The A-Z of Zika drug discovery

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Teaser

Zika clinical outcomes might be nefarious impacting in newborns for a lifetime. There is still no drug available to cure Zika. We provide guidance to help understand and advance the search for a cure.

Abstract (148 words: max 120 words)

Despite the recent outbreak of Zika virus (ZIKV) in the Americas and the global concern that it has raised, there are still no approved treatments and the early stage compounds discovered are likely many years away from approval by regulatory agencies. A comprehensive A-Z review of the recent advances in ZIKV drug discovery efforts is presented, highlighting drug repositioning and computationally guided compounds, including discovered viral and host cell inhibitors. Promising ZIKV molecular targets, including viral proteins such as E glycoprotein, capsid, NS3 helicase and protease, NS5 methyltransferase and polymerase are also described and discussed, as well as targets belonging to the host cell, as new opportunities for ZIKV drug discovery. All this knowledge is not only critical to advancing the fight against the Zika virus and other flaviviruses — it can also help us prepare for the next emerging virus outbreak for which we will have to respond.

Introduction

Zika virus (ZIKV) is still a global health concern. Originally discovered in Africa in 1947 [1], ZIKV became an epidemic 60 years later, reaching several tropical regions of the Americas, Africa and Asia. Despite causing mild symptoms such as fever, rashes and conjunctivitis, the major concern about ZIKV regards the severe neurological disorders, such as microcephaly, craniofacial disproportion, spasticity, seizures, irritability, and other brainstem dysfunctions [2,3]. In 2016, infants head computed tomographic findings, infected in pregnancy, confirmed the causal relationship between microcephaly and Zika infection [3].

A recent study by Yuan and co-workers (2017), demonstrated that a single mutation (S139N) in the pre-membrane (prM) structural protein, increased ZIKV infectivity in neural progenitor cells (NPCs), making the virus more virulent. This mutation arose in the French Polynesia strain [4], and it has contributed to the increased incidence of microcephaly and higher mortality in neonates, according to experimental assays [4]. The disorders caused by Zika infection mainly affect infants, but it can also impact

adults, including by modulating significantly key immuno markers [5]. There have been ZIKV-related cases of Guillain–Barré syndrome [6], myelitis [7], uveitis [8] and meningoencephalitis [9] reported in adults.

Currently neither a specific antiviral drug nor a vaccine are available for treating or preventing ZIKV infection. However, there are several promising drug targets encoded by the virus or present in host cells. There have been several reports on compounds found to have activity against ZIKV and its proteins. Here we present a comprehensive A-Z review of the recent advances in ZIKV drug design, including viral and host cell inhibitors and several experimental and computational techniques that have been applied in these studies. This information will contribute to the design of drugs against ZIKV and related viruses.

Structural features of Zika Virus proteins

ZIKV is a spherical, enveloped virus, with an icosahedral-like symmetry (Figure 1A). Belonging to the genus *Flavivirus* of the *Flaviviridae* family [10], ZIKV carries a positive-sense single-stranded RNA genome, encoding a large polyprotein, which after processing by host and viral proteases yields three structural and seven non-structural proteins (Figure 1B). Envelope protein (E), membrane protein (M, which is expressed as prM, the precursor to M) and capsid (C) are the structural proteins, which form the virion. The non-structural (NS) proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5) are essential for genomic replication and modulation of host immunity [10] (Figure 1B).

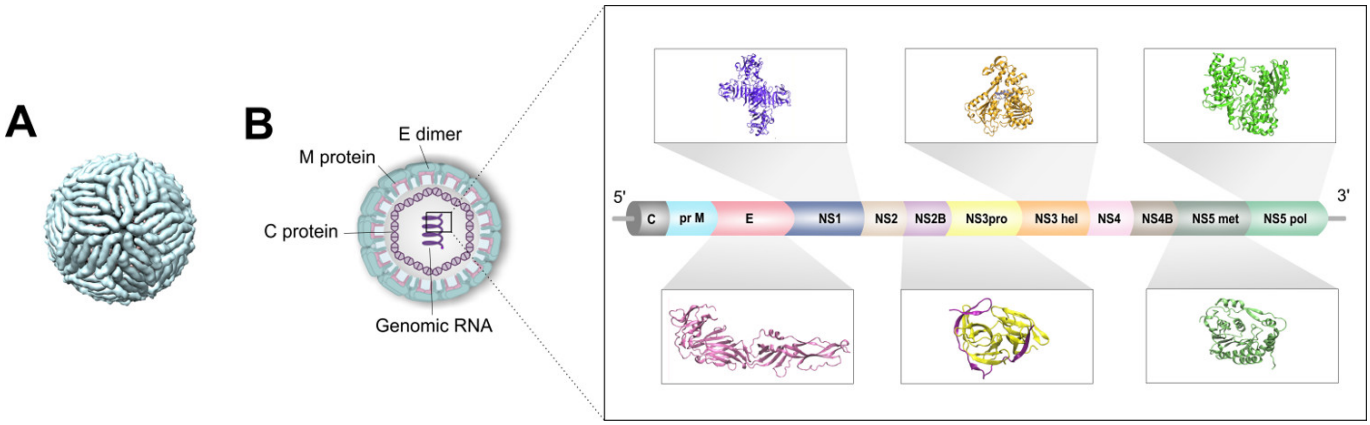

Figure 1. Scheme of ZIKV surface, structural and non-structural proteins. (A) surface-shaded depth cued representation of mature ZIKV (built using UCSF Chimera package [11], http://www.rbvi.ucsf.edu/chimera, based on PDB ID 5IRE), showing the icosahedral-like symmetry arrangement of surface proteins; (B) virion components, highlighting the E, M and C proteins, as well as genomic RNA. ZIKV encodes a large polyprotein, which after processing yields three structural proteins (C, M and E) and seven non-structural proteins (NS1; NS2A; NS2B; NS3 protease and helicase domains; NS4A; NS4B; NS5 methyltransferase and RNA polymerase domains), built using VMD program [12], http://www.ks.uiuc.edu/Research/vmd/. NS5 domains are represented separately, as two distinct targets, but NS5 methyltransferase is attached to the NS5 polymerase domain to form the full-length NS5.

Initially, prior to the availability of ZIKV structures in early March 2016, there were several efforts to develop homology models of the ZIKV proteins based on close homologs such as dengue virus (DENV) and other flaviviruses [13–15]. Since late March 2016, ZIKV protein structures have been determined mostly by X-ray crystallography and have been made available in databases such as the Protein Data Bank (PDB) [16]: NS1, NS2B-NS3 protease, NS3 helicase, NS5 methyltransferase, NS5 polymerase, NS5 full-protein and envelope glycoprotein (Table 1). Among them, NS2B-NS3 protease, NS3 helicase, and NS5 methyltransferase structures are available with ligands/ATP/RNA, which are very useful for ligand binding site identification and which are likely to be more useful in virtual screens than apo conformations.

