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*Teaser Zika clinical outcomes might be nefarious impacting 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.*



# The A–Z of Zika drug discovery

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Despite the recent outbreak of Zika virus (ZIKV), there are still no approved treatments, and early-stage compounds are probably many years away from approval. 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 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 crucial to advancing the fight against the Zika virus and other flaviviruses but also helps us prepare for the next emerging virus outbreak to which we will have to respond.

## Introduction

Zika virus (ZIKV) remains 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

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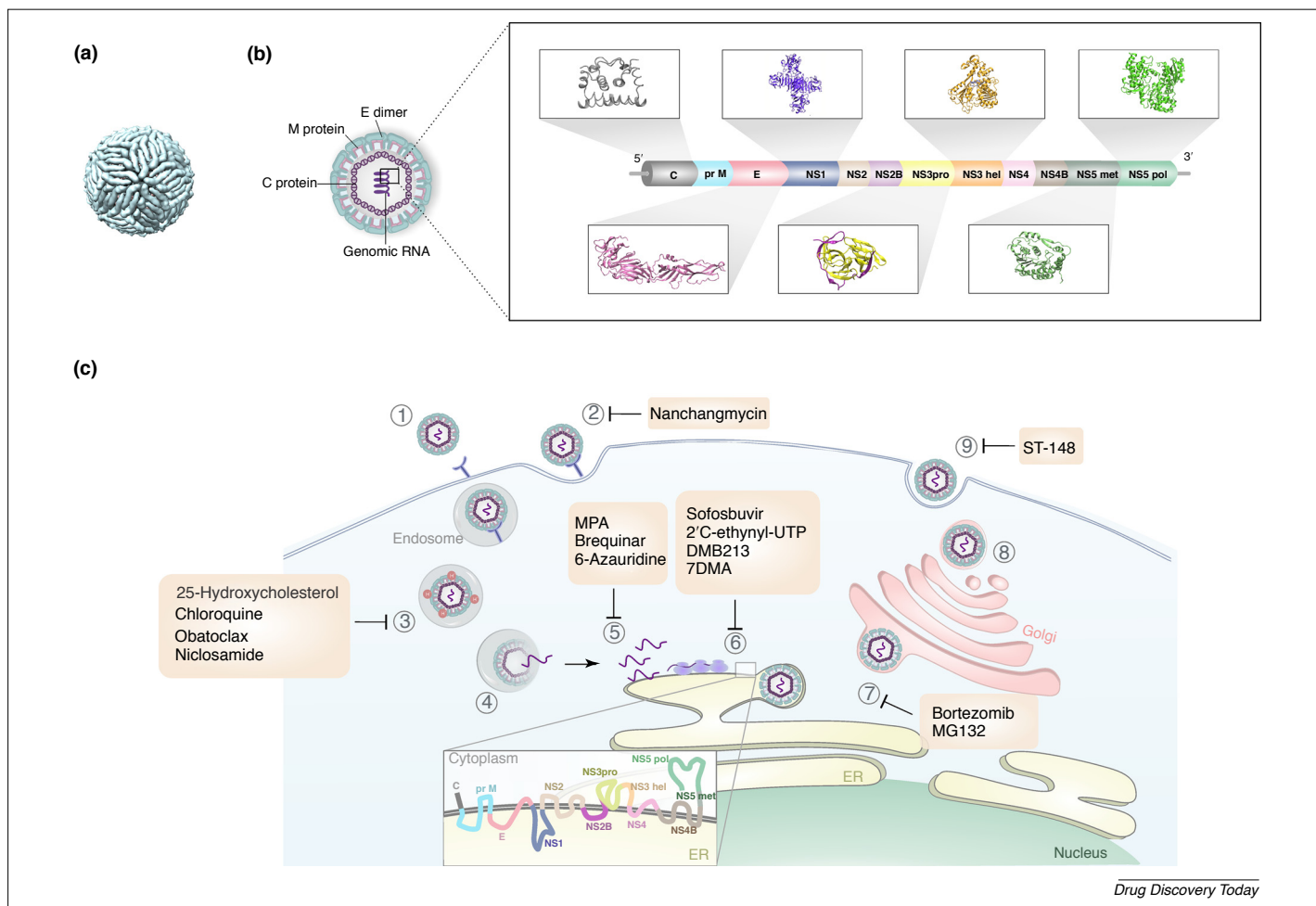
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disproportion, spasticity, seizures, irritability and other brainstem dysfunctions [2,3]. In 2016, infant head computed tomographic findings, of subjects infected during pregnancy, confirmed the causal relationship between microcephaly and Zika infection [3]. A recent study by Yuan and co-workers 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 [4]. This mutation arose in the French Polynesia strain, and it has contributed to the increased incidence of microcephaly and higher mortality in neonates, according to experimental assays [4]. The disorders attributed to Zika infection mainly affect infants but can also impact adults. There have been ZIKV-related cases of Guillain-Barré syndrome [5], myelitis [6], uveitis [7] and meningoencephalitis [8] reported in adults.

