Available online at www.sciencedirect.com

ScienceDirect

Current Opinion in Virology

Strategies for Zika drug discovery

Jing Zou[1] and Pei-Yong Shi[1,2,3,4]

Zika virus (ZIKV) can cause devastating congenital syndrome in fetuses from pregnant women and autoimmune disorder Guillain-Barré syndrome in adults. No clinically approved vaccine or drug is currently available for ZIKV. This unmet medical need has motivated a global effort to develop countermeasures. Several promising ZIKV vaccine candidates have already entered clinical trials. In contrast, antiviral development of ZIKV is lagging behind. Here, we review the overall strategies for ZIKV drug discovery, including (i) repurposing of clinically approved drugs, (ii) viral replication-based phenotypic screening for inhibitors, and (iii) targeted drug discovery of viral proteins. Along with vaccines, the development of antiviral treatment will provide a complementary means to control ZIKV infections.

Addresses
[1] Department of Biochemistry and Molecular Biology, University of Texas Medical Branch, Galveston, TX, USA
[2] Department of Phamarcology and Toxicology, University of Texas Medical Branch, Galveston, TX, USA
[3] Sealy Center for Structural Biology and Molecular Biophysics, University of Texas Medical Branch, Galveston, TX, USA
[4] Sealy Institute for Vaccine Sciences, University of Texas Medical Branch, Galveston, TX, USA

Corresponding author: Shi, Pei-Yong (peshi@utmb.edu)

Current Opinion in Virology 2019, **35**:19–26

This review comes from a themed issue on **Antiviral strategies**

Edited by **Margo A Brinton** and **Richard K Plemper**

For a complete overview see the Issue and the Editorial

Available online 7th March 2019

https://doi.org/10.1016/j.coviro.2019.01.005

Introduction

Zika virus (ZIKV), a member from the mosquito-borne *flavivirus* genus within *Flaviviridae* family, has recently caused continental level epidemics due to the distribution of the mosquito vectors. Besides ZIKV, many other flaviviruses are also important human pathogens that pose constant threats to public health, including the four serotypes of dengue (DENV), yellow fever (YFV), West Nile (WNV), Japanese encephalitis (JEV), and tick-borne encephalitis virus (TBEV). ZIKV was first isolated in 1947 from a Rhesus macaque in Uganda [1]. Outside Africa, ZIKV was first isolated from *Aedes aegypti* mosquitoes in 1966 in Malaysia. Before 2007, ZIKV had silently circulated between primates and mosquitoes in forests in Africa and Southeast Asia, and caused only sporadic human infections with negligible clinical manifestations. In 2007, the first major outbreak of ZIKV occurred on Yap Island, Micronesia, where 49 confirmed and 59 suspected ZIKV cases were identified with common clinical symptoms such as rash, fever, arthralgia, and conjunctivitis [2]. After that, large epidemics occurred in French Polynesia and other regions of the South Pacific in 2013–2014 [3]. Since 2015, 84 countries reported to have ZIKV transmission (https://www.who.int/emergencies/zika-virus/situation-report/10-march-2017/en/). During the 2015 epidemic in South America, clusters of microcephaly [4,5] and neurological disorders [6] were linked to the circulation of ZIKV. Because of the explosive epidemics and teratogenic potential, the World Health Organization (WHO) declared ZIKV to be a Public Health Emergency of International Concern from February to November 2016. Since then, intensive global efforts have been made to understand ZIKV biology and to develop countermeasures. Currently, there is no clinically approved vaccine or therapy to prevent or treat ZIKV infection. In this report, we review the strategies for discovery of small molecule inhibitors of ZIKV.

Viral genome and replication

Flavivirus genome is a single-stranded, positive-sense RNA in the length of ~10.8 kb. It contains a single open-reading-frame that encodes a polyprotein. The polyprotein is cleaved into three structural proteins [capsid (C), pre-membrane (prM), and envelope (E)] and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) by viral and cellular proteases. Structural proteins form viral particles, and nonstructural proteins play roles in the formation of viral replication complex, virion assembly, and evasion of host immune response [7]. Among the ten viral proteins, only NS3 and NS5 have enzymatic activities. NS3 protein contains serine protease activity at the N terminus with NS2B as a cofactor, and RNA triphosphatase and helicase activities at the C terminus [8]. NS5 protein consists of two enzymatic domains: the N terminus is a methyltransferase (MTase) which is required for viral RNA cap methylation and internal RNA methylation [9–12]. The cap methylation facilitates the efficiency of RNA translation and evasion of host immune response [13]; the C terminus of NS5 is a RNA-dependent RNA polymerase (RdRp), which is responsible for viral RNA synthesis [14]. Targeting viral enzymes is a proven antiviral approach, as evidenced by the clinically approved drugs against HIV-1 protease, reverse transcriptase, integrase and hepatitis C virus (HCV) protease and RdRp [15,16].

Phylogenetic analysis classifies ZIKV strains into two major lineages: African and Asian/American [17]. Serologic reactivity indicates that all ZIKV strains fall into a single serotype [18•]. Over the past fifteen years, significant progress has been made on DENV drug discovery. Since ZIKV is closely related to other flaviviruses [2] and shares 54–59% amino acid identity to DENV [19••], lessons learned from DENV drug discovery should be applicable to ZIKV drug discovery [20•,21].

Overall antiviral strategies

Three general strategies have been pursued to identify inhibitors of ZIKV: (i) repurposing of clinically approved drugs. Drug repurposing aims to identify new uses of existing drugs (or compounds in clinical development) for ZIKV therapy. (ii) Viral replication-based phenotypic screening for inhibitors. Infectious cDNA clones and replicons have been developed for various strains of ZIKV. These reverse genetic systems can be used to facilitate antiviral drug discovery [22–24,25•,26–28]. As detailed below, reporter ZIKV and replicon have been adapted to high-throughput screening (HTS) of compound libraries [22,23,25•,29•]. (iii) Targeted drug discovery of viral proteins. The target-based strategy has employed three common approaches: viral enzyme-based HTS, structure-based rational design, and *in silico* docking [30–34,35••,36]. These approaches have been strongly bolstered by the structural information of ZIKV, including the cryo-EM structure of virion [37–39] and the atomic structures of individual viral proteins, including capsid [40,41], envelope [42], NS1 [43,44], NS3 [45–49], and NS5 [50–55].