Insert Table 1

These protein structures are fundamental when performing experimental and computational studies. Figure 2 shows some ZIKV protein 3D structures and their binding sites. The binding site of ZIKV NS2B/NS3 protease (NS2B residues: S81, D83, K54 and NS3pro residues: D75, H51, S135, G133, G151, D129, Y161) (Figure 2A) was based on the co-crystallized fragment 1H-benzo[d]imidazol-1-ylmethanol [17] and a boronate inhibitor [18], which bind in the same binding site. NS3 helicase presents two binding sites, one encompasses the RNA strand and the other is where the ATP molecule binds (Figure 2B). The full-length NS5 protein contains two domains, representing distinct targets: The NS5 methyltransferase and NS5 polymerase. NS5 methyltransferase domain has two substrates: SAM, a co-substrate involved in methyl group transfer, and GTP, a substrate for RNA synthesis. Consequently, the accessibility of these two substrate, together with a conserved catalytic tetrad of Lys61, Asp146, Lys182, Glu218 that forms the active site [19] (Figure 2C). The NS5 polymerase domain has three adjacent binding sites: the active site, the RNA site and the NTP channel [19] (Figure 2D). ZIKV envelope protein is composed by three distinct domains: the β-barrel-shaped domain (DI), the finger-like domain (DII) and the immunoglobulin-like domain (DIII) (Figure 2E). Domain II is responsible for the dimerization [20]. A binding site between DI and DIII, enclosing a hydrophobic cavity around a flexible linker, was computationally predicted [21]. The protein-protein binding site is composed of residues from DI (1, 13, 34–40, 143–146, 163, 183), and DIII (340–345, 354–358, 360–367, 372–375, 378, 388, 390–392, 395) (Figure 2E).

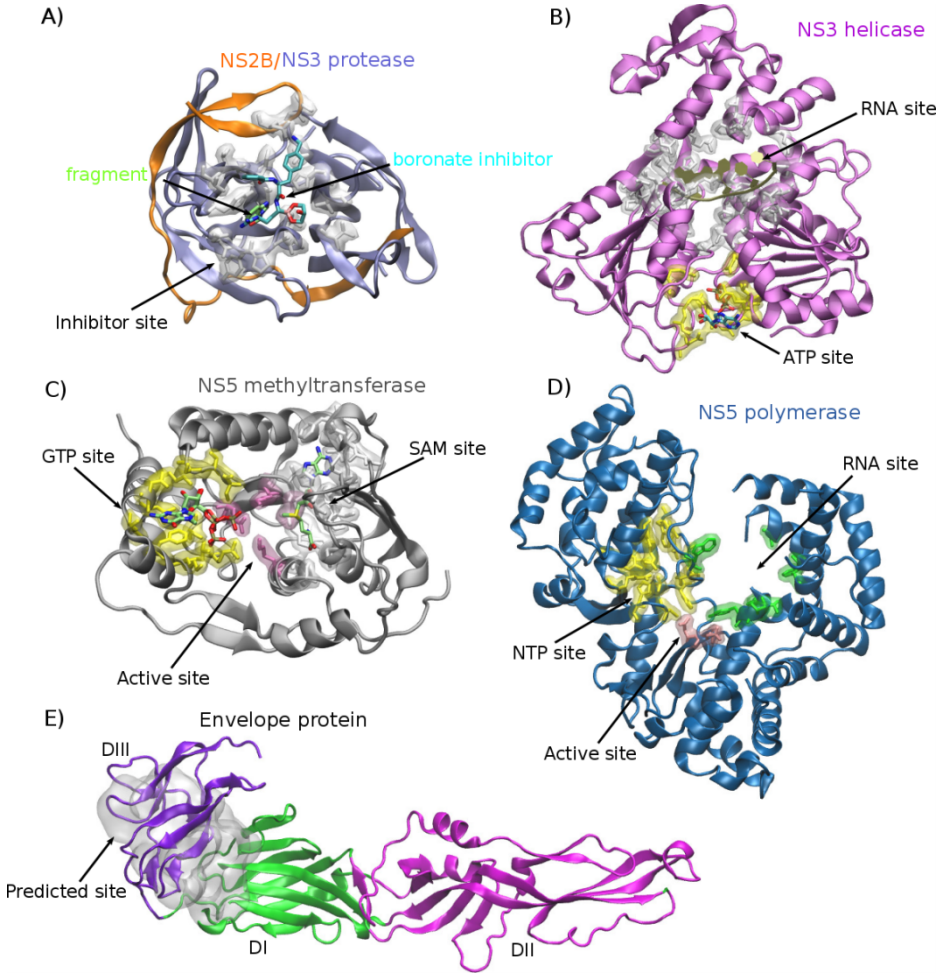

Figure 2. Ribbon-representation of some of the ZIKV protein 3D structures. (A) NS2B/NS3 protease, highlighting the binding site occupied by a boronate inhibitor [18] and the fragment 1H-benzo[d]imidazol-1-ylmethanol [17]; (B) NS3 helicase, highlighting the RNA and ATP binding sites; (C) NS5 methyltransferase domain, highlighting the active site, SAM/SAH and GTP/cap binding sites; (D) NS5 polymerase domain, emphasizing the active site, NTP and RNA binding sites; (E) Envelope protein [20] and its β-barrel-shaped domain I (DI), finger-like domain II (DII), immunoglobulin-like domain III (DIII) and a ligand binding site between DI and DIII [21]. This predicted binding site encloses a hydrophobic cavity around the flexible linker joining DI and DIII. All these 3D structures are available in PDB and the figures were built using VMD program [12], http://www.ks.uiuc.edu/Research/vmd/.

Viral proteins as drug targets

The viral proteins play an important role in the virus's infection and replication processes. The E glycoprotein is associated with virus adsorption, internalization, and fusion with the host cell, as well as with the development of neutralizing humoral immunity. The prM protein has several roles in the flavivirus life cycle, such as assisting in the chaperone-mediated folding of the E protein and preventing premature fusion during virion egress [22]. The main role of the C protein is the assembly and packaging of the viral RNA genome to form the viral nucleocapsid, in addition to acting as elements of viral particle assembly when associated with other proteins on lipid droplets and the endoplasmic reticulum (ER) [23].

The functional role of some nonstructural flavivirus proteins is still not completely understood but can be summarized as follows. NS1 plays several roles, including flaviviral replication and virion maturation [24]. The NS2 subdomain is required for the proper formation of the substrate recognition site of the NS3 protease [25]. The NS3 helicase (NS3h) protein promotes the separation of RNA strands during viral replication and unwinds the RNA secondary structure in the 3' non-translated region [26]. Together with NS2B, NS3 protease is responsible for cleavage and posttranslational modification of the viral polyprotein [18,25]. Flavivirus NS4A and NS4B proteins compose the ER membrane-associated replication complex [25]. DENV NS4B has been shown to interact with NS3h and dissociate it from single-stranded RNA to modulate RNA synthesis [27]. ZIKV NS4A and NS4B cooperatively suppress the host's Akt - mammalian target of rapamycin (Akt-mTOR) pathway, inhibiting neurogenesis and inducing autophagy [28]. For those reasons, the NS4B is an important target whose inhibition could impair viral propagation. Unfortunately, no crystal structures of ZIKV NS4B are available (as of Feb. 14th 2018). NS5 contains a methyltransferase domain, which methylates the RNA cap structure, and an RNA-dependent RNA polymerase domain, which synthesizes the viral RNA and is thus essential to ZIKV survival and establishment of the infection in host cells [19].

The innate immune response is the initial line of host defense, where type I interferon (IFN) production and signaling display a central role. Regarding this, NS5 proteins have been shown to inhibit the IFN signaling to evade host antiviral defense [29]. The NS5 protein uses different mechanisms (depending on the specific flavivirus)

to target this signaling pathway. For Tick-borne encephalitis virus (TBEV) and West Nile virus (WNV), NS5 proteins inhibit signal transducer and activator of transcription (STAT1) phosphorylation and nuclear translocation [30], this action was related to the polymerase domain [31]. For Japanese encephalitis virus (JEV), NS5 blocks tyrosine phosphorylation of tyrosine-protein kinase Tyk2 [32]. For Yellow fever virus (YFV), NS5 can bind to STAT2, but only after IFN treatment, and this prevents STAT2 binding to IFN-stimulated responsive promoter elements present on IFN target genes [33].