Currently, neither a specific antiviral drug nor a vaccine is 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 ZIKV proteins

ZIKV is a spherical, enveloped virus, with an icosahedral-like symmetry [9] (Fig. 1a). Belonging to the genus *Flavivirus* of the



**FIGURE 1**

Scheme of Zika virus (ZIKV) surface, structural and nonstructural proteins. **(a)** Surface-shaded depth-cued representation of mature ZIKV (built using UCSF Chimera package [171], <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 nonstructural proteins (NS1; NS2A; NS2B; NS3 protease and helicase domains; NS4A; NS4B; NS5 methyltransferase and RNA polymerase domains), built using the VMD program [172] (<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. **(c)** ZIKV infectious life cycle: the virus is attached (1) and subsequently internalized (2) by receptor-mediated endocytosis. The virus is then trafficked to early endosomes, where the acidic environment induces fusion (3) between the virus and host membrane resulting in particle disassembly and genome release (4). RNA is replicated and translated into a single polyprotein, which is processed by host and virus-encoded proteins (5). Following translation, a replication complex is assembled and associated to virus-induced membranes where viral replication takes place (6). The progeny RNA (+) strands can either initiate a new translation cycle or be assembled into virions within the endoplasmic reticulum (ER) (7). The resultant immature virions are transported to the trans-Golgi where the immature virions are transformed into mature infectious particles (8) that are released by exocytosis (9). In the pink boxes are the names of the compounds that can inhibit the marked steps of the virus lifecycle.

*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 nonstructural (NS) proteins (Fig. 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 NS proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5) are essential for genomic replication and modulation of host immunity [10] (Fig. 1b). Figure 1c presents an overview of the replication process of ZIKV in the infected cell, showing the marked steps of the replication, which can be inhibited by the compounds discussed below in this review. We also present a special section (see supplementary material online: viral entry and replication mechanisms).

Initially, before 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; see Glossary) and other flaviviruses [11–13]. 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) [14]: 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 or RNA; this is very useful for ligand-binding-site identification in virtual screens versus apo conformations.

These protein structures are fundamental when performing experimental and computational studies. Figure 2 shows some

ZIKV protein 3D structures and their **ligand and or substrate binding pockets**. The **substrate binding pocket** of ZIKV NS2B/NS3 protease (NS2B residues: S81, D83, K54 and NS3pro residues: D75, H51, S135, G133, G151, D129, Y161) (Fig. 2a) was based on the co-crystallized compound 1H-1,3-benzodiazol-1-ylmethanol [15] and a boronate inhibitor [16], which bind to a similar pocket. NS3 helicase presents two binding sites, one encompasses the RNA strand and the other is where the ATP molecule binds (Fig. 2b) [17]. The full-length NS5 protein contains two domains, representing distinct targets: the NS5 methyltransferase and NS5 polymerase [18]. The NS5 polymerase domain has three adjacent binding sites: the active site, the RNA site and the NTP channel [18] (Fig. 2c). The NS5 methyltransferase domain has two substrates: SAM, a co-substrate involved in methyl group transfer, and GTP, a substrate for RNA synthesis [18]. Consequently, the accessibility of these two substrates, together with a conserved catalytic tetrad of Lys61, Asp146, Lys182, Glu218, forms the active site [18] (Fig. 2d). ZIKV envelope protein is composed of three distinct domains: the  $\beta$ -barrel-shaped domain (DI), the finger-like domain (DII) and the immunoglobulin-like domain (DIII) (Fig. 2e). Domain II is responsible for the dimerization [19]. A binding site between DI and DIII, enclosing a hydrophobic cavity around a flexible linker, was computationally predicted [20]. The protein–protein binding pocket is composed of residues from DI and DIII (Fig. 2e). The crystallographic structure of the capsid protein was recently solved [21,22]. It contains four  $\alpha$  helices in each monomer, and it forms a dimer (Fig. 2f). We predicted the capsid pocket druggability using the PockDrug server [23], which identified three pockets: pocket 1 (between  $\alpha$ 2 and  $\alpha$ 3 helices), pocket 2 (between N-terminal and  $\alpha$ 1 helix of the monomers) and pocket 3 (between  $\alpha$ 4 helices of the monomers).

TABLE 1

**Available ZIKV protein 3D structures in the PDB**

ZIKV protein	PDB ID
NS1	5X8Y (mutation), 5GS6, 5K6K, 5IY3
NS2B–NS3 protease	5TFN, 5TFO, 5GXJ, 5GPI (with reverse peptide inhibitor), 5H4I (with a benzimidazole fragment), 5GJ4 (with a peptide), 5T1V (apo form), 5LC0 (with a boronate inhibitor covalently bound), 5YOD (with benzoic acid), 5YOF (with dipeptide inhibitor), 5H6V (with dipeptide inhibitor)
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 analog), 5MRK (with sinefungin), 5M5B, 5GOZ (with GTP and SAH), 5GP1 (with GTP and SAH), 5TFR, 5KQR (with SAM), 5KQS (with SAM and RNA analog)
NS5 polymerase	5UOC, 5WZ3, 5U04, 5TMH
NS5 full	5UOB, 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), 5LBS (with antibody)
Capsid	5YGH, 5ZOR, 5ZOV

**Viral proteins as drug targets**

The viral proteins play an important part in virus 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 lifecycle, such as assisting in the chaperone-mediated folding of the E protein and preventing premature fusion during virion egress [24]. 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) [25].

The function of NS flavivirus proteins is still not completely understood but can be summarized as follows. NS1 has several roles, including flaviviral replication and virion maturation [26]. The NS2B subdomain is required for the proper formation of the substrate recognition site of the NS3 protease [27]. The NS3 helicase (NS3h) protein promotes the separation of RNA strands during viral replication and unwinds the RNA secondary structure in the 3' nontranslated region [28]. Together with NS2B, NS3 protease is responsible for cleavage and posttranslational modification of the viral polyprotein [16,29]. Flavivirus NS4A and NS4B proteins compose the ER membrane-associated replication complex [30–32]. DENV NS4B has been shown to interact with NS3h and dissociate it from single-stranded RNA to modulate RNA synthesis [33]. ZIKV NS4A and NS4B cooperatively suppress the

