: In Memory of Maurizio Botta: His Vision of Medicinal Chemistry

Received: December 31, 2019 Accepted: March 2, 2020



involved in viral replication are minimally vulnerable to mutations induced by drug administration. The implication of DDX3X in the life cycle of DENV is still debated in the literature, and different studies describe DDX3X either as a pro-viral or as an antiviral protein.^{10–12} Indeed, Kumar et al. reported that DDX3X knock down leads to increased viral titers;¹⁰ in contrast, Khadka and co-workers showed that knockdown of DDX3X reduces the expression of DENV reporter gene, thus highlighting a negative role in DENV replication.¹¹

Since an X-ray crystal structure of DDX3X in its active closed conformation was disclosed only very recently,¹³ but was unavailable at the start of this study, we previously generated a homology model that led us to identify two series of RNA-competitive inhibitors.^{5,7} Compound 1 with a ureabased structure was characterized by a promising broadspectrum antiviral activity, good passive permeability, and metabolic stability but limited aqueous solubility. On the contrary the sulfonamide series was characterized by higher solubility values and improved activity against WNV with respect to the hit compound 1 (compounds 2-6).⁸ In order to verify their activity against DENV, the most promising sulfonamide compounds were assayed against DENV-2 through an immunodetection assay. Briefly, human hepatoma cells (Huh-7) were infected with 50 TCID₅₀ of DENV-2 strain in the presence of serial dilution of compounds. After 72 h, supernatants from each well were used to infect preseeded Huh-7 cells in a second cycle of infection. Viral replication was determined after 72 h by an immunoenzymatic assay using a monoclonal antibody targeting a conserved region of viral E antigen. The measurement of the half maximal inhibitory concentration (IC_{50s}) indicated that most of the compounds had an antiviral activity in the low micromolar range, comparable or lower than the activity of the already described hit compound 1 (Chart 1). Since DDX3X inhibitors were demonstrated to interfere with mitochondrial translation causing lower intracellular ATP concentrations,¹⁴ we replaced the MTT assay with a CellTiter-Glo 2.0 (Promega) kit, which determines the number of viable cells by measuring the

Chart 1. Structures of Known DDX3X Inhibitors, Anti-Enzymatic Activities Expressed as Half Maximal Inhibitory Concentrations (IC_{50}) \pm Standard Deviation (SD), Antiviral Activities against DENV-2, and Cytotoxicity on Huh-7 Cells*



[^]Previously published data.

quantity of cellular ATP. According to the reported mechanism of toxicity, the half maximal cytotoxic concentrations (CC_{50}) of compounds decreased with respect to those previously obtained using the MTT assay in Huh-7 cells. Nonetheless, all compounds showed selectivity indexes (SI) higher than ten, with the exception of **2** (SI = 3). Among known DDX3X inhibitors (Chart 1), compound **5** exhibited the most promising anti-WNV activity and resulted to be the most active of the series also against DENV infection with an IC₅₀ of 1.6 μ M and low cytotoxicity (SI = 94).⁸ The trifluoromethyl derivative **2** presented lower antiviral activities in the low micromolar range and low toxicity similarly to compound **5**, while methoxy derivative **6** shows low antiviral activity and higher standard deviation, probably due to its lower solubility.

In order to investigate the cellular mechanisms of our inhibitors during viral replication, we analyzed the effects of compound 3 in the number of viable cells, in the expression of viral dsRNA and DENV not structural proteins 3 and 5 (NS3 and NS5). Huh-7 cells were infected with DENV-2 strain at the concentration of 100 TCID₅₀. Cells were treated with increasing concentrations of compound 3 (0.1, 1, 10, 20 μ M), and at 72 h post infection (p.i.) cells were fixed, stained with specific fluorescent antibodies, and analyzed by confocal microscopy.