A recent study showed that ZIKV NS5 expression resulted in proteasomal degradation of the IFN-regulated transcriptional activator STAT2 from humans, but not mice, resembling the DENV NS5 mode of action [34,35]. Particularly for ZIKV, a docking study was carried out to predict the protein-protein interactions between NS5, seven in absentia homologues protein (SIAH2) and STAT2 proteins. The study suggests that (a) NS5 recruits SIAH2 for the ubiquitination-dependent degradation of STAT2, and (b) the NS5 amino acid residues involved in interaction with SIAH2 and/or STAT2 were found to be conserved across related flaviviruses [36]. Another structural study of ZIKV NS5 indicated that the small molecule inhibitor-binding site of DENV3 NS5 is structurally conserved in ZIKV NS5, indicating a potential mechanism for functional inhibition of ZIKV NS5 [37].

Envelope glycoprotein inhibitors

A polyphenol present in green tea, epigallocatechingallate (EGCG), has been shown to have antiviral activity for many viruses, such as human immunodeficiency virus (HIV), herpes simplex virus (HSV), influenza virus and hepatitis C virus (HCV) [38]. Recently, Carneiro and co-workers (2016), through *in vitro* assays (Vero E6 cells), showed that EGCG also inhibited the entry of ZIKV into the host cell by at least 1-log (>90%) at higher concentrations (>100μM). The authors supposed that the inhibition is probably related to the direct interaction of the EGCG with lipid envelope, leading to a subsequent destruction of the virus particle [38]. Conversely, EGCG contains the catechol group, which is a well-known PAIN substructure (Pan-Assay Interference compound) that promiscuously / non-specifically inhibits many different targets [39–41].

A subsequent computational study by Sharma and co-workers (2017), using induced fit docking, molecular dynamics (MD) and drug-like properties calculations, revealed that EGCG binds to a hydrophobic site located between DI and DIII, close to a flexible linker (see Figure 1E). Using MD simulations, they showed that EGCG blocks the major conformational change during the membrane fusion process [21]. Another natural product, nanchangmycin, from *Streptomyces nanchangensis,* proved to be a potent inhibitor of ZIKV in high-throughput screening (HTS) assays using human cells, [42] with an IC_{50} 0.1 μM (Figure 3A). To determine if nanchangmycin was blocking a pre- or post-entry step in the viral life cycle, the authors treated cells with the drug and removed the drug 4 h post-infection (hpi) and monitored the level of infection (24 hour later). Nanchangmycin inhibited infection, which suggests that it blocks an early step in the viral life cycle [42]. Unfortunately, nanchangmycin contains a reactive Michael acceptor, which is also a well-known PAIN substructure [43].

Capsid protein inhibitors

Although the capsid protein has emerged as a promising target for antiflaviviral agents, few capsid inhibitors have been identified until now. The compound ST-148 has been shown to interact with the capsid protein and was identified as a potent inhibitor of all four serotypes of DENV *in vitro* and *in vivo*, reducing viraemia and viral load in vital organs [22]. Characterization of the mode of action of ST-148 showed that it directly targeted the capsid protein and presented bimodal antiviral activity affecting both assembly/release and entry of infectious DENV particles [44]. Another example of a flaviviral capsid inhibitor is the biotinylated derivative of compound SL209, which inhibited HCV infection, blocking the core dimerization with an IC_{50} of 2.80 μM [23]. Both studies support the hypothesis that inhibitors of viral capsid formation might constitute a new class of antiviral agents, therefore may also be applicable to ZIKV.

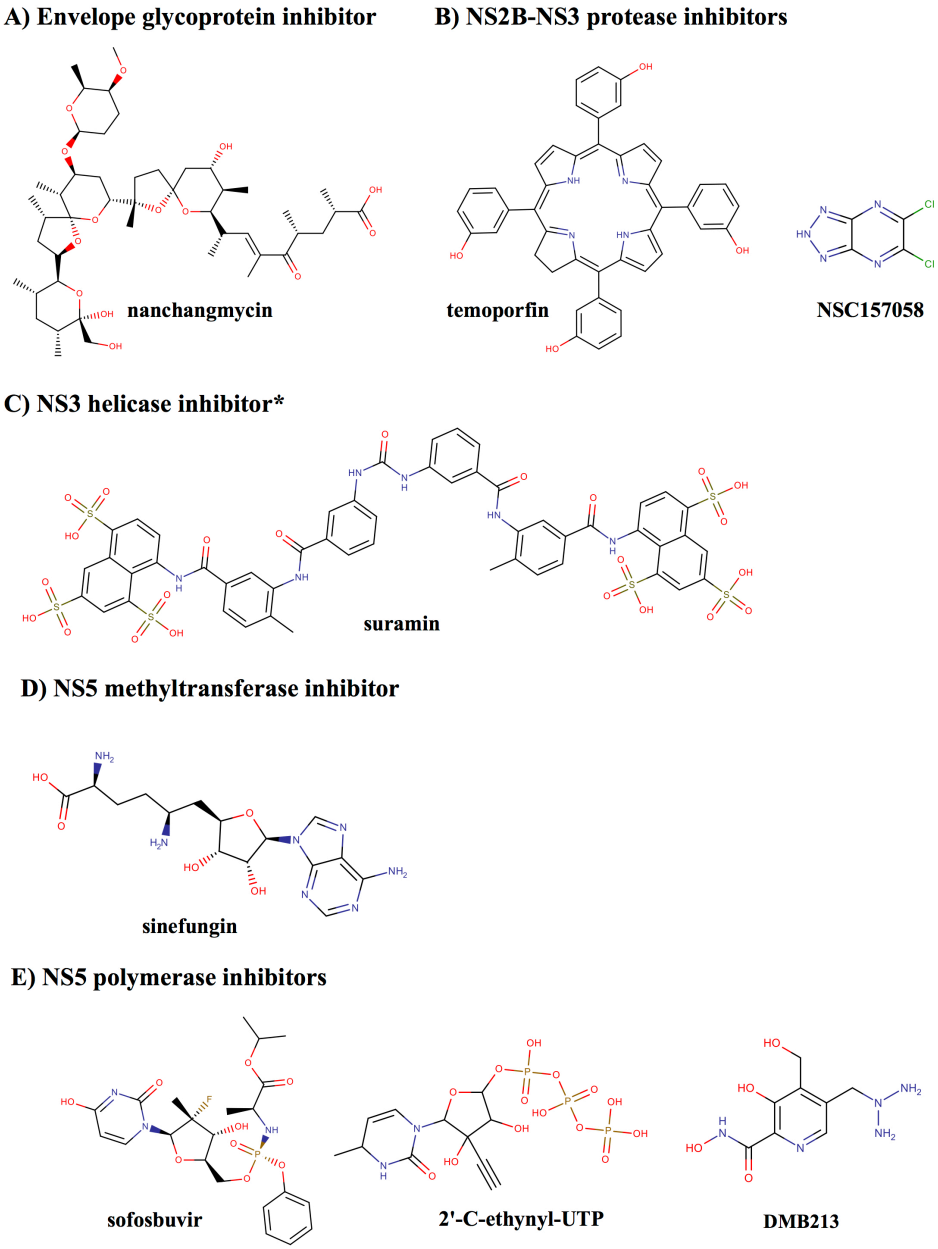

Figure 3. Chemical structures of selected ZIKV protein inhibitors. (A) Envelope glycoprotein inhibitor: nanchangmycin (IC_{50} = 0.1 μM) [42]; (B) NS2B-NS3 protease inhibitors: temoporfin (IC_{50} = 1.1 μM) [45], and NSC157058 (IC_{50} = 0.82 μM) [46]; (C) NS3 helicase inhibitor*: suramin (EC_{50} = 0.42 μM), which was tested only in cell-based assay in ZIKV [47]; (D) NS5 methyltransferase inhibitor: sinefungin (IC_{50} = 1.18 μM) [48,49]; (E) NS5 polymerase inhibitors: sofosbuvir (IC_{50} =7.3 μM) [50], 2-C-ethynyl-UTP (IC_{50} = 0.46 μM) [51], and DMB213 (IC_{50} = 5.2 μM) [50].