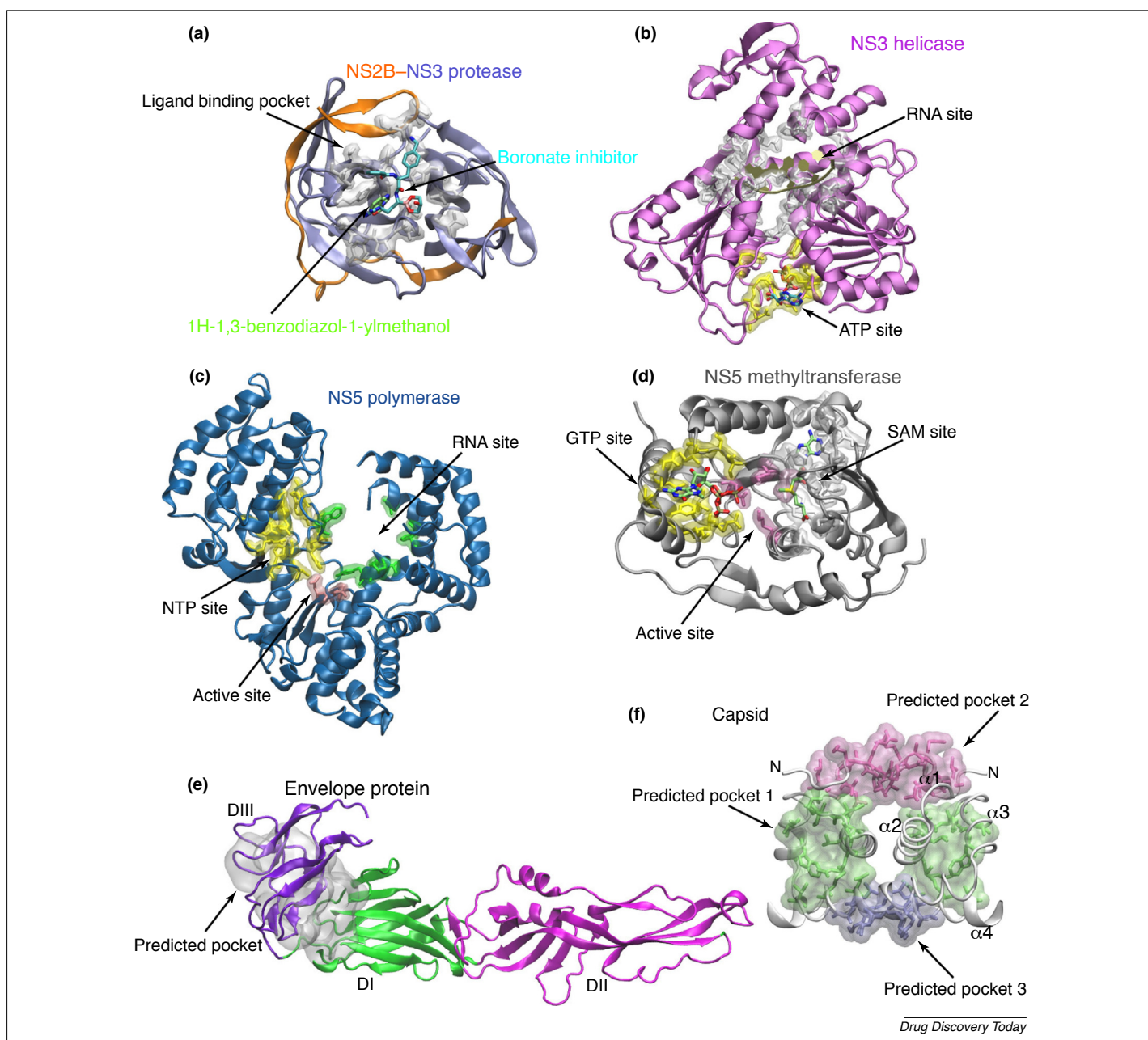


FIGURE 2

Ribbon-representation of some of the ZIKV protein 3D structures. **(a)** NS2B-NS3 protease, highlighting the substrate/ligand-binding pocket occupied by a boronate inhibitor [16] and the compound 1H-1,3-benzodiazol-1-ylmethanol [15]. **(b)** NS3 helicase, highlighting the RNA and ATP binding sites [17]. **(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 [19] and its  $\beta$ -barrel-shaped domain I (DI), finger-like domain II (DII), immunoglobulin-like domain III (DIII) and a ligand-binding pocket between DI and DIII [20]. This predicted binding pocket encloses a hydrophobic cavity around the flexible linker joining DI and DIII. **(f)** Capsid protein [21,22] and the predicted ligand-binding pockets. All these 3D structures are available in PDB and the figures were built using the VMD program [172] (<http://www.ks.uiuc.edu/Research/vmd/>).

host's Akt/mammalian target of rapamycin (mTOR) pathway, inhibiting neurogenesis and inducing autophagy [34]. For these reasons, NS4B is an important target and its inhibition could impair viral propagation. Unfortunately, no crystal structures of ZIKV NS4B are available (as of 25<sup>th</sup> June 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 [18].

The innate immune response is the initial line of host defense, where type I interferon (IFN) production and signaling have a central role. Regarding this, NS5 proteins have been shown to inhibit the IFN signaling to evade host antiviral defense [35]. 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 [36], this action was related to

the polymerase domain [37]. For Japanese encephalitis virus (JEV), NS5 blocks tyrosine phosphorylation of tyrosine protein kinase Tyk2 [38]. 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 [39].

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 [40,41]. Particularly for ZIKV, a docking study was carried out to predict the protein–protein interactions between NS5, seven in absentia homolog (SIAH2) protein and STAT2 proteins. The study suggests that: (i) NS5 recruits SIAH2 for the ubiquitination-dependent degradation of STAT2; and (ii) the NS5 amino acid residues involved in interaction with SIAH2 and/or STAT2 were found to be conserved across related flaviviruses [42]. 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 [43]. In principle, small molecules interfering with the function of any ZIKV viral proteins have potential in restricting virus replication and preventing the progress of ZIKV-infection-related pathogenesis and diseases. Here, we summarize several inhibitors targeting viral proteins that have been identified from high-throughput cell-based screening, *in silico* docking or compound library screening. Some of them have even undergone *in vivo* testing.