As reported in Figure 1, compound 3 reduced the number of NS3 (white bars), NS5 (blue bars), and viral dsRNA (yellow bars) positive cells. In addition, the treatment with compound 3 was associated with a higher number of viable cells, contrasting the cytopathic effect associated with DENV-2 infection. Taking into account these results, extensive molecular dynamic studies were carried out to design a small series of derivatives (for details see Supporting Information). The novel urea and sulfonamide compounds 7-24 were thus designed and synthesized. Full synthetic protocols are described in the Supporting Information. Compounds were tested for their ability to inhibit the helicase activity of DDX3X using our FRET-based biochemical assay previously published.^{5,7,8} As reported in Chart 2, several derivatives showed activities from the low micromolar to the submicromolar range. As suggested by docking and molecular dynamic studies, the replacement of methyl with small and electron withdrawing groups such as fluorine is well tolerated in ortho (7 and 11) and in meta positions (compounds 8, 9, and 10), while the para substituted derivative 12 is about ten-times less active. In contrast with the sulfonamide series the ethoxymethyl introduction was less tolerated than the other substitutions, with compound 9 being about 100-times less active. Taking into account our previously published studies^{7,8} the butyl side chain on the triazole ring was replaced with other linear and not hindered substituents, such as isopentyl and 3-oxobutyl. The replacement of butyl with oxobutyl slightly decreased the activity (compound 10); in contrast, the substitution with isopentyl led to compounds with comparable activity (11 and 8). The methyl moiety was replaced with the bioisosteric trifluoromethyl group, and as already observed for the sulfonamide series it was well tolerated (compounds 13, 14, 16-18, and 20). Among the series of trifluoromethyl derivatives, 13 with a methoxy group in the meta position with respect to the triazole ring was less active than the corresponding ortho derivative 14, with 14 being able to form an additional hydrogen bond between the oxygen of the methoxy group and Gly325. Since bulky electron donating



Figure 1. Effects of compound 3 on the viral replication of DENV-2. As reported in the panels, compound 3 reduced the number of NS3 (white bars), NS5 (blue bars), and viral dsRNA (yellow bars) positive cells. Compound 3 had a protective effect and recovered the number of viable cells (red bars, bottom panel).

groups were well accepted in the sulfonamide series,⁸ we synthesized an ortho methylsulfonamido derivative in which ethoxymethyl was replaced with a butyl side chain. As a result **15** showed a high inhibitory activity with an IC₅₀ value of about 0.1 μ M. Compound **16** with a fluoro-methyl substitution at C(4) triazole position was about ten-times less active than derivatives **19** and **20**, confirming that longer lipophilic side chains are preferred; in contrast, derivative **17** was about 20-times less active, probably due to the perfluorobutyl side chain, which according to docking analysis is accommodated outside the binding site.

The sulfonamide series demonstrates promising antienzymatic activities, with compounds 21, 22, and 24 being characterized by activities of 0.06, 0.005, and 0.08 μ M. Morpholino derivative 21 maintains all the key interactions of the sulfonamide series, in particular a hydrogen bond between triazole and Arg276 and two hydrogen bonds between sulfonamide and Arg480 and Arg276, and establishes an additional hydrogen bond between the morpholine oxygen and Arg351. Ester 22 was extremely active; however, the corresponding carboxylic acid 23 was not evaluated due to its precipitation during the assays. Finally, an ortho methylsulfonamido derivative was synthesized (24); its activity value of 0.08 is comparable to that of urea compound 15. Even in this case, 24 maintains all the key interactions and forms an additional hydrogen bond between the oxygen of sulfonamide and Arg351.

The antiviral activity and cytotoxicity of 12 selected compounds were tested using the immunodetection assay as described in the Supporting Information. Their IC_{50s} values were in the low micromolar to submicromolar range and were comparable to or lower than those of ribavirin and sofosbuvir, two broad spectrum antivirals used in our tests as reference compounds.^{15,16} As shown in Table 1, the best result was represented by compound 7, characterized by an IC₅₀ of 0.9 μ M and low cytotoxicity, with its selectivity index being equal to 222. Compound 9 was found inactive probably due to its lower DDX3X inhibition, while derivative 10 had a very promising IC₅₀ value of 0.3 μ M and a selectivity index of 19.

Fluorinated derivative 11 showed high selectivity index, but it was about four-times less active than the corresponding isopentyl derivative 8.