NS2B-NS3 protease inhibitors

Natural products like polyphenols are known to have antiviral activity against influenza virus (Flu), coronavirus (CoV), DENV and others [52–55]. In this way, many natural products have been tested against NS2B-NS3 protease, and some of them were able to inhibit ZIKV protease activity. In one study, 22 compounds were tested at (the relatively high concentration of) 100 μM, and seven compounds were able to inhibit more than 50% of the protease activity [56]. The IC_{50} of these compounds were determined and ranged from 22 to 112 μM [56]. In another study, five flavonoids and one natural phenol were tested [57]. The flavonoids were myricetin (IC_{50} = 1.3 μM), quercetin (IC_{50} = 2.4 μM), luteolin (IC_{50} = 2.7 μM), isorhamnetin (IC_{50} = 15.5 μM), apigenin (IC_{50} = 56.3 μM), and the natural phenol curcumin (IC_{50} = 3.5 μM). These polyphenols inhibit the enzyme in a non-competitive mode, which means that they could perhaps be allosteric inhibitors. Docking studies showed that the compounds are predicted to bind to a pocket on the back of the active site of ZIKV NS2B-NS3 [57]. However, curcumin, quercetin, and other flavonoids have been shown to be promiscuous inhibitors, e.g., via colloidal aggregation [58–60]; curcumin also contains reactive Michael acceptors and quercetin has a catechol, a well-known PAIN substructure, which may make these compounds less favorable.

An HTS assay was developed to test compounds that inhibit the interaction between NS3 and the NS2B *N*-terminal fragment [45]. Then, a library of FDA-approved drugs and investigational drugs (2,816 drugs) was screened using this assay, and 23 compounds produced an IC_{50} below 15 μM, of which 12 were considered PAINs. The remaining 11 compounds were tested for their protease inhibition activities, and three could inhibit with IC_{50} values ranging from 1.1 to 15.9 μM. The three compounds were tested against ZIKV, DENV, WNV, JEV, and YFV. They presented inhibitory activity close to the nanomolar level, whereas temoporfin (Figure 3B) displayed an EC_{50} (half maximal effective concentration) for all flaviviruses tested at low nanomolar concentration. The therapeutic indices for all three compounds were high, since their CC_{50} (concentration that inhibits 50% of mammalian cell proliferation) was considerably higher than their EC_{50}. The compounds were also able to inhibit ZIKV replication in placental epithelial and neuronal cells and to inhibit viral polyprotein cleavage. Temoporfin was tested in a viraemia mouse model and a lethal mouse model and was able to inhibit viremia and protect 83% of the mice; the mice that survived did

not present any signs of neurological disorder [45]. A similar study was done using structure-based virtual screening of 8,277 compounds from the DrugBank database, and the top 100 candidates were identified [61]. From these, eight clinically approved compounds belonging to different drug classes were selected for further validation studies. From the eight selected compounds, five were validated as NS2B-NS3 protease inhibitors. Then, the compounds that had favorable safety profiles and that could be administrated to pregnant women were selected (n=3) for phenotypic screening. Two compounds were able to inhibit ZIKV replication. Since the compound novobiocin had a higher selectivity index, it was tested *in vivo*. Dexamethasone immunosuppressed mice with disseminated ZIKV infection and novobiocin treatment had a significantly higher survival rate, lower mean blood and tissue viral loads, and less severe histopathological changes than untreated controls [61].

A similar strategy is to appropriate other flavivirus active compounds to inhibit ZIKV, since they are closely related in terms of the sequence and structure of their proteins [62]. Based upon a literature review, 11 drugs were selected to be tested in ZIKV if they could match the following criteria: (i) known toxicity profile in humans and pregnancy, (ii) known broad spectrum antiviral activity, (iii) known antiviral activity against flavivirus/another RNA virus, (iv) coverage of a broad range of indications and drug classes. Bromocriptine presented inhibitory activity in a cytopathic effect inhibition assay, virus yield reduction assay, and plaque yield reduction assay. The drug was predicted to bind to NS2B-NS3 protease through molecular docking studies and also presented inhibition of the enzyme's proteolytic activity [62]. Twenty-seven HCV NS2B-NS3 protease inhibitors were tested in ZIKV NS2B-NS3: 10 presented IC_{50} values below 50 μM. The best two inhibitors were tested to determine their inhibition type, and both presented competitive inhibition profiles, meaning that they might bind at the enzyme's active site [63]. Apoptin, a 60 amino acid bovine trypsin inhibitor, previously tested against WNV protease, also inhibits ZIKV protease (IC_{50} = 70nM), and molecular modeling studies predicted that the inhibitor probably blocks the interactions of NS3 and NS2B. On the other hand, in our analysis of crystal structures of WNV protease with bovine pancreatic trypsin inhibitor (PDB ID 2IJO) [64] and of DENV protease bound to aprotinin (PDB ID 3U1J) [65], these types of inhibitors bind to and occlude the substrate site. A focused library of protease inhibitors that bind to WNV exosites was tested, and about 700 structurally similar compounds were screened [46]. The best inhibitors were NSC157058 (IC_{50} = 0.82 μM) (Figure 3B),

NSC86314 (IC_{50} = 0.97 μM), and NSC716903 (IC_{50} = 1.12 μM). They were also tested against furin, a human serine protease with a cleavage sequence preference similar to those of flaviviral proteases, and they showed no effect at 100 μM. These compounds decreased the viral yield in neuronal precursor cells. The best inhibitor, NSC157058, was tested using *in vivo* mouse model and decreased the viremia by 10-fold; unfortunately, it has a very unfavorable pharmacokinetics profile [46].

Brecher and coworkers (2017) developed an assay to analyze the conformational changes in DENV NS2B-NS3 protease using luciferase [66]. They performed a virtual screening (VS) assay using an allosteric site conformation that is present only in the enzyme's inactive state. Twenty-nine compounds were selected from a VS pipeline to be tested in a protease inhibition trial, of which three were subsequently selected to be tested in the allosteric assay. Only NSC135618 was able to inhibit the protease. The same compound also inhibited viral replication of DENV, ZIKV, WNV, and YFV [66].

Crystal structure elucidations and MD studies have been undertaken to further understand the interactions and conformational changes of the protein bound to its inhibitors. The inhibitor complex seen in the crystal structures may provide a model for assemblies formed at the site of polyprotein processing, guiding future drug discovery processes against ZIKV protease [17,18,67].

NS3 helicase inhibitors

Helicase displays both Adenosine triphosphate (ATPase) and RNA triphosphatase (RTPase) activities. There are few studies regarding NS3h inhibitors, mainly using VS, but not experimentally validated. In an ATPase activity analysis of NS3 helicase, the ATPase inhibitors resveratrol and quercetin were tested at 150 μM; their inhibitory effects were 51% and 15% ATPase activity inhibition, respectively [68]. Suramin (Figure 3C), an antiparasitic drug, was previously characterized as a DENV NS3 helicase inhibitor with a non-competitive mode [69], and recently it was shown to inhibit ZIKV replication with EC50 value of 0.42 μM [47]. Albulescu and co-workers (2017) suggested through experimental assays that suramin affected ZIKV binding/ entry, virion biogenesis and attachment to host cells [47]. The authors also suggest that suramin might inhibit the NS3h activity or might affect packaging by binding to positively charged residues on the capsid protein [47].