#### Envelope glycoprotein inhibitors

A polyphenol present in green tea, epigallocatechingallate (EGCG), has been shown to have antiviral activity for many viruses, such as HIV, herpes simplex virus (HSV), influenza virus and hepatitis C virus (HCV) [44]. Recently, Carneiro and co-workers, through *in vitro* assays (in 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 (>100  $\mu\text{M}$ ). The authors proposed that the inhibition is probably related to the direct interaction of EGCG with the lipid envelope, leading to a subsequent destruction of the virus particle [44]. Conversely, EGCG contains the catechol group, which is a well-known PAIN substructure (pan-assay interference compound) that promiscuously or nonspecifically inhibits many different targets [45–47].

A subsequent computational study by Sharma and co-workers, using induced-fit docking, molecular dynamics (MD) and drug-like property calculations, revealed that EGCG binds to a hydrophobic site located between DI and DIII, close to a flexible linker (Fig. 1e). Using MD simulations, they showed that EGCG blocks the major conformational change during the membrane fusion process [20]. Another natural product, nanchangmycin, from *Streptomyces nanchangensis*, proved to be a potent inhibitor of ZIKV in HTS assays using human cells [48], with an  $\text{IC}_{50}$  0.1  $\mu\text{M}$  (Fig. 3a). To determine whether nanchangmycin was blocking a pre- or post-entry step in the viral lifecycle, the authors treated cells with the drug and removed the drug 4 h post-infection (hpi) and monitored the level of infection (24 h later). Nanchangmycin inhibited infection, which suggests that it blocks an early step in the viral lifecycle [48]. Unfortunately, nanchangmycin contains a reactive Michael acceptor, which is also a well-known PAINS

substructure [49]. Fernando and co-workers, through homology modeling, docking and MD simulation approaches, predicted two compounds: ZINC33683341 and ZINC49605556, could inhibit ZIKV E protein. These two small molecules preferentially bind to the glycan-binding domain, related to the virulence of the virus. The antiviral activity of ZINC33683341 was confirmed by cell culture assay (in Vero cells) [50].