Repurposing of clinically approved drugs

Drug repurposing screening has emerged as a rapid strategy for drug development. Compared with screening compound libraries for new inhibitors, drug repurposing has the advantages of speed and cost-saving in identifying active drugs. The repurposed drugs may be readily advanced to clinical trials because they have already been approved for human use. So far, at least four groups have performed drug-repurposing screening for ZIKV [56•,57•,58•,59•].

Barrows *et al.* screened a library of FDA-approved 774 drugs for inhibitors of ZIKV MEX_1_7 strain on Huh-7 cells [58•]. A high-content imaging assay was used to quantify the E protein-positive cells after ≥24 h of compounds treatment in a 96-well format. The screen identified 45 inhibitors, among which 8 drugs (i.e. ivermectin, daptomycin, MPA, sertraline, pyrimethamine, cyclosporine A, azathioprine, and mefloquine) were further validated on cell lines (Huh-7, HeLa, and JEG3), primary human amnion epithelial cells, and neural stem cells. The 8 FDA-approved drugs were shown to inhibit ZIKV in a cell-type independent manner.

Adcock *et al.* screened an NIH Clinical Compound Collection Library of 727 compounds for inhibitors of two ZIKV strains, MR766 and PRVABC59 [56•]. The HTS was based on monitoring the cytopathic effect (CPE) in a 384-well format. Cell viability was measured by the intracellular ATP level. Compounds that protect cells from the ZIKV-induced CPE are potential antiviral inhibitors. The authors identified three hits (6-azauridine, finasteride, and mevastatin), among which 6-azauridine exhibited the highest potency with EC_{50s} of 3.9 μM and 3.2 μM against PRVABC59 and MR766 strains, respectively. Since 6-azauridine is known to inhibit other flaviviruses by inhibiting cellular pyrimidine synthesis, the authors tested other pyrimidine synthesis inhibitors and found that Brequinar and CID 91632869 had potent anti-ZIKV activity (EC_{50s} of 0.8–2.2 μM) without obvious cytotoxicity. This is not surprising because inhibitors of pyrimidine synthesis pathway were known to have pan-antiviral activity in cell culture, but no efficacy in animals because the salvage pathway restored the pyrimidine levels from food [60].

Pascoalino *et al.* [59•] screened 725 FDA-approved drugs on Huh-7 cells using a high content imaging method in a 384-well format, similar to that described in Ref. [58•]. The screening identified 29 inhibitors, among which 22 drugs were confirmed with a secondary dose titration assay. On the basis of the selective index, maximum activity, and EC_{50}, five most promising hits were Lovastatin (EC_{50} 20 μM), 5-fluorouracil (EC_{50} 14 μM), 6-azauridine (EC_{50} 2.3 μM), palonosetron (EC_{50} 16 μM), and kitasamycin (EC_{50} 42 μM).

Xu *et al.* performed a repurposing screening of ~6,000 compounds that included FDA-approved drugs, clinical trial compounds, and pharmacologically active compounds in human neural progenitor cells (hNPCs), SNB-19 cells, and astrocytes [57•]. The HTS measured caspase-3 activity in the primary assay and intracellular ATP activity in the secondary cell viability assay [61]. Two classes of compounds were identified: (i) compounds with anti-ZIKV activity and (ii) compounds with neuroprotective activity. Interestingly, combination treatments using one antiviral compound and one neuroprotective compound showed an additive protection of human neural progenitors and astrocytes from ZIKV-induced cell death.

The above inhibitors identified from the repurposing effort need further testing in ZIKV animal models. More importantly, it is critical to evaluate if the drug exposure levels are sufficiently high in humans to achieve therapeutic effect. It should be noted that lovastatin has been tested in a dengue clinical trial and showed no clinical benefits [62]. Because the unique patient populations are pregnant women, the risk of treatments using re-purposed drugs has to be weighed against the risk of no

treatment. Re-purposed drugs such as cancer drugs would not be appropriate to give to pregnant women.

Viral replication-based phenotypic screening

HTS can be used for drug repurposing screening [56•,57•,58•,59•] and for new compound library screening. Three viral replication-based assays have been developed for phenotypic HTS: (a) ZIKV infection assay; (b) ZIKV replicon assay; (c) Zika virus-like particle (VLP) infection assay. All the three assays have previously been successfully adopted for HTS of large compound libraries for DENV and WNV [63,64,65•].