Molecular dynamics simulations of NS3h where performed to study the proteins flexibility and conformations preferences; they showed that the RNA binding loop is influenced by the presence of the RNA strand, being more stable and in a closed conformation, when RNA is bound to the protein [70]. The different conformations observed during MD, showed a distinct region beneath/behind the ATP site, which could perhaps be an allosteric site, and may help guide the discovery or design of new inhibitors. These conformations and strategies are guiding new virtual screening experiments in the OpenZika project, which is being performed on IBM's World Community Grid (WCG). OpenZika has been virtually screening millions of compounds against all of the ZIKV protein structures (and targets from related flaviviruses) using molecular docking (on WCG) and QSAR modelling filtering (performed in-house) [71]. This project is ongoing and several compounds are currently being assayed were identified in these virtual screens.

Other flaviviral NS3 helicase inhibitors, such as ivermectin, [72] and benzoxazole, that have activity against YFV NS3h and DENV NS3h, respectively [73], are also interesting compounds to be tested against ZIKV NS3h.

NS4B inhibitors

There are few studies regarding ZIKV NS4B protein inhibition thus far. An aminothiazole derivative (NITD-618) was identified as a specific dengue virus inhibitor, capable of inhibiting all four serotypes (but not closely related flaviviruses); it was identified in an HTS of 1.8 million compounds using a DENV replicon containing the luciferase gene [74]. In another screen using the NIH clinical collection, a naltrindole analogue (SDM25N) was found to inhibit DENV, and specific NS4B point mutations (F164L and P104L) conferred resistance against the compound, indicating that NS4B is likely the targeted protein [75].

NS5 methyltransferase inhibitors

Different types of DENV methyltransferase (MTase) inhibitors were also tested for ZIKV MTase. The classes of inhibitors tested were *S*-adenosyl-*L*-methionine (SAM) analogs, RNA Cap analogs and compounds targeting an allosteric site of DENV MTase. Analogs S-adenosyl-L-homocysteine (SAH) and the SAM analog sinefungin (Figure 3D) presented IC_{50} values of 0.43 μM and 1.18 μM, respectively. The Cap analogs

inhibited the enzyme with IC_{50} values ranging from 72 to 491 μM. The allosteric compounds presented 50% inhibition values of 24 to 221 μM [48]. The structure of ZIKV MTase with the SAM analogue sinefungin was recently elucidated, and the construction of an inhibitor connecting sinefungin with a Cap analogue attached by a linker was proposed, which could perhaps grant higher affinity to the protein [49].

A virtual screen using a hydrophobic site close to the SAM pocket was performed with more than 20,000 compounds [76]. The 10 compounds with the best scores were selected for experimental screening: 4 were able to inhibit viral growth in concentrations below 20 μM and the best inhibitor presented an IC_{50} value of 4.8 μM [76].

Jain and co-workers (2017) developed a SAM analog that intrudes the guanosine Triphosphate GTP/Cap pocket of ZIKV MTase [77]. They started from the crystallographic structure bound to SAM and developed the analog, attaching a 4-fluorophenyl group that gave the compound the capacity to bind to a part of the RNA tunnel and to occupy the Cap methylation site [77].

NS5 polymerase inhibitors

Nucleoside analogs have been used in other viruses as NS5 polymerase inhibitors, such as the prodrug sofosbuvir that is part of combination therapies that can cure HCV. Some nucleoside analogs like 2′-C- and 2′-O-methyl–substituted nucleosides, 2′-C-fluoro-2′-C-methyl–substituted nucleosides, 3′-O-methyl–substituted nucleosides, 3′-deoxynucleosides, derivatives with a 4′-C-azido substitution, heterobase-modified nucleosides, and neplanocins were tested against ZIKV, and the most promising inhibitors were the 2-C-methylated nucleosides, with IC_{50} values less than 10 μM [78].

A similar observation was found when ribonucleoside triphosphate analogs were tested against NS5 polymerase: the 2’-C-methyl and 2’-C-ethinyl substituted ribonucleoside triphosphates were the best inhibitors. The compounds were tested for their abilities to be incorporated into the RNA chain and to terminate its elongation. The analogs showing both abilities were 2'-F-2-C-ME-UTP (IC_{50} = 90.76 μM), 2'-C-ME-UTP (IC_{50} = 5.78 μM) and 2'-C-ethynyl_UTP (IC_{50} = 0.46 μM) (Figure 3E) [51]. Adenosine triphosphate analogs were tested in NS5 polymerase in another study, and the compounds that led to the strongest enzyme inhibition were the 2’-C-methylated

adenosine triphosphate [79]. The best inhibitors presented IC_{50} values of 5.6 μM and 7.9 μM [79].

Using a drug repurposing strategy, Xu and co-workers (2017) tested compounds already used for HCV NS5 polymerase: sofosbuvir (Figure 3E), a nucleoside inhibitor, and DMB213 (Figure 3E), a non-nucleoside inhibitor [50]. Both compounds inhibited ZIKV NS5 polymerase with IC_{50} values of 7.3 μM and 5.2 μM, respectively. Mutations that confer resistance to nucleoside analog inhibitors in HCV also led to resistance to sofosbuvir in ZIKV, which was not the case for DMB213 [50]. In a recent study, known flaviviral NS5 polymerase inhibitors were tested on cell-based assays in ZIKV. The compound 7-deaza-2'-C-methyladenosine (7DMA) inhibited ZIKV proliferation in Vero cells and in an animal model, it decreased viremia and delayed morbidity and mortality caused by ZIKV [80]. Although the authors haven't tested this compound against the ZIKV protein, it is a very promising candidate [80].

Four known HCV NS5 inhibitors were docked to a NS5 homology model: sofosbuvir, R7128, MK-0608 and IDX-184 [81]. The latter two presented lower docking scores, indicating that they perhaps interact better with the target [81]. A pharmacophore-based strategy was used to search for NS5 inhibitors [14]. The researchers constructed models for NS5 polymerase and NS5 methyltransferase using the molecules ribavirin and BG323. They used the models to conduct a virtual screening of the ZINC database and found 23 candidates for NS5 polymerase and 18 candidates for methyltransferase. These candidates were docked to their respective targets, and 3 potential leads were selected for each protein, based on the docking scores. The compounds with the best docking scores were ZINC39563464 for NS5 polymerase and ZINC64717952 for NS5 MTase [14].

An allosteric site for DENV NS5 polymerase has been elucidated, and a fragment-based screening of this site used the crystallographic structure [82]. Promising allosteric inhibitors were synthesized, and they inhibited the enzyme with IC_{50} values of 1-2 μM [82]. The same strategy could likely be used for ZIKV NS5 allosteric inhibitors design, since the viral proteins have conserved sequences and similar structures.

Host proteins as drug targets

ZIKV, as well as other *flaviviridae* members, possesses a small genome and requires the host cell machinery to carry out several core functions that are essential to

viral replication. In addition to the inhibition of viral polymerases, an attractive broad-spectrum strategy is to target host cell processes, since they are often employed by multiple viruses and are less prone to the development of drug-resistance [83].

The recently described inhibitors of the most commonly targeted cellular functions in ZIKV can be found in Table S1 in the **Supplementary Material** and are reviewed below.