Compounds 19 and 20 had favorable antiviral activities and cytotoxicity, comparable to those of the fluorinated compound 8. Sulfonamide 21 and 22, despite their potent DDX3X inhibitory concentrations showed activities of 5.3 and 4.3 μ M, respectively. The low activity of 22 is probably due to its cellular hydrolysis into the corresponding carboxylic acid 23, which was found toxic and inactive. Sulfonamide 24 and urea 15 showed IC₅₀ of 2.5 and 8.3 μ M, respectively, and their selectivity indexes were lower than those of the other compounds of the series.

The exact implication of DDX3X in the life cycle of flaviviruses such as WNV or DENV is actually poorly understood. Nevertheless, in the last few years, immuno-fluorescence studies have been performed by different groups to investigate the DDX3X role during WNV¹⁷ and JEV¹⁸ infection by analyzing its colocalization with cellular and viral proteins.

In order to better understand the mode of action of our compounds in DENV infected cells, we planned the synthesis of a fluorescent inhibitor to be used as a probe in time course confocal microscopy experiments. Fluoresceine isothiocyanate (FITC) was selected as fluorophore; computational analysis led us to choose sulfonamide 3 for further modifications and the side chain on C(4) triazole position as an optimal position to insert FITC. The synthesis of the fluorescent probe (25) was accomplished according to Scheme 1, and first entailed the synthesis of azide 29. Sulfonamide 28 was synthesized starting from 2-trifluoromethylaniline 26 and 3-nitrosulfonide chloride 27. Subsequent Pd on charcoal catalyzed hydrogenation furnished the corresponding aniline that was converted into azide 29 through diazotization reaction.⁸ Alkyne 32 was synthesized starting from ethanolamine 30, that was protected with di-tert-butyl dicarbonate and converted into the terminal alkyne 31 by reaction with propargyl bromide. Acidic deprotection of the amino group and reaction with FITC furnished the fluorescent alkyne 32. Click reaction between Chart 2. Structures of Novel DDX3X Inhibitors Synthesized, Anti-enzymatic Activities Expressed as Half Maximal Inhibitory Concentrations (IC₅₀), Calculated Using a FRET-Based Assay



azide **29** and terminal alkyne **32** led to the desired FITC-labeled derivative **25**.

Compound **25** was tested for its ability to inhibit DENV-2 replication in Huh-7 cells, its IC_{50} was $28.2 \pm 4.6 \ \mu$ M, and its CC_{50} was 140.0 μ M. Starting from this result, we studied the time course localization of our fluorescent probe investigating its colocalization with DDX3X and viral protein NS5. Huh-7 cells were infected with DENV-2 at the TCID₅₀ of 100, fixed at 0, 6, 12, and 24 h.p.i., permeabilized and immunostained. Confocal microscopy analysis revealed that DDX3X is primarily localized into the cytoplasm, while during viral infections DDX3X is recruited to perinuclear spots, particularly between 6 and 24 h.p.i. (Figure 2 and Figure S1). The recruitment of DDX3X to these structures could be a potential shared strategy employed by RNA viruses, such as HCV and WNV, that exploit DDX3X function.^{17–19} Similarly, DENV interacts with other components within stress granules and processing bodies sites, such as DDX6, a member of the same

 Table 1. Antiviral Activity of Selected Compounds against

 DENV-2 Infected Cells

Cpd ID	$IC_{50} \pm SD (\mu M)^a$	$\mathrm{CC}_{50}^{\ c} \pm \mathrm{SD} (\mu \mathrm{M})^{b}$	SI ^c
7	0.9 ± 0.1	200 ± 12	222.2
8	2.5 ± 0.8	200 ± 23	80.0
9	>145	145 ± 18	
10	0.3 ± 0.1	20 ± 4	64.7
11	10.5 ± 9.1	200 ± 18	19.0
15	2.5 ± 0.2	35 ± 6	14.0
19	1.4 ± 0.8	170 ± 24	121.4
20	2.7 ± 0.1	170 ± 19	62.9
21	5.3 ± 5.0	100 ± 9	18.9
22	4.3 ± 2.3	100 ± 14	23.2
23	>7	7.0 ± 2	
24	8.3 ± 3.7	87 ± 11	10.5
ribavirin	4.0 ± 0.6	100 ± 14	25.0
sofosbuvir	3.8 ± 1.1	200 ± 17	52.6