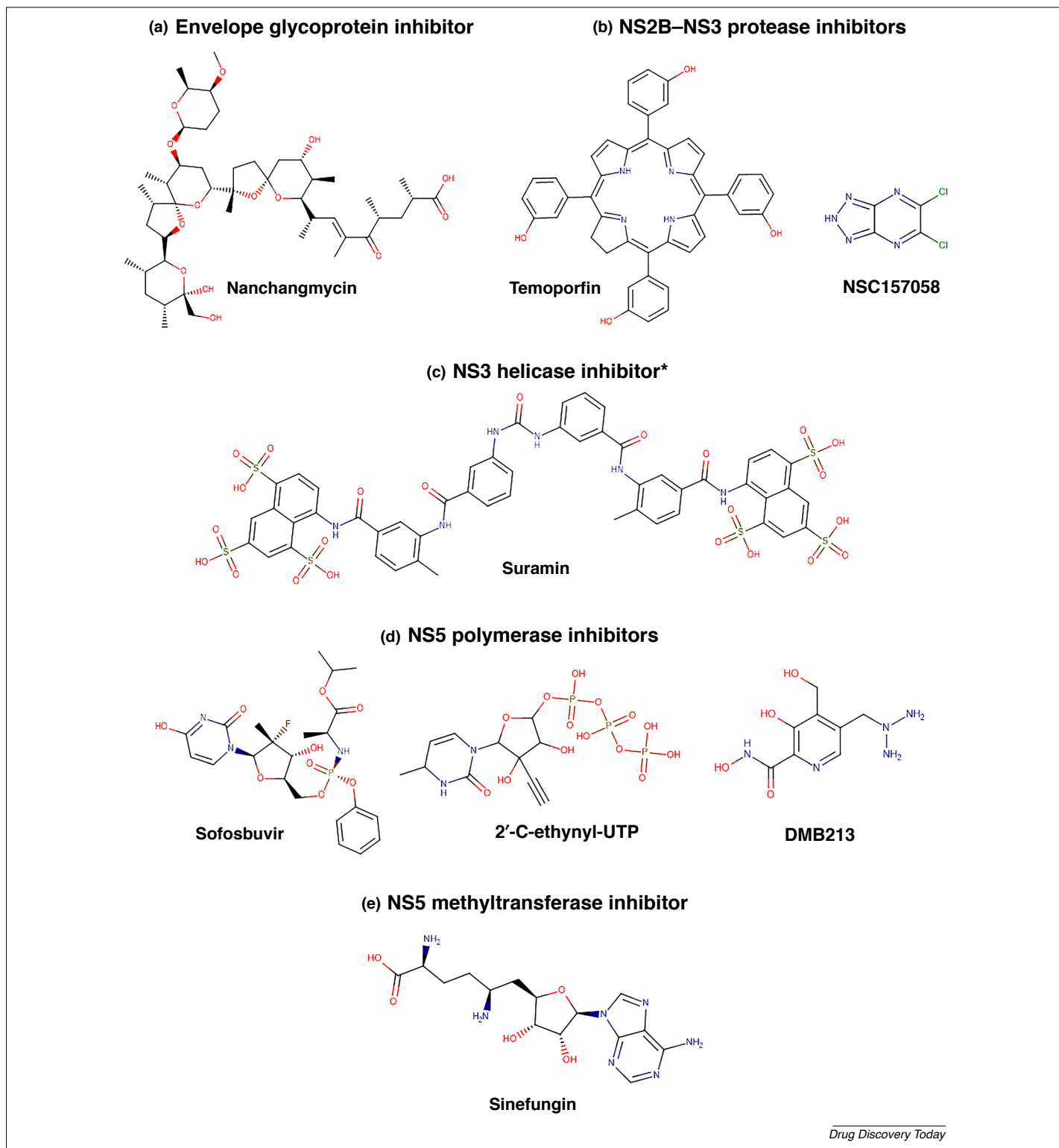
#### Capsid protein inhibitors

Although the capsid protein has emerged as a promising target for antiviral agents, few capsid inhibitors have been identified to date. 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 viremia and viral load in vital organs [24]. Characterization of the mode-of-action of ST-148 showed that it directly targeted the capsid protein and presented bimodal antiviral activity affecting assembly and/or release and entry of infectious DENV particles [51]. Another example of a flaviviral capsid inhibitor is the biotinylated derivative of compound SL209, which inhibited HCV infection, blocking the core dimerization with an  $\text{IC}_{50}$  of 2.80  $\mu\text{M}$  [52]. Both studies support the hypothesis that inhibitors of viral capsid formation might constitute a new class of antiviral agents, and therefore could also be applicable to ZIKV.

#### NS2B–NS3 protease inhibitors

Natural products like polyphenols are known to have antiviral activity against influenza virus, coronavirus (CoV), DENV and others [53–56]. 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  $\mu\text{M}$ , and seven compounds were able to inhibit >50% of the protease activity [57]. The  $\text{IC}_{50}$ s of these compounds were determined and ranged from 22 to 112  $\mu\text{M}$  [57]. In another study, five flavonoids and one natural phenol were tested [58]. The flavonoids were myricetin ( $\text{IC}_{50}$  = 1.3  $\mu\text{M}$ ), quercetin ( $\text{IC}_{50}$  = 2.4  $\mu\text{M}$ ), luteolin ( $\text{IC}_{50}$  = 2.7  $\mu\text{M}$ ), isorhamnetin ( $\text{IC}_{50}$  = 15.5  $\mu\text{M}$ ), apigenin ( $\text{IC}_{50}$  = 56.3  $\mu\text{M}$ ) and the natural phenol curcumin ( $\text{IC}_{50}$  = 3.5  $\mu\text{M}$ ). These polyphenols inhibit the enzyme in a noncompetitive 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 [58]. However, curcumin, quercetin and other flavonoids have been shown to be promiscuous inhibitors, for example via colloidal aggregation [59–61]; curcumin also contains reactive Michael acceptors and quercetin has a catechol, a well-known PAINS substructure, which might 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 [62]. Then, a library of FDA-approved drugs and investigational drugs (2816 drugs) was screened using this assay, and 23 compounds produced an  $\text{IC}_{50}$  below 15  $\mu\text{M}$ , of which 12 were considered PAINS. The remaining 11 compounds were tested for their protease inhibition activities, and three could inhibit with  $\text{IC}_{50}$  values ranging from 1.1 to 15.9  $\mu\text{M}$ . The three compounds were tested against ZIKV, DENV, WNV, JEV and YFV. They presented inhibitory activity close to the nanomolar level, whereas temoporfin (Fig. 3b) displayed an  $\text{EC}_{50}$  (half-maximal effective concentration)

**FIGURE 3**

Chemical structures of selected Zika virus (ZIKV) protein inhibitors. **(a)** Envelope glycoprotein inhibitor: nanchangmycin ( $IC_{50} = 0.1 \mu M$ ) [48]. **(b)** NS2B–NS3 protease inhibitors: temoporfin ( $IC_{50} = 1.1 \mu M$ ) [62] and NSC157058 ( $IC_{50} = 0.82 \mu M$ ) [66]. **(c)** NS3 helicase inhibitor: suramin ( $EC_{50} = 0.42 \mu M$ ), which was tested only in a cell-based assay in ZIKV [75]. **(d)** NS5 polymerase inhibitors: sofosbuvir ( $IC_{50} = 7.3 \mu M$ ) [105], 2'-C-ethynyl-UTP ( $IC_{50} = 0.46 \mu M$ ) [99] and DMB213 ( $IC_{50} = 5.2 \mu M$ ) [105]. **(e)** NS5 methyltransferase inhibitor: sinefungin ( $IC_{50} = 1.18 \mu M$ ) [90,91].

for all flaviviruses tested at low nanomolar concentrations. The therapeutic indices for all three compounds were high, because their  $CC_{50}$  (concentration that inhibits 50% of mammalian cell proliferation) was considerably higher than their  $EC_{50}$ . The com-

pounds were also able to inhibit ZIKV replication in placental epithelial and neuronal cells and to inhibit viral polyprotein cleavage. Temoporfin was tested in a viremia 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 [62]. A similar study was done using structure-based virtual screening (VS) of 8277 compounds from the DrugBank database, and the top 100 candidates were identified [63]. 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 novobiocin, lopinavir, ritonavir and rifampicin which had favorable safety profiles and could be administered to pregnant women were selected for phenotypic screening. Two compounds were able to inhibit ZIKV replication. Because 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, as well as less severe histopathological changes than untreated controls [63].