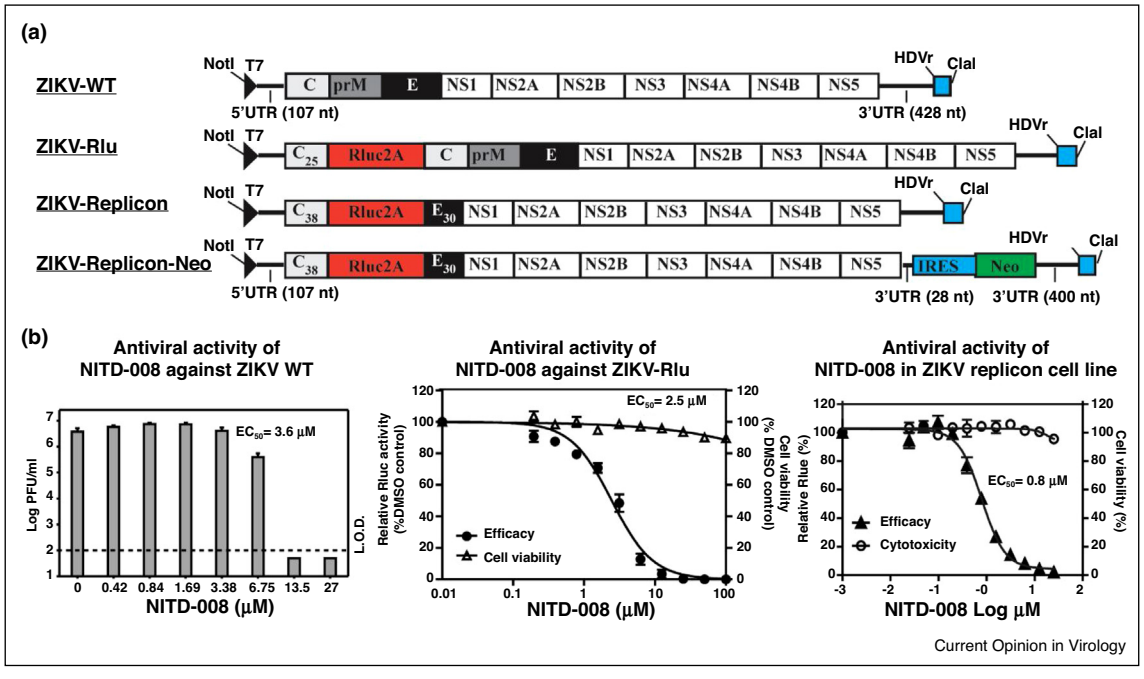
ZIKV infection assay

ZIKV infection assay captures each step of a viral replication cycle, including virus attachment, entry, fusion, nucleocapsid uncoating, RNA translation, RNA synthesis, virion assembly, and virus release. Inhibitors of any of these replication steps could be identified through such ZIKV infection HTS. As described in the preceding section, wild-type ZIKV infection of various human cells was used to screen FDA-approved drugs [56•,57•,58•,59•]. Besides the wild-type ZIKV, recombinant ZIKV expressing a reporter gene (luciferase, EGFP, or mCherry) is a valuable tool for drug discovery, since the reporter gene could be used as a surrogate to indicate viral replication levels and drug inhibition activities [25•,63, 66, 67]. Shan *et al.* engineered a luciferase ZIKV (Figure 1a). Comparable EC_{50} values were obtained for NIT-D008 (a known adenosine analog inhibitor of flaviviruses [68]) when tested in the wild-type and reporter ZIKV (Figure 1b). Similar to ZIKV, a luciferase reporter DENV-2 was previously used for HTS with a robust Z' factor of 0.79 [63]. These results indicate that reporter ZIKV could be used for drug screening.

ZIKV replicon assay

Flavivirus replicon contains an in-frame deletion in the structural genes that are replaced by a reporter gene (luciferase or EGFP; Figure 1a) [29•]. Due to the lack of structural genes, flavivirus replicon can translate proteins and replicate, but could not form infectious virions. Thus, compared with an infectious virus-based assay, the replicon assay can be performed in biosafety level 2 laboratory, which is advantageous when working with BSL3 flaviviruses, such as WNV, JEV, and TBEV. Flavivirus replicons have been well established for antiviral screening and for studying viral replication [64,69–76]. For ZIKV, Xie *et al.* developed a transient luciferase replicon and a stable cell line harboring a luciferase replicon (Figure 1a) [29•]. The stable replicon cell line was further formatted into a 96-well HTS and validated with NITD008 for HTS (Figure 1a and b).

Figure 1



Viral replication-based HTS platforms.
(a) Schematic diagrams of wild-type ZIKV infectious clone (ZIKV-WT) [25•], renilla luciferase reporter virus (ZIKV-Rlu) [25•], renilla luciferase replicon (ZIKV-Replicon), and dual reporter replicon with luciferase and neomycin resistance genes (ZIKV-Replicon-Neo) [29•]. **(b)** Different antiviral platforms were validated for antiviral testing by nucleoside inhibitor NITD-008 [25•,29•]. Left panel: WT ZIKV infection assay on Vero cells; Middle panel: luciferase ZIKV infection assay on Vero cells; Right panel: luciferase replicon cell line assay on Huh-7 cells.

Zika virus-like particle (VLP) infection assay

VLPs are prepared through *trans* supply of viral structural proteins in cells containing replicon RNA. Since the replicon does not encode viral structural proteins, VLP infection is single-round, and captures the steps of viral entry, translation, and RNA synthesis, but not virion assembly and release [65•,77]. VLPs have been successfully used for antiviral screening, vaccine development, and serological diagnosis of flaviviruses, including WNV, JEV, YFV, DENV, and ZIKV [64,65•,77–81]. Recently, Garg *et al.* generated VLPs by expressing ZIKV structural proteins (CPrME or PrME) and a WNV-EGFP replicon [81], such VLP infection assay could be used to screen for inhibitors of ZIKV entry. So far, no study has been reported using the ZIKV VLPs for drug screening. HTS experience from DENV drug discovery demonstrated that, among the three assays discussed in (i)–(iii), the VLP HTS yielded the least productive hits [20•].

One advantage of the cell-based infection assays is to identify compounds with cellular antiviral activity. However, target deconvolution could be challenging for these inhibitors. This is because flaviviruses encode only ten viral proteins and require hundreds of cellular proteins to support viral replication. Consequently, it is likely that most inhibitors identified from the viral infection-based assays exert their antiviral activities through interfering with host factors. Compared with viral targets, interference with host factors for antiviral therapy has a higher safety liability of perturbing the physiological functions of the host proteins. In accordance with the past experience in DENV drug discovery, the cell-based infection assay has a higher chance to identify inhibitors with antiviral efficacy in animal models [68,82,83].

Targeted drug discovery of viral proteins

Flavivirus NS3 and NS5 have enzymatic activities that could be targeted for antiviral discovery. The structures of ZIKV NS2B–NS3 protease alone and in complex with an inhibitor have been solved [45–49,84,85]. The crystal structures of ZIKV NS5 MTase and RdRp domains have also been solved [50–55]. These structural information has formed a solid foundation for structure-based rational design and in silicon docking.

Protease

Designing inhibitors of flavivirus protease with good oral bioavailability has proven to be difficult in the past fifteen years. This is partially because of the flat surface and highly charged active site of the NS2B–NS3 proteases [86,87]. Although several inhibitors have been found to potently inhibit WNV and DENV proteases *in vitro*, none of them showed *in vivo* activity due to lack cell permeability and poor pharmacokinetics [88–92]. For WNV and DENV NS2B–NS3 proteases, only the structures of closed conformation in complex with ligands or inhibitors have been solved. For ZIKV NS2B–NS3 protease, structures of both open conformation (without an inhibitor) and closed conformation (with an inhibitor) are available, providing a solid foundation for drug discovery [87]. Chan *et al.* identified bromocriptine as a ZIKV protease inhibitor [93]. It inhibited ZIKV in cell culture (EC_{50} 12 μM) and protease activity (IC_{50} 22 μM). Bromocriptine is an agonist of dopamine receptors two and three, and is used to treat galactorrhea and Parkinson's diseases. This compound was previously reported to have anti-DENV and anti-TBEV activities [94].