 ${}^{a}\text{IC}_{50}$: mean \pm standard deviation of half maximal inhibitory concentration calculated in Huh7 cells from at least two experiments; ${}^{b}\text{CC}_{50}$: half maximal cytotoxic concentration, evaluated on Huh-7 cells. ${}^{c}\text{SI}$: Selectivity index, calculated as the ratio between CC₅₀ and IC₅₀.

helicase family as DDX3X. Thus, proper assembly of these subcellular structures has functional consequences for DENV replication and infectivity.^{20,21} Moreover, our analysis showed that DDX3X increases its expression during infection, with a maximum expression at 48 and 72 h (CV panels in Figure S1). As shown in Figure 2 (left panels), 25 colocalized with DDX3X at the perinuclear region in the first 6 h of treatment and then induced DDX3X cytoplasmatic localization as in uninfected Huh7 cells (Figure S1, CC panel). The same experiment was performed by analyzing cellular localization of the viral protein NS5 using a specific antibody detected using Alexaflour-labeled secondary antibody (Figure 2, right panels). In the first 6 h, NS5 had a cytoplasmatic localization and was colocalized with 25 and then moved in the nucleus. As shown in Figure S2 and Figure S3, 25 was associated with an increased number of viable cells and with a significant reduction of the total number of NS5 positive cells at 48 and 72 h.

In the present work, we focused our efforts in expanding the structure activity relationship (SAR) around the two series of already discovered DDX3X inhibitors, concentrating our work on the search of novel promising compounds active against DENV infection. As a result, we discovered novel DDX3X helicase inhibitors with improved antiviral activity, comparable to or lower than those reported for known broad spectrum antivirals such as ribavirin or sofosbuvir. Notably, the most promising derivative, compound 10, is about 9-times more active than the previous hit (compound 1). In addition, we investigated the mechanism of action of our compounds in infected cells, using the novel fluorescent derivative 25. Immunofluorescence analysis confirms that 25, during the first hours of DENV infection, colocalized with DDX3X, promoting the reduction of NS5 positive cells and recovering the cell number, over time (until 72 h).

The low cytotoxicity of compounds, evaluated by measuring ATP concentration, indicates once again that our compounds are characterized by high cellular tolerability. Overall, results reported herein confirm that DDX3X inhibitors represent a

Scheme 1. Synthesis of the Fluorescent Inhibitor 25^a



"Reagents and conditions: (*i*) Pyr, 5 h, r.t. (*ii*) (a) H₂, Pd/C, MeOH, 2 h; (b) *t*-BuONO, CH₃CN, 20 min. 0 °C; (c) TMSN₃, CH₃CN, 2 h, r.t.; (*iii*) (a) Di-*tert*-butyldicarbonate, DCM, 3 h, 0 °C to r.t.; (b) NaH, DMF, 0 °C to r.t., then propargyl bromide, 9 h, r.t.; (*iv*) (a) HCl (3 N), MeOH, r.t., 2 h; (b) TEA, DCM, 1 h, r.t., then FITC, 12 h, r.t.; (*v*) CuSO₄·SH₂O, sodium ascorbate, H₂O, *t*-BuOH (1:1), MW, 300 W, 10 min, 120 °C.



Figure 2. Immunofluorescence analysis. DENV-2 infected cells were treated with DDX3X fluorescent inhibitor **25** at a concentration of 20 μ M. Cells were stained at different time-points with a DDX3 antibody (detected using Alexaflour568 labeled secondary antibody, left panels) and DENV NS5 antibody (detected using Alexaflour (568) labeled secondary antibody, right panels) and examined by confocal microscopy. Individual antibody stained as well as merged images are shown as indicated. Each experiment was repeated at least two times.

safe and promising class of antivirals, supporting their evaluation in an animal model of DENV infection.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsmedchemlett.9b00681.