Host Cell Nucleoside Biosynthesis Inhibitors

Viruses rely on the supply of nucleosides from the host cell to maintain proper RNA replication. Furthermore, there is evidence that inhibition of nucleoside biosynthesis triggers the activation of antiviral interferon-stimulated genes in human cells [84]. Thus, host enzymes involved in the de novo biosynthesis of nucleosides, such as inosine monophosphate dehydrogenase (IMPDH) and dihydroorotate dehydrogenase (DHODH), are interesting targets for broad-spectrum antiviral therapy.

IMPDH catalyzes the oxidative conversion of inosine 5'-monophosphate into xanthosine 5'-monophosphate, which is the first committed and rate-limiting step of the guanine nucleotide biosynthetic pathway [85]. Known inhibitors of IMPDH include ribavirin, 5-Ethynyl-1-beta-D-ribofuranosylimidazole-4-carboxamide (EICAR) and mycophenolic acid (MPA).

Ribavirin, one of the first clinically used broad-spectrum antivirals, is commonly employed in combination therapies to treat HCV and is thought to have multiple mechanisms of action, including the inhibition of the viral polymerase and host IMPDH [86]. It was found to inhibit the virus-induced cytopathic effects (CPE) of several flaviviruses, including ZIKV (EC_{50} = 142.9 μg/mL) [87]. A recent study tested the activity of known broad-spectrum antivirals, including ribavirin, which yielded a poor inhibition of virus-induced CPE (EC_{50} >50 μM for MR766 strain), concluding it was not a suitable candidate for ZIKV therapeutics [88]. Other nucleoside biosynthesis inhibitors were also tested in the same study, among which were MPA (EC_{50} = 0.11 μM), brequinar (EC_{50} = 0.08 μM) and 6-azauridine (EC_{50} = 0.98 μM). The latter two compounds inhibit dihidroorotate dehydrogenase (DHODH) and orotidylic acid (OMP) decarboxylase, respectively, which are two enzymes involved in pyrimidine biosynthesis [88].

Barrows and co-workers (2016) also tested MPA, which presented potent anti-ZIKV activity, inhibiting ZIKV infection in HuH-7, HeLa, JEG3, hNSC and HAEC cells [89]. Their screen also detected other nucleoside biosynthesis inhibitors, such as azathiopurine, mercaptopurine hydrate, mycophenolate mofetil (a prodrug of MPA), and thioguanine.

Another cell-based screen carried out by Pascoalino and co-workers (2016) [90] also identified 6-azauridine (EC_{50} = 2.3 ± 0.1 μM) and another pyrimidine biosynthesis inhibitor: 5-fluorouracil (EC_{50} = 14.3 ± 8.6 μM), which inhibits thymidylate synthase, the enzyme that catalyzes the final step of thymidine biosynthesis. 5-fluorouracil and floxuridine also showed dose-dependent inhibition of ZIKV replication in a study by Tiwari et al [91].

Although the "pregnancy categories" labeling system is being replaced [92,93], it is worth noting that these compounds were generally classified in pregnancy category D by the FDA [89], and thus have presented "positive evidence of human fetal risk". This is not unexpected, since they deplete the cellular pool of nucleotides, which certainly affects proper development of the fetus.

Host Cell Lipid Biosynthesis Inhibitors

Flaviviral infection has been associated with alterations in the lipid homeostasis [94] and membrane structure of infected cells [95]. DENV infection, for example, is known to induce dramatic relocalization of the fatty acid synthase (FAS) to the sites of viral replication [96,97].

Cholesterol has been identified as an important modulator of the host response to several flaviviruses, but the exact mechanism by which this modulation occurs is not yet fully understood. Nevertheless, inhibition of the cholesterol biosynthesis pathway represents an attractive therapeutic approach. Several enzymes involved in cholesterol biosynthesis, such as mevalonate decarboxylase, 3-hidroxi-3-methyl-glutaril-CoA (HMG-CoA) synthase and squalene synthetase were found to be important for efficient replication of DENV in A549 and K562 cell lines [98].

Lovastatin, an HMG-CoA reductase inhibitor, was tested against ZIKV as part of a cell-based screen of 725 FDA-approved drugs, and its activity was confirmed through a dose-response assay (EC_{50} = 20.7 ± 8.6 μM) [90]. Its anti-flaviviral activity

was previously reported in HCV [99] and DENV [98]. Moreover, Sarkey and coworkers (2017) demonstrated that lovastatin attenuated nervous system injury in an animal model and may be used in inflammatory peripheral nerve diseases, including Guillain-Barré Syndrome, which can be a consequence of ZIKV infection [100]. Nevertheless, although it is considered safe, treatment with Lovastatin showed no evidence of beneficial impact on Dengue infections in a randomized, double-blind, placebo-controlled trial [101]. Mevastatin also had anti-ZIKV activity at concentrations of 1 – 5 μM, but it was not dose-responsive at concentrations above 5 μM [88]. Mevastatin is known to induce apoptosis [102], which could perhaps explain the lack of antiviral efficacy at higher concentrations.

Nordihydroguaiaretic acid (NDGA) and its derivative tetra-O-methyl nordihydroguaiaretic acid (M_4N) were tested against ZIKV and showed inhibition in the low-micromolar range (IC_{50} values of 9.1 and 5.7 μM, respectively) [103]. They are polyphenols, whose mechanism of action is not fully elucidated (and could involve promiscuous inhibition; see the aforementioned section on PAINS in the NS2B-NS3 protease section), but NDGA has been shown to affect HCV replication through the reduction of the amount of lipid droplets, thought to be mediated by the sterol regulatory element-binding protein (SREBP) pathway [104]. Early studies have established NDGA as a 5-lipoxygenase inhibitor [105], but it has been shown to bind several other molecular partners, such as glucose transporters (GLUT1) [106], tyrosine kinases [107,108] and even transthyretin [109]. The molecular mechanisms and clinical applications of these compounds are thoroughly reviewed by Lü and co-workers (2010) [110].

Host kinase inhibitors

Protein kinases catalyze the addition of phosphate groups on several molecular entities, such as proteins, lipids and carbohydrates, thus controlling many cellular processes. Since viral replication requires the hijacking of several cellular mechanisms, it is expected to be hindered by the modulation of kinase activity. Indeed, host cell kinases have been implicated in the replication of several RNA virus families [111]. Tang and co-workers (2016) have carried out a transcriptome analysis of human neural progenitor cells (hNPCs) and enrichment analysis of their supplemental data

indicates that several of the up-regulated genes are related to protein kinase activity [112].

Xu and co-workers (2016) performed a two-step drug repurposing screen by initially measuring the caspase-3 activity and subsequently measuring cell viability with the primary hits. Using this approach, they were able to detect PHA-690509, an investigational cyclin-dependent kinase inhibitor (CDKi), which inhibited ZIKV infection with an EC_{50} value of 1.72 μM [113]. They then tested an additional 27 CDKis and identified nine that could inhibit ZIKV replication. Among them, seliciclib (a purine analog, also called roscovitine) and RGB-286147 inhibited ZIKV infection at submicromolar concentrations [113]. The authors concluded that these results suggest that one or more host CDKs may be important for ZIKV replication, as flaviviruses are not known to encode any CDK [113].