Brecher *et al.* developed a split luciferase complementation (SLC) assay to monitor the conformational switch of NS2B during protease catalysis and to characterize allosteric inhibitors [95]; the rationale was that binding of an active-site inhibitor to the protease would lead to a conformational change of NS2B, which could be measured by the SLC change. They screened a National Cancer Institute library using the SLC assay, and identified NSC135618 as a broad spectrum inhibitor of flavivirus protease. NSC135618 inhibited DENV-2, ZIKV, WNV, and YFV *in vivo* with EC_{50s} of low micromolar range [95].

Lee *et al.* screened 71 HCV protease inhibitors in a ZIKV protease assay. They identified several compounds with anti-ZIKV protease activity (e.g. compound 3 with IC_{50} 4 μM in a protease assay and K_D 9 μM in an SPR binding assay) [84]. Results from *in silico* docking and binding kinetics suggest that the compounds competitively interact with the substrate-binding site on the ZIKV protease.

Helicase

Limited progress has been made towards developing flavivirus helicase inhibitors despite of the availability of high-resolution structures [96,97]. Several groups performed HTS against flavivirus helicase, but failed to identify specific inhibitors [98–100], probably because of the flat nature of the RNA substrate binding site on the helicase [101]. Nevertheless, Suramin, an FDA-approved anti-parasitic drug, was reported to have anti-ZIKV activity in cell culture (EC_{50} 1.9–3.9 μM); *in silico* modeling suggests that the compound binds to ZIKV helicase [102]. However, the exact mode-of-action remains to be determined because Suramin also seemed to inhibit virus adsorption and entry [102].

MTase

NS5 is one of the most conserved flavivirus proteins. Because of its MTase and RdRp activities, flavivirus NS5 has been the major target for drug discovery [50,53]. For targeting flavivirus NS5 MTase, the challenge is to design inhibitors that selectively suppress viral MTase without affecting the host MTases. This is because the core domain of flavivirus MTase is similar to those of host MTases [20•]. To overcome the challenge, Lim *et al.* rationally designed analogs of *S*-adenosyl

homocysteine (SAH, a by-product of methylation reaction) that specifically bind to a cavity unique to the flavivirus MTase [103–107]. Although the structure-based rational design yielded inhibitors with selective activity against viral MTase, the compounds lack membrane permeability and, therefore, did not show robust antiviral activity in cell culture [104]. These results challenge the use of SAH or its analog Sinefungin as a starting point for rational design of flavivirus MTase inhibitors. To identify new inhibitors targeting this flavivirus MTase-conserved cavity, Stephen *et al.* performed a virtual screening of 28 341 compounds and identified 10 hits, 4 of which showed cell culture activities with EC_{50s} 4.8–17.6 μM [33]. Medicinal chemistry effort is needed to improve the potency and other properties of these inhibitors.

RdRp

Both nucleoside analog inhibitors (NIs) and non-nucleoside inhibitors (NNIs) have been identified for ZIKV RdRp [68,108–117]. Eyer *et al.* screened 29 nucleoside analogs and found three 2′-*C*-methylated nucleosides that inhibited ZIKV on Vero cells with EC_{50s} 5.3–10.5 μM [118]. Sofosbuvir is a uridine nucleotide drug approved for treatment of HCV patients. Sofosbuvir was reported to inhibit ZIKV on different cell lines through blocking viral RdRp activity [119]. However, since Sofosbuvir is designed to target liver for HCV inhibition, it remains to be determined if the exposure levels of Sofosbuvir are sufficiently high in ZIKV-replicating sites (other than liver) to achieve antiviral efficacy in humans.

Using *in silico* screening of ZIKV RdRp, Pattnaik *et al.* identified a promising compound TPB with potent cell culture activity (EC_{50} 94 nM and CC_{50} 19.4 μM). When dosed at 25 m/kg, TPB suppressed viremia by nearly 40-folds in a mouse model [35••]. It remains to be demonstrated that TPB directly binds to and suppresses ZIKV RdRp *in vitro*.

Envelope

E protein is an attractive target since it mediates viral entry and fusion. Using rational design, Yu *et al.* reported a synthetic peptide derived from the stem region of ZIKV E protein, designated Z2, which inhibited ZIKV in cell culture and in mice. The Z2 peptide disrupted the integrity of virion membrane, penetrated the placental barrier, and inhibited vertical transmission of ZIKV in pregnant mice [120••]. For targeting E protein, therapeutic antibodies will be a highly attractive approach to deliver clinical candidates, as recently reviewed in Ref. [121].

Conclusion

The devastating disease outcomes of congenital syndromes in pregnant women and Guillain-Barre syndrome in adults have inspired global effort to combat ZIKV. Significant progresses have been made towards the development of ZIKV countermeasures in the past three years [19••,122••,123]. The success of clinically approved drugs against HIV-1 protease and reverse transcriptase, HBV polymerase, and HCV protease and polymerase [15,16,124•] justifies the antiviral focus on flavivirus NS3 and NS5. Meanwhile, lessons should be learned from previous experience on drug discovery for other flaviviruses [20•]. Although the number of ZIKV infection in humans has significantly declined in the Americas, the recent outbreak of ZIKV in India [125] underscores the urgency to continue the development of effective countermeasures against this emerging pathogen.

Funding

J.Z. was awarded with postdoctoral fellowships from the McLaughlin Endowment at University of Texas Medical Branch. P.-Y.S. lab was supported by a Kleberg Foundation Award and NIH grants AI127744 and AI136126.

Conflict of interest statement

Nothing declared.

Acknowledgements

We thank our colleagues at the University of Texas Medical Branch for helpful discussion and support during the course of the study. Because of size limit, we apologize for not being able to cite more references.