Full synthetic procedures and spectral characterization, data for all intermediates and final compounds, experimental details of the enzymatic assays, antiviral assays, the cytotoxicity experiment, and the immunofluorescence experiments (PDF)

AUTHOR INFORMATION

Corresponding Author

Elena Dreassi – Dipartimento Farmaco Chimico Tecnologico, Università degli Studi di Siena, 53100 Siena, Italy; orcid.org/0000-0001-8987-940X; Email: elena.dreassi@ unisi.it

Authors

- Annalaura Brai Dipartimento Farmaco Chimico Tecnologico, Università degli Studi di Siena, 53100 Siena, Italy; orcid.org/0000-0001-6395-9348
- Adele Boccuto Dipartimento di Biotecnologie Mediche, Università degli Studi di Siena, 53100 Siena, Italy
- Martina Monti Dipartimento di Medicina Molecolare e dello Sviluppo, Università degli Studi di Siena, 53100 Siena, Italy; orcid.org/0000-0002-2173-7269
- Serena Marchi Dipartimento di Medicina Molecolare e dello Sviluppo, Università degli Studi di Siena, 53100 Siena, Italy
- Ilaria Vicenti Dipartimento di Biotecnologie Mediche, Università degli Studi di Siena, 53100 Siena, Italy
- Francesco Saladini Dipartimento di Biotecnologie Mediche, Università degli Studi di Siena, 53100 Siena, Italy; orcid.org/0000-0002-9934-377X
- **Claudia Immacolata Trivisani** Dipartimento Farmaco Chimico Tecnologico, Università degli Studi di Siena, 53100 Siena, Italy
- Alessandro Pollutri Dipartimento Farmaco Chimico Tecnologico, Università degli Studi di Siena, 53100 Siena, Italy

- **Claudia Maria Trombetta** Dipartimento di Medicina Molecolare e dello Sviluppo, Università degli Studi di Siena, 53100 Siena, Italy
- **Emanuele Montomoli** Dipartimento di Medicina Molecolare e dello Sviluppo, Università degli Studi di Siena, 53100 Siena, Italy; VisMederi Srl, 53100 Siena, Italy
- Valentina Riva Institute of Molecular Genetics IGM-CNR "Luigi Luca Cavalli-Sforza", 27100 Pavia, Italy
- Anna Garbelli Institute of Molecular Genetics IGM-CNR "Luigi Luca Cavalli-Sforza", 27100 Pavia, Italy
- Emanuele Maria Nola Institute of Molecular Genetics IGM-CNR "Luigi Luca Cavalli-Sforza", 27100 Pavia, Italy
- Maurizio Zazzi Dipartimento di Biotecnologie Mediche, Università degli Studi di Siena, 53100 Siena, Italy
- Giovanni Maga Institute of Molecular Genetics IGM-CNR "Luigi Luca Cavalli-Sforza", 27100 Pavia, Italy; @ orcid.org/ 0000-0001-8092-1552
- ^O**Maurizio Botta** Dipartimento Farmaco Chimico Tecnologico, Università degli Studi di Siena, 53100 Siena, Italy; Sbarro Institute for Cancer Research and Molecular Medicine, Center for Biotechnology, College of Science and Technology, Temple University, Philadelphia, Pennsylvania 19122, United States; o orcid.org/0000-0003-0456-6995

Complete contact information is available at: https://pubs.acs.org/10.1021/acsmedchemlett.9b00681

Author Contributions

Annalaura Brai designed and synthesized compounds and wrote the original manuscript. AP synthesized compounds. Adele Boccuto, IV, and FS performed DENV and cytotoxicity assays, VR, AG, and EMN purified DDX3X and performed enzymatic assays. MM, SM, and CT performed immunofluorescence assays. CIT performed in silico studies. ED analyzed compounds. MZ supervised antiviral and cytotoxicity assays. EM supervised immunofluorescence assays. GM supervised enzymatic assays. GM and MB had the original idea. MB supervised the project.

Author Contributions

 $^{\nabla}$ Annalaura Brai and Adele Boccuto equally contributed to this work.

Notes

The authors declare no competing financial interest. $^{\circ}$ Deceased on August 2, 2019.