A similar strategy is to appropriate other flavivirus active compounds to inhibit ZIKV, because they are closely related in terms of the sequence and structure of their proteins [64]. 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 or 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 proteolytic activity of the enzyme [64]. Twenty-seven HCV NS3–NS4A protease inhibitors were tested in ZIKV NS2B–NS3; ten presented  $IC_{50}$  values below 50  $\mu$ M. The best two inhibitors were tested to determine their inhibition type, and they presented competitive inhibition profiles, meaning that they might bind at the active site [65]. Aprotinin, a 58 amino acid bovine trypsin inhibitor, previously tested against WNV protease, also inhibits ZIKV protease ( $IC_{50}$  = 70 nM), and molecular modeling studies predicted that the inhibitor probably blocks the interactions of NS3 and NS2B [66]. By contrast, in our analysis of crystal structures of WNV protease with bovine pancreatic trypsin inhibitor (PDB ID 2IJO) [67] and of DENV protease bound to aprotinin (PDB ID 3U1J) [68], 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 ~700 structurally similar compounds were screened [66]. The best inhibitors were NSC157058 ( $IC_{50}$  = 0.82  $\mu$ M) (Fig. 3b), NSC86314 ( $IC_{50}$  = 0.97  $\mu$ M) and NSC716903 ( $IC_{50}$  = 1.12  $\mu$ M). 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  $\mu$ M. These compounds decreased the viral yield in neuronal precursor cells. The best inhibitor, NSC157058, was tested using an *in vivo* mouse model and decreased the viremia by tenfold; unfortunately, it has a very unfavorable pharmacokinetics profile [66].

Brecher *et al.* developed an assay to analyze the conformational changes in DENV NS2B–NS3 protease using luciferase [69]. They performed a 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 [69]. 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 could provide a model for assemblies formed at the site of poly-protein processing, guiding future drug discovery processes against ZIKV protease [15,16,70].

### NS3 helicase inhibitors

Helicase displays ATPase and RNA triphosphatase (RTPase) activities. There are few studies regarding NS3h inhibitors, mainly using VS, but they are not experimentally validated. Although we have recently had some promising preliminary results in our own virtual screens against ZIKV NS3h, developing drugs that target ZIKV NS3h could involve significant hurdles. For instance, the accessible pocket(s) within the active site of the NS3h–RNA complex is relatively shallow, and previous efforts to discover specific inhibitors of DENV NS3h have been challenging [71]. A potential strategy to deal with these issues could involve targeting other sites of ZIKV NS3h, such as the putative allosteric site that we discovered in our MD simulations [72]. We are currently performing new VS against snapshots of ZIKV NS3h that we generated with MD to test this strategy. Other challenges are related to: (i) the development of potent inhibitors, as a result of the lack of the specific amino acid interaction with ATP nucleobase or ribose ring; and (ii) the discovery of specific inhibitors, because the host helicase is within the same SF2 superfamily as NS3h [71].

In an ATPase activity analysis of NS3 helicase, the ATPase inhibitors resveratrol and quercetin were tested at 150  $\mu$ M; their inhibitory effects were 51% and 15% ATPase activity inhibition, respectively [73]. Suramin (Fig. 3c), an antiparasitic drug, was previously characterized as a DENV NS3 helicase inhibitor with a noncompetitive mode of action [74], and recently it was shown to inhibit ZIKV replication with  $EC_{50}$  value of 0.42  $\mu$ M [75]. Albulescu and co-workers suggested through experimental assays that suramin affected ZIKV binding and/or entry, virion biogenesis and attachment to host cells [75]. 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 [75].

MD simulations of NS3h were performed to study the proteins flexibility and conformations preferences [72]. This work 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 [72]. The different conformations observed during MD showed a distinct region beneath or behind the ATP site, which could perhaps be an allosteric site, and could help guide the discovery or design of new inhibitors. These conformations and strategies are guiding new VS 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 modeling filtering (performed in-house) [76]. This project is ongoing and several compounds are currently being



assayed that were identified in these virtual screens. Other flaviviral NS3 helicase inhibitors such as ivermectin [77] and benzoxazole have activity against YFV NS3h and DENV NS3h, respectively [78]. Ivermectin was also reported to inhibit DENV NS5 interaction with its nuclear transporter importin (IMP)  $\alpha/\beta$  *in vitro* [79,80]. All these NS3h agents are also interesting compounds to be tested against ZIKV NS3h.

#### NS4B inhibitors

The NS4B protein is known to have transmembrane domains and is placed in the ER [31]. The biological function of this protein is not fully established, but it is part of the replication complex (RC), and some studies have demonstrated that NS4B induces the formation of a membrane web that forms a scaffold for the formation of the RC [81]. NS4B also interacts with other viral proteins, such as NS1, NS2B, NS3 and NS4A [33,82–84]. For those reasons, NS4B is a good target to develop inhibitors. Unfortunately, the search for NS4B inhibitors faces many challenges, such as poor pharmaceutical properties of the inhibitors, difficulties to find pan-flaviviral inhibitors and also a high risk of developing resistant viruses [85].

There are few studies regarding ZIKV NS4B protein inhibition thus far. An aminothiazole derivative (NITD-618) was identified as a specific DENV 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 [86]. NITD-618 faced problems with pharmacokinetics in *in vivo* studies because of its high lipophilicity, and medicinal chemistry attempts to overcome this issue resulted in loss of antiviral selectivity against DENV [86]. In another screen using the NIH clinical collection, a naltrindole analog (SDM25N) was found to inhibit DENV, and specific NS4B point mutations (F164L and P104L) conferred resistance against the compound, indicating that NS4B is probably the targeted protein [87]. A spiropyrazolopyridone compound was found to inhibit DENV serotypes 2 and 3 but not 1 and 4. This fact is correlated with the low identity between NS4B of flaviviruses and even between different serotypes of DENV [88]. The compound was able to suppress viremia in a mouse model of DENV serotype 2 infection. Two compounds (CCG-3394 and CCG-4088) were hits from a screen against YFV NS4B, and mutations on the K128 residue conferred resistance to YFV against both of these compounds [89].