References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

1. Dick GW: **Zika virus. II. Pathogenicity and physical properties**. *Trans R Soc Trop Med Hyg* 1952, **46**:521-534.
2. Duffy MR *et al.*: **Zika virus outbreak on Yap Island, Federated States of Micronesia**. *N Engl J Med* 2009, **360**:2536-2543.
3. Cao-Lormeau VM *et al.*: **Zika virus, French polynesia, South Pacific, 2013**. *Emerg Infect Dis* 2014, **20**:1085-1086.
4. de Paula Freitas B *et al.*: **Ocular findings in infants with microcephaly associated with presumed Zika virus congenital infection in Salvador, Brazil**. *JAMA Ophthalmol* 2016, **134**:529-535.
5. Leal MC *et al.*: **Hearing loss in infants with microcephaly and evidence of congenital Zika virus infection – Brazil, November 2015–May 2016**. *MMWR Morb Mortal Wkly Rep* 2016, **65**:917-919.
6. Oehler E *et al.*: **Zika virus infection complicated by Guillain-Barre syndrome—case report, French Polynesia, December 2013**. *Euro Surveill* 2014, **19**.
7. Pierson TC, Diamond MS: **Flaviviruses**. In *Fields Virology*, 6th ed., vol 1. Edited by Knipe DM, Howley PM. 2013:747-794.
8. Li H *et al.*: **The serine protease and RNA-stimulated nucleoside triphosphatase and RNA helicase functional domains of dengue virus type 2 NS3 converge within a region of 20 amino acids**. *J Virol* 1999, **73**:3108-3116.
9. Egloff MP *et al.*: **An RNA cap (nucleoside-2′-O-)-methyltransferase in the flavivirus RNA polymerase NS5: crystal structure and functional characterization**. *EMBO J* 2002, **21**:2757-2768.
10. Ray D *et al.*: **West Nile virus 5′-cap structure is formed by sequential guanine N-7 and ribose 2′-O methylations by nonstructural protein 5**. *J Virol* 2006, **80**:8362-8367.

11. Zhou Y *et al.*: **Structure and function of flavivirus NS5 methyltransferase**. *J Virol* 2007, **81**:3891-3903.

12. Dong H *et al.*: **2′-O Methylation of internal adenosine by flavivirus NS5 methyltransferase**. *PLoS Pathog* 2012, **8**: e1002642.

13. Daffis S *et al.*: **2′-O Methylation of the viral mRNA cap evades host restriction by IFIT family members**. *Nature* 2010, **468**:452-456.

14. Yap TL *et al.*: **Crystal structure of the dengue virus RNA-dependent RNA polymerase catalytic domain at 1.85-angstrom resolution**. *J Virol* 2007, **81**:4753-4765.

15. Wyles DL: **Antiviral resistance and the future landscape of hepatitis C virus infection therapy**. *J Infect Dis* 2013, **207(Suppl. 1)**:S33-S39.

16. De Clercq E: **Anti-HIV drugs: 25 compounds approved within 25 years after the discovery of HIV**. *Int J Antimicrob Agents* 2009, **33**:307-320.

17. Weaver SC *et al.*: **Zika virus: history, emergence, biology, and prospects for control**. *Antiviral Res* 2016, **130**:69-80.

18. • Dowd KA *et al.*: **Broadly neutralizing activity of Zika virus-immune sera identifies a single viral serotype**. *Cell Rep* 2016, **16**:1485-1491.
This study demonstrated that Zika virus has only one serotype by neutralizing assay.

19. •• Diamond MS, Ledgerwood JE, Pierson TC: **Zika virus vaccine development: progress in the face of new challenges**. *Annu Rev Med* 2019, **70**:121-135.
Good review about the development of ZIKV vaccine.

20. • Lim SP *et al.*: **Ten years of dengue drug discovery: progress and prospects**. *Antiviral Res* 2013, **100**:500-519.
This review summarized the experience and lessons learned from dengue drug discovery, which could be potentially applied for anti-ZIKV drug discovery.

21. Noble CG *et al.*: **Strategies for development of dengue virus inhibitors**. *Antiviral Res* 2010, **85**:450-462.

22. Gadea G *et al.*: **A robust method for the rapid generation of recombinant Zika virus expressing the GFP reporter gene**. *Virology* 2016, **497**:157-162.

23. Mutso M *et al.*: **Reverse genetic system, genetically stable reporter viruses and packaged subgenomic replicon based on a Brazilian Zika virus isolate**. *J Gen Virol* 2017, **98**:2712-2724.

24. Schwarz MC *et al.*: **Rescue of the 1947 Zika virus prototype strain with a cytomegalovirus promoter-driven cDNA clone**. *mSphere* 2016, **1**.

25. • Shan C *et al.*: **An infectious cDNA clone of Zika virus to study viral virulence, mosquito transmission, and antiviral inhibitors**. *Cell Host Microbe* 2016, **19**:891-900.
A systematic study about how to generate an infectious clone of ZIKV and how to characterize the recombinant virus using different platforms.

26. Tsetsarkin KA *et al.*: **A full-length infectious cDNA clone of Zika virus from the 2015 epidemic in Brazil as a genetic platform for studies of virus-host interactions and vaccine development**. *mBio* 2016, **7**.

27. Weger-Lucarelli J *et al.*: **Development and characterization of recombinant virus generated from a new world Zika virus infectious clone**. *J Virol* 2017, **91**.

28. Widman DG *et al.*: **A reverse genetics platform that spans the Zika virus family tree**. *mBio* 2017, **8**.

29. • Xie X *et al.*: **Zika virus replicons for drug discovery**. *EBioMedicine* 2016, **12**:156-160.
The study developed a ZIKV replicon platform for anti-ZIKV drug discovery.

30. Byler KG, Ogungbe IV, Setzer WN: **In-silico screening for anti-Zika virus phytochemicals**. *J Mol Graph Model* 2016, **69**:78-91.

31. Shiryaev SA *et al.*: **Characterization of the Zika virus two-component NS2B-NS3 protease and structure-assisted identification of allosteric small-molecule antagonists**. *Antiviral Res* 2017, **143**:218-229.