AXL (from the Greek word *anexelekto*, or uncontrolled) is a tyrosine kinase receptor (TKR) thought to mediate viral attachment to the host cells [114,115]. Its function is also responsible for a down-regulation of interferon production [116]. Although this recognition may be unrelated to its kinase activity, AXL inhibitors were found to inhibit ZIKV infection rates. Rausch and coworkers have screened a library of ~2000 compounds on human osteosarcoma cells (U2OS) and found 19 ZIKV inhibitors, eight of which had protein kinases as targets, including five TKR and, among these, two AXL inhibitors [42]. The same AXL inhibitors did not exhibit the same activity on different cell lines (HBMEC and Jeg-3), indicating that the effect is cell type specific. The authors also point out that Jeg-3 cells are highly permissive to ZIKV infection, despite showing no detectable AXL expression levels, which suggests that this receptor is not essential for infection [42]. This hypothesis is corroborated by an *in vivo* study [117].

Intracellular membrane traffic is a mechanism that is also exploited by viruses and depends heavily on the enzymatic activity of protein kinases, which regulate the vesicle traffic via the phosphorylation of a specific subunit of the associated adaptor proteins (APs) [118]. Bekerman and coworkers (2017) have tested the antiviral activity of erlotinib and sunitinib for several different flaviviruses (and other genera), including ZIKV (EC_{50} values of 6.28 and 0.51 μM, respectively) [118]. They present several lines of evidence which indicate that the likely targets are AP2-associated kinase (AAK1) and G-associated kinase (GAK), with other possible candidates being AXL, KIT proto-oncogene receptor tyrosine kinase (KIT) and proto-oncogene RET [118].

Monel and co-workers (2017) described in depth the vacuolization of ZIKV-infected cells and suggested that they undergo a paraptosis-like death [119], which is associated with the activity of phophoinositide 3-kinases (PI3K) [120] and membrane associated protein kinases (MAPK) [121]. They therefore tested the activity of several kinase inhibitors and verified that they could prevent the onset of vacuoles, particularly the specific class-1 PI3K (ZSTK474) and AKT (triciribine) inhibitors. However, none of the tested inhibitors blocked ZIKV infection [119].

Protein Metabolism Disruptors

Viral replication extensively hijacks the endoplasmic reticulum functions. ER is intimately associated with the intracellular membrane network remodeling, and it is the framework where the viral polyprotein is expressed and processed. These events generate a considerable amount of stress on this organelle [122].

Correct expression and processing of nascent proteins are paramount for efficient viral replication. Several host proteins are responsible for the monitoring of proper protein synthesis, folding and degradation. Impairment of these functions results in reduced viral assembly and budding. Examples are the endoplasmic reticulum membrane complex (EMC), α-glucosidase, cyclophilin and proteasome elements. Some inhibitors have been investigated under this context and are reviewed hereinafter. EMC, an ensemble of 9 components that are thought to assist protein folding, has been shown to be up-regulated in ZIKV-infected cells. Several proteins of this complex are multi-pass membrane proteins [123].

Another key-player related to protein metabolism is α-glucosidase, an ER resident enzyme that catalyzes the removal of glucose units from N-linked oligosaccharides. This processing is crucial for the nascent glycoprotein maturation and subsequent correct folding. Glycoproteins that fail to be processed are subjected to abnormal accumulation in the ER and ER-associated degradation (ERAD), in which nascent misfolded proteins are retrotranslocated back to the cytosol, ubiquitinated and degraded by the proteasome [124].

α-Glucosidase inhibitors exhibit broad antiviral activity against multiple genera [125,126], but celgosvir (6-O-butanoyl castanospermine) showed poor activity against ZIKV in a CPE-based assay in Vero 76 cells infected with either MR766 or PRVABC59 strains (EC_{50} > 50 μM in both cases) [88].

Xin and co-workers (2017) have carried out a quantitative proteomic analysis of C6/36 cells infected with ZIKV and found up-regulated genes from two protein-related pathways: the ubiquitin-proteasome system (UPS) and the unfolded protein response (UPR) [127]. They subsequently tested the dose-dependent effect of bortezomib (EC_{50} = 5.525 nM) and MG132 (EC_{50} = 1.151 μM), two proteasome inhibitors, in ZIKV-infected Vero cells. They also found that bortezomib can reduce viral load and signs of pathology in ZIKV-infected mice [127]. Bortezomib was also detected in one of the previous screens, but it showed moderate toxicity in the hNSC cell line, as could be expected based on its mechanism and primary use as an anticancer agent [89].

Cyclophilins are cytoplasmic proteins responsible for the isomerization of proline peptide bonds from trans to cis conformation, thus facilitating protein folding [128]. Their activities were shown to be essential for HIV and HCV replication. Cyclosporine A targets cyclophilins and was found to inhibit HCV [129], WNV, DENV, and YFV infections [130]. Barrows and co-workers (2016) have detected cyclosporine as a potential hit, but it displayed controversial results, even enhancing the infection at 1μM compared to the control [89].

Endocytosis/Endosomal Fusion Blocking Agent

Most flaviviruses, including ZIKV, are endocytosed by a clathrin-mediated mechanism and undergo pH-dependent fusion processes, in which the endosomal membrane fuses with the viral envelope through the action of the envelope protein. In order for these steps to occur, the E protein must undergo conformational changes and expose its fusional loop [131].

Li and co-workers (2017) have identified that 25-hydroxycholesterol (25HC) is likely capable of blocking viral entry by modifying host-cell membrane properties, with a calculated EC_{50} of 188 nM. This compound was also able to (a) reduce viremia in mice and in nonhuman primates and (b) protect mice embryos from microcephaly [132].

The antimalarial drug chloroquine is known to exhibit broad-spectrum antiviral activity [133]. It is a weak base (pK_1=8.1, pK_2=10.1), and the pH of lysosomes in the presence of chloroquine increases from ~4 to ~6 [134]. This increase is thought to be responsible for the inhibition of the pH-dependent fusion step of viral entry.

Chloroquine was tested against ZIKV, showing activity in Vero cells (9.82 ± 2.79 μM), human brain microvascular endothelial cells (14.20 ± 0.18 μM) and human neural stem cells (12.36 ± 2.76 μM). It reduced the number of infected cells *in vitro,*

inhibited virus production and cell death promoted by ZIKV infection, and displayed relatively minor cytotoxic effects (CC_{50} ranging from 94.95 to 134.54 μM). Moreover, chloroquine partially reversed morphological changes induced by ZIKV in mouse neurospheres [135]. Chloroquine was found to reduce viral burden in the placenta of ZIKV-infected C57BL/6 pregnant mice [136]. However, it also showed controversial results in another study [88]. Quinacrine (EC_{50} = 2.27 ± 0.14 μM), Mefloquine (EC_{50} 3.95 ± 0.21 μM), and GSK369796 (EC_{50} = 2.57 ± 0.09 μM), other antimalarial drugs with a similar mechanism of action, also were recently tested against ZIKV [137].

The anti-cancer drug obatoclax also promotes rapid neutralization of lysosomal pH, showing activity against YFV (EC_{50} < 0.125 μM), WNV (EC_{50} = 0.10 ± 0.04 μM) and ZIKV (EC_{50} = 0.13 ± 0.01 μM). The authors suggest that the anticancer and antiviral activities are independent and rely on different mechanisms [138]. Obatoclax was also tested in retinal pigment cells (RPE) and displayed an EC_{50} value of 0.04 ± 0.01 μM in the recovery of infected-cell viability [139]. In addition, it was also detected in a drug screen in Jeg-3 cells with an EC_{50} value of 0.08 μM [42].

Saliphenylhalamide (SaliPhe) is another known viral entry blocker, which acts through the inhibition of the vacuolar ATPase. It was tested in Vero 76 cells, yielding an EC_{50} value of 0.62 μM for MR766 strain and 0.49 μM for the PRVABC59 strain. It was also tested by Kuivanen and co-workers (2017) in RPE cells, yielding an EC_{50} value of 0.05 ± 0.02 μM [139].