#### 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. *S*-adenosyl-L-homocysteine (SAH) and the SAM analog sinefungin (Fig. 3d) presented IC<sub>50</sub> values of 0.43  $\mu$ M and 1.18  $\mu$ M, respectively. The Cap analogs inhibited the enzyme with IC<sub>50</sub> values ranging from 72 to 491  $\mu$ M. The allosteric compounds presented 50% inhibition values of 24 to 221  $\mu$ M [90]. The structure of ZIKV MTase with the SAM analog sinefungin was recently elucidated, and the construction of an inhibitor connecting sinefungin with a Cap analog attached by a linker was proposed, which could perhaps grant higher affinity to the protein [91]. VS using a hydrophobic site close to the SAM pocket was performed with >20 000 compounds [92]. The ten compounds with the best scores

were selected for experimental screening: four were able to inhibit viral growth in concentrations below 20  $\mu$ M and the best inhibitor presented an IC<sub>50</sub> value of 4.8  $\mu$ M [92].

Jain and co-workers developed a SAM analog that encroaches into the guanosine triphosphate (GTP)/Cap pocket of ZIKV MTase [93]. 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 [93]. Designing ZIKV-specific MTase inhibitors is a challenge, as is the case for DENV and other flaviviruses, mainly owing to the selectivity of these compounds to SAM-binding or SAM-utilizing proteins present in the host cell, because of the similarity to human RNA and DNA MTases [29]. Moreover, another issue with the design of competitive MTase inhibitors is the high cellular SAM concentration [94]. Still, these issues have been overcome in the field of kinases [95], indicating that it could also be feasible for MTases [94].

#### NS5 polymerase inhibitors

NS5 RdRp remains one of the most promising targets, as demonstrated by the large number of marketed nucleoside inhibitor (NI) and non-nucleoside inhibitor (NNI) drugs available for the treatment of HIV-1, cytomegalovirus and hepatitis B and C viruses [96,97]. Nucleoside analogs, such as the prodrug sofosbuvir, have been used as part of certain combination therapies that can treat HCV. Some NIs, 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<sub>50</sub> values <10  $\mu$ M [98].

A similar observation was found when ribonucleoside triphosphate analogs were tested against NS5 polymerase: the 2'-C-methyl- and 2'-C-ethynyl-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<sub>50</sub> = 90.76  $\mu$ M), 2'-C-ME-UTP (IC<sub>50</sub> = 5.78  $\mu$ M) and 2'-C-ethynyl\_UTP (IC<sub>50</sub> = 0.46  $\mu$ M) (Fig. 3e) [99]. ATP analogs were tested in NS5 polymerase in another study, and the compounds that led to the strongest enzyme inhibition were the 2'-C-methylated ATPs [100]. The best inhibitors presented IC<sub>50</sub> values of 5.6  $\mu$ M and 7.9  $\mu$ M [100].

An adenosine analog called BCX4430 inhibited ZIKV in cell culture, as well as in a mouse model [101]. This compound already presented broad-spectrum activity against a wide range of RNA viruses: West Nile, tick-borne yellow fever, Marburg and Ebola [102,103]. Previous studies suggested that BCX4430 acts on NS5 polymerase, promoting chain termination of viral RNA synthesis [102]. In a recent study, another adenosine analog and a known flaviviral NS5 polymerase inhibitor was 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 [104]. Although the authors have not tested this compound against the ZIKV protein, it represents a very promising candidate [104].

Nucleoside inhibitors against the polymerase have made great progress; however, the major challenges for the NI-based antiviral approach include toxicity and the added complication of being a prodrug (which requires host kinases to convert it to the triphosphate form of NI) [94]. Using a drug repurposing strategy, Xu *et al.* tested compounds already used for HCV NS5 polymerase: sofosbuvir (Fig. 3e), a NI, and DMB213 (Fig. 3e), a NNI [105]. Both compounds inhibited ZIKV NS5 polymerase with IC<sub>50</sub> 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 [105].

Mumtaz and co-workers tested sofosbuvir against ZIKV using Vero, A549 and Huh7 cells, measuring the level of the active sofosbuvir metabolite by mass spectrometry [106]. Sofosbuvir showed a cell-type-dependent activity, inhibiting ZIKV with an IC<sub>50</sub> of ~4 μM (but only in Huh7 cells) [106]. This correlated with differences in the intracellular concentration of the active metabolite of sofosbuvir, which was higher in Huh7 cells compared with the other cells. These results highlight the importance of a careful selection of cell system for repurposing trials of prodrugs to evaluate antiviral activity [106].

A pharmacophore-based strategy was used to search for NS5 inhibitors [12]. The researchers constructed models for NS5 polymerase and NS5 methyltransferase using the molecules ribavirin and BG323. They used the models to conduct VS 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 three 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 [12]. These results remain to be confirmed through validation assays.

Lim *et al.* performed a fragment-based screening by X-ray crystallography targeting the DENV NS5 polymerase and elucidated an allosteric binding pocket at the base of the thumb subdomain, close to the enzyme active site [107]. Promising allosteric inhibitors were developed through structure-guided design, and they were active in DENV polymerase biochemical and cell-based assays, inhibiting the enzyme with IC<sub>50</sub> values of 1–2 μM [107]. The same strategy could probably be used for ZIKV NS5 allosteric inhibitor design, because the viral proteins have conserved sequences and similar structures.

#### Host proteins as drug targets

ZIKV, as well as other *flaviviridae* members, has 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 the function of viral proteins, an attractive broad-spectrum strategy is to target host cell processes, because they are often employed by multiple viruses and are less prone to the development of drug resistance [108]. The recently described inhibitors of the most commonly targeted cellular functions in ZIKV can be found in Table S1 (see supplementary material online) and are reviewed below. Moreover, we also present a special section regarding the antiviral effects of the type I and II interferons in the supplementary material.