32. Yuan S *et al.*: **Structure-based discovery of clinically approved drugs as Zika virus NS2B-NS3 protease inhibitors that potently inhibit Zika virus infection in vitro and in vivo**. *Antiviral Res* 2017, **145**:33-43.

33. Stephen P *et al.*: **Structural insight into NS5 of Zika virus leading to the discovery of MTase inhibitors**. *J Am Chem Soc* 2016, **138**:16212-16215.

34. Elfiky AA, Elshemey WM: **Molecular dynamics simulation revealed binding of nucleotide inhibitors to ZIKV polymerase over 444 nanoseconds**. *J Med Virol* 2018, **90**:13-18.

35. •• Pattnaik A *et al.*: **Discovery of a non-nucleoside RNA polymerase inhibitor for blocking Zika virus replication through in silico screening**. *Antiviral Res* 2018, **151**:78-86.
The study identified TPB as a potent ZIKV inhibitor by *in silico* screening targeting ZIKV RdRp and experiments *in vitro* and *in vivo*.

36. Ramharack P, Soliman MES: **Zika virus NS5 protein potential inhibitors: an enhanced in silico approach in drug discovery**. *J Biomol Struct Dyn* 2018, **36**:1118-1133.

37. Kostyuchenko VA *et al.*: **Structure of the thermally stable Zika virus**. *Nature* 2016, **533**:425-428.

38. Sirohi D *et al.*: **The 3.8 A resolution cryo-EM structure of Zika virus**. *Science* 2016, **352**:467-470.

39. Prasad VM *et al.*: **Structure of the immature Zika virus at 9 A resolution**. *Nat Struct Mol Biol* 2017, **24**:184-186.

40. Li T *et al.*: **Structural insight into the Zika virus capsid encapsulating the viral genome**. *Cell Res* 2018, **28**:497-499.

41. Shang Z *et al.*: **Crystal structure of the capsid protein from Zika virus**. *J Mol Biol* 2018, **430**:948-962.

42. Barba-Spaeth G *et al.*: **Structural basis of potent Zika-dengue virus antibody cross-neutralization**. *Nature* 2016, **536**:48-53.

43. Xu X *et al.*: **Contribution of intertwined loop to membrane association revealed by Zika virus full-length NS1 structure**. *EMBO J* 2016, **35**:2170-2178.

44. Brown WC *et al.*: **Extended surface for membrane association in Zika virus NS1 structure**. *Nat Struct Mol Biol* 2016, **23**:865-867.

45. Zhang Z *et al.*: **Crystal structure of unlinked NS2B-NS3 protease from Zika virus**. *Science* 2016, **354**:1597-1600.

46. Lei J *et al.*: **Crystal structure of Zika virus NS2B-NS3 protease in complex with a boronate inhibitor**. *Science* 2016, **353**:503-505.

47. Phoo WW *et al.*: **Structure of the NS2B-NS3 protease from Zika virus after self-cleavage**. *Nat Commun* 2016, **7**:13410.

48. Jain R *et al.*: **Structure of the NS3 helicase from Zika virus**. *Nat Struct Mol Biol* 2016, **23**:752-754.

49. Li Y *et al.*: **Structural dynamics of Zika virus NS2B-NS3 protease binding to dipeptide inhibitors**. *Structure* 2017, **25**:1242-1250.e3.

50. Upadhyay AK *et al.*: **Crystal structure of full-length Zika virus NS5 protein reveals a conformation similar to Japanese encephalitis virus NS5**. *Acta Crystallogr F Struct Biol Commun* 2017, **73**:116-122.

51. Godoy AS *et al.*: **Crystal structure of Zika virus NS5 RNA-dependent RNA polymerase**. *Nat Commun* 2017, **8**:14764.

52. Zhao B *et al.*: **Structure and function of the Zika virus full-length NS5 protein**. *Nat Commun* 2017, **8**:14762.

53. Wang B *et al.*: **The structure of Zika virus NS5 reveals a conserved domain conformation**. *Nat Commun* 2017, **8**:14763.

54. Zhou H *et al.*: **The conformational changes of Zika virus methyltransferase upon converting SAM to SAH**. *Oncotarget* 2017, **8**:14830-14834.

55. Coutard B *et al.*: **Zika virus methyltransferase: structure and functions for drug design perspectives**. *J Virol* 2017, **91**.

56. Adcock RS *et al.*: **Evaluation of anti-Zika virus activities of broad-spectrum antivirals and NIH clinical collection compounds using a cell-based, high-throughput screen assay**. *Antiviral Res* 2017, **138**:47-56.
Repurposing of drugs by screening an NIH Clinical Compound Collection Library of 727 compounds for inhibitors of two ZIKV strains, MR766 and PRVABC59.

57. Xu M *et al.*: **Identification of small-molecule inhibitors of Zika virus infection and induced neural cell death via a drug repurposing screen**. *Nat Med* 2016, **22**:1101-1107.
Repurposing of drugs by screening ~6000 compounds that included FDA-approved drugs, clinical trial compounds, and pharmacologically active compounds in human neural progenitor cells, SNB-19 cells, and astrocytes.

58. Barrows NJ *et al.*: **A screen of FDA-approved drugs for inhibitors of Zika virus infection**. *Cell Host Microbe* 2016, **20**:259-270.
Repurposing of drugs by screening a library of FDA-approved 774 drugs for inhibitors of ZIKV MEX_1_7 strain on Huh-7 cells.

59. Pascoalino BS *et al.*: **Zika antiviral chemotherapy: identification of drugs and promising starting points for drug discovery from an FDA-approved library**. *F1000Res* 2016, **5**:2523.
Repurposing of drugs by screening 725 FDA-approved drugs on Huh-7 cells.

60. Wang QY *et al.*: **Inhibition of dengue virus through suppression of host pyrimidine biosynthesis**. *J Virol* 2011, **85**:6548-6556.

61. Tang H *et al.*: **Zika virus infects human cortical neural progenitors and attenuates their growth**. *Cell Stem Cell* 2016, **18**:587-590.

62. Whitehorn J *et al.*: **Lovastatin for the treatment of adult patients with dengue: a randomized, double-blind, placebo-controlled trial**. *Clin Infect Dis* 2016, **62**:468-476.