Niclosamide blocks the acidification of endosomes, albeit using a different mechanism that is not yet fully elucidated [140,141]. It was detected in a screen against ZIKV in SNB-19 cells showing an EC_{50} value of 0.37 μM based on the measurement of intracellular viral RNA [113]. Niclosamide is an FDA-approved drug, formerly designated in pregnancy category B and broadly used in the treatment of intestinal helminthiasis. Drugs in pregnancy category B have not undergone controlled studies in pregnant women, but they have failed to demonstrate a risk to the fetus in animal reproduction studies.

Inhibitors of endosomal sorting complexes required for transport (ESCRT) machinery may be promising targets to ZIKV drug discovery and are presented and discussed in **Supplementary Material**. Moreover, ZIKV inhibitors with unclassified or unconfirmed mechanism of action are also discussed in the **Supplementary Material**.

Available screening assays for anti-ZIKV hit discovery

One of the key steps in the drug discovery pipeline is the development of screening assays to assess the antiviral activity of compounds [142]. Viruses depend on cell machinery to replicate, and for this reason, *in vitro* assays are developed using host cells for culture and viral replication. ZIKV has recently been shown to infect different cells in multiple species. These findings show that a diversity of cell lines can be used to study ZIKV infection, providing a good framework for the drug discovery process [143]. We also present and discuss the available screening assays for anti-ZIKV hit discovery in the **Supplementary Material.**

Concluding remarks

Although the scientific community has devoted considerable efforts to the search for a vaccine and antiviral drugs to prevent and treat ZIKV infection, there are still no approved treatments or prevention for this flavivirus. In a relatively short time we have gone from having no structures of proteins for this virus to a wealth of data. Due to the current state of known anti-ZIKV compounds, there is likely some way to go until clinical trials of the discovered candidates are undertaken, especially when considering that some patients will have underlying medical conditions, including immunosuppressed and pregnant women. For instance, many of the compounds are clearly unsuitable for these patients. Moreover, one of the frequent issues we see with many of the published screens are that they used unrealistic concentrations (e.g. 100 μM) or compounds that could never be used in pregnant women (e.g. cytotoxic anticancer compounds). Much of the work would have benefited from having experienced medicinal chemists involved to advise. Perhaps this could have prevented some inefficient efforts. This would also help to avoid compounds like PAINS, which were reported as hits from several screens.

Ultimately, the recent advances in the discovery of anti-ZIKV agents, ZIKV protein structures and host target protein structures, and our understanding of the disease itself are not only critical to advancing the fight against the Zika virus—they can also be useful for the next emerging virus outbreak for which we will have to respond. We should also heed the lessons learned from ZIKV drug discovery so we can be more successful and avoid dead ends.

Conflicts of interest

All authors except SE (CEO of Collaborations Pharmaceuticals, Inc.) do not have any conflicts of interest to declare. These views and reflections are those of the authors.

Glossary (virus abbreviations)

CoV (Coronavirus)

DENV (Dengue virus)

HCV (Hepatitis C virus)

HIV (Human immunodeficiency virus)

HSV (Herpes simplex virus)

Flu (Influenza virus)

JEV (Japanese encephalitis virus)

TBE (Tick-borne encephalitis virus)

WNV (West Nile virus)

YFV (Yellow fever virus)

ZIKV (Zika virus)

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Figure Captions

Figure 1. Scheme of ZIKV surface, structural and non-structural proteins. (A) surface-shaded depth cued representation of mature ZIKV (built using UCSF Chimera package [11], http://www.rbvi.ucsf.edu/chimera, based on PDB ID 5IRE), showing the icosahedral-like symmetry arrangement of surface proteins; (B) virion components, highlighting the E, M and C proteins, as well as genomic RNA. ZIKV encodes a large polyprotein, which after processing yields three structural proteins (C, M and E) and seven non-structural proteins (NS1; NS2A; NS2B; NS3 protease and helicase domains; NS4A; NS4B; NS5 methyltransferase and RNA polymerase domains), built using VMD program [12], http://www.ks.uiuc.edu/Research/vmd/. NS5 domains are represented separately, as two distinct targets, but NS5 methyltransferase is attached to the NS5 polymerase domain to form the full-length NS5.

Figure 2. Ribbon-representation of some of the ZIKV protein 3D structures. (A) NS2B/NS3 protease, highlighting the binding site occupied by a boronate inhibitor [18] and the fragment 1H-benzo[d]imidazol-1-ylmethanol [17]; (B) NS3 helicase, highlighting the RNA and ATP binding sites; (C) NS5 methyltransferase domain, highlighting the active site, SAM/SAH and GTP/cap binding sites; (D) NS5 polymerase domain, emphasizing the active site, NTP and RNA binding sites; (E) Envelope protein [20] and its β-barrel-shaped domain I (DI), finger-like domain II (DII), immunoglobulin-like domain III (DIII) and a ligand binding site between DI and DIII [21]. This predicted binding site encloses a hydrophobic cavity around the flexible linker joining DI and DIII. All these 3D structures are available in PDB and the figures were built using VMD program [12], http://www.ks.uiuc.edu/Research/vmd/.

Figure 3. Chemical structures of selected ZIKV protein inhibitors. (A) Envelope glycoprotein inhibitor: nanchangmycin (IC_{50} = 0.1 μM) [42]; (B) NS2B-NS3 protease inhibitors: temoporfin (IC_{50} = 1.1 μM) [45], and NSC157058 (IC_{50} = 0.82 μM) [46]; (C) NS3 helicase inhibitor*: suramin (EC_{50} = 0.42 μM), which was tested only in cell-based assay in ZIKV [47]; (D) NS5 methyltransferase inhibitor: sinefungin (IC_{50} = 1.18 μM) [48,49]; (E) NS5 polymerase inhibitors: sofosbuvir (IC_{50} =7.3 μM) [50], 2-C-ethynyl-UTP (IC_{50} = 0.46 μM) [51], and DMB213 (IC_{50} = 5.2 μM) [50].

Tables:

Table 1. Available ZIKV protein 3D structures in the PDB (searched on February 9th, 2018).

ZIKV protein	PDB ID
NS1	5X8Y (mutation), 5GS6, 5K6K, 5IY3
NS2B-NS3 protease	5TFN, 5TFO, 5GXJ, 5GPI, 5H4I (with a benzimidazole fragment), 5GJ4 (with a peptide), 5T1V, 5LC0 (with a boronate inhibitor covalently bound)
NS3 helicase	5VI7, 5Y4Z (with AMPPNP), 5JPS, 5MFX (with RNA), 5TXG, 5JWH, 5K8I, 5K8L, 5K8T (with GTP), 5K8U, 5GJC (with ATP), 5JRZ, 5JMT, 5GJB (with ssRNA)
NS5 methyltransferase	5VIM, 5ULP (with SAM analog), 5WXB (with SAH), 5WZ1 (with SAM), 5WZ2 (with SAM and RNA analogue), 5MRK (with sinefungin), 5M5B, 5GOZ (with GTP and SAH), 5GP1 (with GTP and SAH), 5TFR, 5KQR (with SAM), 5KQS (with SAM and RNA analogue)
NS5 polymerase	5U0C, 5WZ3, 5U04
NS5 full	5U0B, 5TFR
Envelope	5JHM, 5LBV (with an antibody), 5JHL (with an antibody), 5KVD (with antibody), 5KVE (with antibody), 5KVF (with antibody), 5KVG (with antibody), 5GZN (with antibody), 5GZO (with antibody), 5VIG (with antibody), 5VIC (with antibody)