ACKNOWLEDGMENTS

We are grateful to First Health Pharmaceuticals B.V., the exclusive license holder of the DDX3 inhibitors discussed in this paper, for their economic support of MB, MZ, FS, and ABo. Financial support was also provided by Tuscany Region (DD3242/2009 Bando Salute 2009) to MB. GM and VR were partially supported by the CNR project "Identification of novel therapeutic molecules against Zika and other emerging viruses.

DEDICATION

This work is dedicated to Professor Maurizio Botta, who transmitted his devotion for science to generations of researchers, extending, during the years, his critical but hopeful vision of medicinal chemistry.

ABBREVIATIONS

TMSN₃, trimethylsilylazide; tBuONO, *tert*-butylnitrite; FRET, fluorescence resonance energy transfer; FITC, fluoresceine

isothiocyanate; TCID50, half maximal tissue culture infectious dose; DENV, Dengue Virus

REFERENCES

(1) Dengue and severe dengue. https://www.who.int/news-room/ fact-sheets/detail/dengue-and-severe-dengue (accessed 2019-10-21).

(2) Rigau-Perez, J. G; Clark, G. G; Gubler, D. J; Reiter, P.; Sanders, E. J; Vance Vorndam, A Dengue and Dengue Haemorrhagic Fever. *Lancet* **1998**, 352 (9132), 971–977.

(3) Thomas, S. J.; Yoon, I.-K. A Review of Dengvaxia®: Development to Deployment. *Hum. Vaccines Immunother.* **2019**, *15*, 1–20.

(4) Maga, G.; Falchi, F.; Garbelli, A.; Belfiore, A.; Witvrouw, M.; Manetti, F.; Botta, M. Pharmacophore Modeling and Molecular Docking Led to the Discovery of Inhibitors of Human Immunodeficiency Virus-1 Replication Targeting the Human Cellular Aspartic Acid-Glutamic Acid-Alanine-Aspartic Acid Box Polypeptide 3. *J. Med. Chem.* **2008**, *51* (21), 6635–6638.

(5) Fazi, R.; Tintori, C.; Brai, A.; Botta, L.; Selvaraj, M.; Garbelli, A.; Maga, G.; Botta, M. Homology Model-Based Virtual Screening for the Identification of Human Helicase DDX3 Inhibitors. *J. Chem. Inf. Model.* **2015**, *55* (11), 2443–2454.

(6) Radi, M.; Falchi, F.; Garbelli, A.; Samuele, A.; Bernardo, V.; Paolucci, S.; Baldanti, F.; Schenone, S.; Manetti, F.; Maga, G.; et al. Discovery of the First Small Molecule Inhibitor of Human DDX3 Specifically Designed to Target the RNA Binding Site: Towards the next Generation HIV-1 Inhibitors. *Bioorg. Med. Chem. Lett.* **2012**, 22 (5), 2094–2098.

(7) Brai, A.; Fazi, R.; Tintori, C.; Zamperini, C.; Bugli, F.; Sanguinetti, M.; Stigliano, E.; Esté, J.; Badia, R.; Franco, S.; et al. Human DDX3 Protein Is a Valuable Target to Develop Broad Spectrum Antiviral Agents. *Proc. Natl. Acad. Sci. U. S. A.* **2016**, *113* (19), 5388–5393.

(8) Brai, A.; Martelli, F.; Riva, V.; Garbelli, A.; Fazi, R.; Zamperini, C.; Pollutri, A.; Falsitta, L.; Ronzini, S.; Maccari, L.; et al. DDX3X Helicase Inhibitors as a New Strategy To Fight the West Nile Virus Infection. *J. Med. Chem.* **2019**, *62* (5), 2333–2347.

(9) Brai, A.; Ronzini, S.; Riva, V.; Botta, L.; Zamperini, C.; Borgini, M.; Trivisani, C. I.; Garbelli, A.; Pennisi, C.; Boccuto, A.; et al. Synthesis and Antiviral Activity of Novel 1,3,4-Thiadiazole Inhibitors of DDX3X. *Molecules* **2019**, *24* (21), 3988.