63. Zou G *et al.*: **Development and characterization of a stable luciferase dengue virus for high-throughput screening**. *Antiviral Res* 2011, **91**:11-19.

64. Puig-Basagoiti F *et al.*: **High-throughput assays using a luciferase-expressing replicon, virus-like particles, and full-length virus for West Nile virus drug discovery**. *Antimicrob Agents Chemother* 2005, **49**:4980-4988.

65. Qing M *et al.*: **A high-throughput assay using dengue-1 virus-like particles for drug discovery**. *Antiviral Res* 2010, **86**:163-171.
This study developed a DENV-1 VLP platform and applied it into HTS.

66. Shan C *et al.*: **Evaluation of a novel reporter virus neutralization test for serological diagnosis of Zika and dengue virus infection**. *J Clin Microbiol* 2017, **55**:3028-3036.

67. Shan C *et al.*: **A rapid Zika diagnostic assay to measure neutralizing antibodies in patients**. *EBioMedicine* 2017, **17**:157-162.

68. Yin Z *et al.*: **An adenosine nucleoside inhibitor of dengue virus**. *Proc Natl Acad Sci U S A* 2009, **106**:20435-20439.

69. Zhang QY *et al.*: **Development of a stable Japanese encephalitis virus replicon cell line for antiviral screening**. *Arch Virol* 2017, **162**:3417-3423.

70. Lo MK, Tilgner M, Shi PY: **Potential high-throughput assay for screening inhibitors of West Nile virus replication**. *J Virol* 2003, **77**:12901-12906.

71. Ng CY *et al.*: **Construction and characterization of a stable subgenomic dengue virus type 2 replicon system for antiviral compound and siRNA testing**. *Antiviral Res* 2007, **76**:222-231.

72. Jones CT, Patkar CG, Kuhn RJ: **Construction and applications of yellow fever virus replicons**. *Virology* 2005, **331**:247-259.

73. Usme-Ciro JA *et al.*: **Generation of a DNA-launched reporter replicon based on dengue virus type 2 as a multipurpose platform**. *Intervirology* 2016, **59**:275-282.

74. Alcaraz-Estrada SL *et al.*: **Construction of a dengue virus type 4 reporter replicon and analysis of temperature-sensitive mutations in non-structural proteins 3 and 5**. *J Gen Virol* 2010, **91**:2713-2718.

75. Khromykh AA, Westaway EG: **Subgenomic replicons of the flavivirus Kunjin: construction and applications**. *J Virol* 1997, **71**:1497-1505.

76. Gehrke R *et al.*: **Heterologous gene expression by infectious and replicon vectors derived from tick-borne encephalitis virus and direct comparison of this flavivirus system with an alphavirus replicon**. *J Gen Virol* 2005, **86**:1045-1053.

77. Kojima A *et al.*: **Stable high-producer cell clone expressing virus-like particles of the Japanese encephalitis virus e protein for a second-generation subunit vaccine**. *J Virol* 2003, **77**:8745-8755.

78. Scholle F *et al.*: **trans-Packaged West Nile virus-like particles: infectious properties in vitro and in infected mosquito vectors**. *J Virol* 2004, **78**:11605-11614.

79. Queiroz SRA *et al.*: **Development and characterization of a packaging cell line for pseudo-infectious yellow fever virus particle generation**. *Rev Soc Bras Med Trop* 2018, **51**:66-70.

80. Matsuda M *et al.*: **High-throughput neutralization assay for multiple flaviviruses based on single-round infectious particles using dengue virus type 1 reporter replicon**. *Sci Rep* 2018, **8**:16624.

81. Garg H *et al.*: **Development of virus-like-particle vaccine and reporter assay for Zika virus**. *J Virol* 2017, **91**.

82. Wang QY *et al.*: **Discovery of dengue virus NS4B inhibitors**. *J Virol* 2015, **89**:8233-8244.

83. Byrd CM *et al.*: **A novel inhibitor of dengue virus replication that targets the capsid protein**. *Antimicrob Agents Chemother* 2013, **57**:15-25.

84. Lee H *et al.*: **Identification of novel small molecule inhibitors against NS2B/NS3 serine protease from Zika virus**. *Antiviral Res* 2017, **139**:49-58.

85. Chen X *et al.*: **Mechanisms of activation and inhibition of Zika virus NS2B-NS3 protease**. *Cell Res* 2016, **26**:1260-1263.

86. Poulsen A, Kang C, Keller TH: **Drug design for flavivirus proteases: what are we missing?** *Curr Pharm Des* 2014, **20**:3422-3427.

87. Kang C, Keller TH, Luo D: **Zika virus protease: an antiviral drug target**. *Trends Microbiol* 2017, **25**:797-808.

88. Yin Z *et al.*: **Peptide inhibitors of dengue virus NS3 protease. Part 2: SAR study of tetrapeptide aldehyde inhibitors**. *Bioorg Med Chem Lett* 2006, **16**:40-43.

89. Yin Z *et al.*: **Peptide inhibitors of dengue virus NS3 protease. Part 1: warhead**. *Bioorg Med Chem Lett* 2006, **16**:36-39.

90. Bodenreider C *et al.*: **A fluorescence quenching assay to discriminate between specific and nonspecific inhibitors of dengue virus protease**. *Anal Biochem* 2009, **395**:195-204.

91. Nitsche C *et al.*: **Retro peptide-hybrids as selective inhibitors of the dengue virus NS2B-NS3 protease**. *Antiviral Res* 2012, **94**:72-79.

92. Xu S *et al.*: **Critical effect of peptide cyclization on the potency of peptide inhibitors against dengue virus NS2B-NS3 protease**. *J Med Chem* 2012, **55**:6881-6887.

93. Chan JF *et al.*: **Novel antiviral activity and mechanism of bromocriptine as a Zika virus NS2B-NS3 protease inhibitor**. *Antiviral Res* 2017, **141**:29-37.

94. Kato F *et al.*: **Novel antiviral activity of bromocriptine against dengue virus replication**. *Antiviral Res* 2016, **131**:141-147.