(10) Kumar, R.; Singh, N.; Abdin, M. Z.; Patel, A. H.; Medigeshi, G. R. Dengue Virus Capsid Interacts with DDX3X-A Potential Mechanism for Suppression of Antiviral Functions in Dengue Infection. *Front. Cell. Infect. Microbiol.* **2018**, 7 (JAN). DOI: 10.3389/fcimb.2017.00542.

(11) Khadka, S.; Vangeloff, A. D.; Zhang, C.; Siddavatam, P.; Heaton, N. S.; Wang, L.; Sengupta, R.; Sahasrabudhe, S.; Randall, G.; Gribskov, M.; et al. A Physical Interaction Network of Dengue Virus and Human Proteins. *Mol. Cell. Proteomics* **2011**, *10* (12), M111.012187.

(12) Doñate-Macián, P.; Jungfleisch, J.; Pérez-Vilaró, G.; Rubio-Moscardo, F.; Perálvarez-Marín, A.; Diez, J.; Valverde, M. A. The TRPV4 Channel Links Calcium Influx to DDX3X Activity and Viral Infectivity. *Nat. Commun.* **2018**, *9* (1). DOI: 10.1038/s41467-018-04776-7.

(13) Song, H.; Ji, X. The Mechanism of RNA Duplex Recognition and Unwinding by DEAD-Box Helicase DDX3X. *Nat. Commun.* **2019**, *10* (1), 3085.

(14) Heerma van Voss, M. R.; Vesuna, F.; Bol, G. M.; Afzal, J.; Tantravedi, S.; Bergman, Y.; Kammers, K.; Lehar, M.; Malek, R.; Ballew, M.; et al. Targeting Mitochondrial Translation by Inhibiting DDX3: A Novel Radiosensitization Strategy for Cancer Treatment. *Oncogene* **2018**, *37* (1), 63–74.

(15) Chang, J.; Schul, W.; Butters, T. D.; Yip, A.; Liu, B.; Goh, A.; Lakshminarayana, S. B.; Alonzi, D.; Reinkensmeier, G.; Pan, X.; et al. Combination of α -Glucosidase Inhibitor and Ribavirin for the Treatment of Dengue Virus Infection in Vitro and in Vivo. *Antiviral Res.* **2011**, *89* (1), 26–34. (16) Xu, H.-T.; Colby-Germinario, S. P.; Hassounah, S. A.; Fogarty, C.; Osman, N.; Palanisamy, N.; Han, Y.; Oliveira, M.; Quan, Y.; Wainberg, M. A. Evaluation of Sofosbuvir (β -D-2'-Deoxy-2'- α -Fluoro-2'- β -C-Methyluridine) as an Inhibitor of Dengue Virus Replication. *Sci. Rep.* **2017**, 7 (1), 6345.

(17) Chahar, H. S.; Chen, S.; Manjunath, N. P-Body Components LSM1, GW182, DDX3, DDX6 and XRN1 Are Recruited to WNV Replication Sites and Positively Regulate Viral Replication. *Virology* **2013**, 436 (1), 1–7.

(18) Li, C.; Ge, L.; Li, P.; Wang, Y.; Dai, J.; Sun, M.; Huang, L.; Shen, Z.; Hu, X.; Ishag, H.; et al. Cellular DDX3 Regulates Japanese Encephalitis Virus Replication by Interacting with Viral Un-Translated Regions. *Virology* **2014**, *449*, 70–81.

(19) Ariumi, Y. Multiple Functions of DDX3 RNA Helicase in Gene Regulation, Tumorigenesis, and Viral Infection. *Front. Genet.* **2014**, *5*, 423.

(20) Reineke, L. C.; Lloyd, R. E. Diversion of Stress Granules and P-Bodies during Viral Infection. *Virology* **2013**, 436, 255–267 February 20.

(21) Ward, A. M.; Bidet, K.; Yinglin, A.; Ler, S. G.; Hogue, K.; Blackstock, W.; Gunaratne, J.; Garcia-Blanco, M. A. Quantitative Mass Spectrometry of DENV-2 RNA-Interacting Proteins Reveals That the DEAD-Box RNA Helicase DDX6 Binds the DB1 and DB2 3' UTR Structures. *RNA Biol.* **2011**, 8 (6), 1173–1186.