95. Brecher M *et al.*: **A conformational switch high-throughput screening assay and allosteric inhibition of the flavivirus NS2B-NS3 protease**. *PLoS Pathog* 2017, **13**:e1006411.

96. Luo D *et al.*: **Insights into RNA unwinding and ATP hydrolysis by the flavivirus NS3 protein**. *EMBO J* 2008, **27**:3209-3219.

97. Tian H *et al.*: **Structural basis of Zika virus helicase in recognizing its substrates**. *Protein Cell* 2016, **7**:562-570.

98. Mastrangelo E *et al.*: **Ivermectin is a potent inhibitor of flavivirus replication specifically targeting NS3 helicase activity: new prospects for an old drug**. *J Antimicrob Chemother* 2012, **67**:1884-1894.

99. Tomlinson SM, Watowich SJ: **Use of parallel validation high-throughput screens to reduce false positives and identify novel dengue NS2B-NS3 protease inhibitors**. *Antiviral Res* 2012, **93**:245-252.

100. Byrd CM *et al.*: **Novel benzoxazole inhibitor of dengue virus replication that targets the NS3 helicase**. *Antimicrob Agents Chemother* 2013, **57**:1902-1912.

101. Lescar J *et al.*: **Towards the design of antiviral inhibitors against flaviviruses: the case for the multifunctional NS3 protein from dengue virus as a target**. *Antiviral Res* 2008, **80**:94-101.

102. Tan CW *et al.*: **Polysulfonate suramin inhibits Zika virus infection**. *Antiviral Res* 2017, **143**:186-194.

103. Barral K *et al.*: **Development of specific dengue virus 2′-O- and N7-methyltransferase assays for antiviral drug screening**. *Antiviral Res* 2013, **99**:292-300.

104. Lim SP *et al.*: **Small molecule inhibitors that selectively block dengue virus methyltransferase**. *J Biol Chem* 2011, **286**:6233-6240.

105. Lim SP *et al.*: **A scintillation proximity assay for dengue virus NS5 2′-O-methyltransferase-kinetic and inhibition analyses**. *Antiviral Res* 2008, **80**:360-369.

106. Graves TL, Zhang Y, Scott JE: **A universal competitive fluorescence polarization activity assay for S-adenosylmethionine utilizing methyltransferases**. *Anal Biochem* 2008, **373**:296-306.

107. Brecher M *et al.*: **Identification and characterization of novel broad-spectrum inhibitors of the flavivirus methyltransferase**. *ACS Infect Dis* 2015, **1**:340-349.

108. Amraiz D, Zaidi NU, Fatima M: **Development of robust in vitro RNA-dependent RNA polymerase assay as a possible platform for antiviral drug testing against dengue**. *Enzyme Microb Technol* 2016, **92**:26-30.

109. Xu HT *et al.*: **Identification of a pyridoxine-derived small-molecule inhibitor targeting dengue virus RNA-dependent RNA polymerase**. *Antimicrob Agents Chemother* 2016, **60**:600-608.

110. Chen YL *et al.*: **Inhibition of dengue virus RNA synthesis by an adenosine nucleoside**. *Antimicrob Agents Chemother* 2010, **54**:2932-2939.

111. Chen YL *et al.*: **Inhibition of dengue virus by an ester prodrug of an adenosine analog**. *Antimicrob Agents Chemother* 2010, **54**:3255-3261.

112. Nelson DR *et al.*: **Balapiravir plus peginterferon alfa-2a (40KD)/ribavirin in a randomized trial of hepatitis C genotype 1 patients**. *Ann Hepatol* 2012, **11**:15-31.

113. Nguyen NM *et al.*: **A randomized, double-blind placebo controlled trial of balapiravir, a polymerase inhibitor, in adult dengue patients**. *J Infect Dis* 2013, **207**:1442-1450.

114. Niyomrattanakit P *et al.*: **Inhibition of dengue virus polymerase by blocking of the RNA tunnel**. *J Virol* 2010, **84**:5678-5686.

115. Yin Z *et al.*: **N-Sulfonylanthranilic acid derivatives as allosteric inhibitors of dengue viral RNA-dependent RNA polymerase**. *J Med Chem* 2009, **52**:7934-7937.

116. Niyomrattanakit P *et al.*: **A fluorescence-based alkaline phosphatase-coupled polymerase assay for identification of inhibitors of dengue virus RNA-dependent RNA polymerase**. *J Biomol Screen* 2011, **16**:201-210.

117. Noble CG *et al.*: **Conformational flexibility of the dengue virus RNA-dependent RNA polymerase revealed by a complex with an inhibitor**. *J Virol* 2013, **87**:5291-5295.

118. Eyer L *et al.*: **Nucleoside inhibitors of Zika virus**. *J Infect Dis* 2016, **214**:707-711.

119. Sacramento CQ *et al.*: **The clinically approved antiviral drug sofosbuvir inhibits Zika virus replication**. *Sci Rep* 2017, **7**:40920.

120. •• Yu Y *et al.*: **A peptide-based viral inactivator inhibits Zika virus infection in pregnant mice and fetuses**. *Nat Commun* 2017, **8**:15672.
A synthetic peptide Z2 derived from the stem region of ZIKV E protein inhibited ZIKV in cell culture and in mice.

121. Wang Q, Yan J, Gao GF: **Monoclonal antibodies against Zika virus: therapeutics and their implications for vaccine design**. *J Virol* 2017, **91**.

122. •• Shan C, Xie X, Shi PY: **Zika virus vaccine: progress and challenges**. *Cell Host Microbe* 2018, **24**:12-17.
Good review about the progress and challenges of ZIKV vaccine.

123. Xie X *et al.*: **Small molecules and antibodies for Zika therapy**. *J Infect Dis* 2017, **216(Suppl. 10)**:S945-S950.

124. • De Clercq E, Li G: **Approved antiviral drugs over the past 50 years**. *Clin Microbiol Rev* 2016, **29**:695-747.
This review summarized and classified the approved antiviral drugs for the treatment of the human infectious diseases, which is a good guideline for further antiviral drug development.

125. Saxena SK *et al.*: **Zika virus disease in India – update October 2018**. *Travel Med Infect Dis* 2019, **27**:121-122.