#### 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 [109]. 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 [110]. 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 [111]. It was found to inhibit the virus-induced cytopathic effects (CPE) of several flaviviruses, including ZIKV (EC<sub>50</sub> = 142.9 μg/ml) [112]. A recent study tested the activity of known broad-spectrum antivirals, including ribavirin, which yielded a poor inhibition of virus-induced CPE (EC<sub>50</sub> > 50 μM for MR766 strain), concluding it was not a suitable candidate as a ZIKV therapeutic [113]. Other nucleoside biosynthesis inhibitors were also tested in the same study, among which were MPA (EC<sub>50</sub> = 0.11 μM), brequinar (EC<sub>50</sub> = 0.08 μM) and 6-azauridine (EC<sub>50</sub> = 0.98 μM). The latter two compounds inhibit dihydroorotate dehydrogenase (DHODH) and orotidylic acid (OMP) decarboxylase, respectively, which are two enzymes involved in pyrimidine biosynthesis [113]. It is important to highlight that several nucleotide biosynthesis inhibitors suppress viral growth through innate immunity [109]. In a study by Lucas-Hourani and co-workers, the authors showed that pyrimidine deprivation, using brequinar and another compound DD264, is not directly responsible for the antiviral activity of pyrimidine biosynthesis inhibitors but it rather involves the induction of a metabolic stress and the subsequent triggering of the cellular immune response [109]. Barrows and co-workers also tested MPA, which presented potent anti-ZIKV activity, inhibiting ZIKV infection in HuH-7, HeLa, JEG3, hNSC and HAEC cells [114]. 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 *et al.* [115] identified 6-azauridine (EC<sub>50</sub> = 2.3 ± 0.1 μM) and another pyrimidine biosynthesis inhibitor: 5-fluorouracil (EC<sub>50</sub> = 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.* [116]. Although the 'pregnancy categories' labeling system is being replaced [117,118], it is worth noting that these compounds were generally classified in pregnancy category D by the FDA [114], and thus have presented 'positive evidence of human fetal risk'. This is not unexpected, because 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 [119] and membrane structure of infected cells [120].

DENV infection, for example, is known to induce dramatic relocalization of the fatty acid synthase (FAS) to the sites of viral replication [121,122]. 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-hydroxy-3-methylglutaryl-CoA (HMG-CoA) synthase and squalene synthetase were found to be important for efficient replication of DENV in A549 and K562 cell lines [123].

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 ( $EC_{50} = 20.7 \pm 8.6 \mu\text{M}$ ) [115]. Its antiviral activity was previously reported in HCV [124] and DENV [123]. Moreover, Sarkey and co-workers demonstrated that lovastatin attenuated nervous system injury in an animal model and could be used in inflammatory peripheral nerve diseases, including Guillain–Barré syndrome, which can be a consequence of ZIKV infection [125]. 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 [126]. Mevastatin also had anti-ZIKV activity at concentrations of 1–5  $\mu\text{M}$ , but it was not dose-responsive at concentrations above 5  $\mu\text{M}$  [113]. Mevastatin is known to induce apoptosis [127], 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  $\mu\text{M}$ , respectively) [128]. 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 [129]. Early studies have established NDGA as a 5-lipoxygenase inhibitor [130], but it has been shown to bind several other molecular partners, such as glucose transporter 1 (GLUT1) [131], tyrosine kinases [132,133] and even transthyretin [134]. The molecular mechanisms and clinical applications of these compounds have been thoroughly reviewed by Lü and co-workers [135].

#### 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. Because 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 [136]. Tang and co-workers have carried out a transcriptome analysis of human neural progenitor cells (hNPCs) and enrichment analysis of their supplementary data indicates that several of the upregulated genes are related to protein kinase activity [137].

Xu *et al.* 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  $\mu\text{M}$  [138]. 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 [138]. The authors concluded that these results suggest that one or more host CDKs might be important for ZIKV replication, because flaviviruses are not known to encode any CDK [138].

AXL (from the Greek word *anexelekto*, or uncontrolled) is a tyrosine kinase receptor (TKR) thought to mediate viral attachment to the host cells [139,140]. Its function is also responsible for a downregulation of interferon production [141]. Although this recognition might be unrelated to its kinase activity, AXL inhibitors were found to inhibit ZIKV infection rates. Rausch *et al.* 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 TKRs and, among these, two AXL inhibitors [48]. 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 [48]. This hypothesis is corroborated by an *in vivo* study [142].

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) [143]. Bekerman and co-workers 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  $\mu\text{M}$ , respectively) [143]. They present several lines of evidence that 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 [143].

Monel *et al.* described in-depth the vacuolization of ZIKV-infected cells and suggested that they undergo a paraptosis-like death [144], which is associated with the activity of phosphoinositide 3-kinase (PI3K) [145] and membrane-associated protein kinase (MAPK) [146]. 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 (tricitriline) inhibitors. However, none of the tested inhibitors blocked ZIKV infection [144].

#### Protein metabolism disruptors

Viral replication extensively hijacks the ER 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 [147]. 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 ER membrane complex (EMC),  $\alpha$ -glucosidase, cyclophilin and proteasome elements. Some inhibitors have been investigated under this context. EMC, an ensemble of nine components that are thought to assist protein folding, has been shown to be upregulated in ZIKV-infected cells. Several proteins of this complex are multi-pass membrane proteins [148]. Another key player related to protein metabolism is  $\alpha$ -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 retro-translocated back to the cytosol, ubiquitinated and degraded by the proteasome [149].  $\alpha$ -Glucosidase inhibitors exhibit broad antiviral activity against multiple genera [150,151], 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 ( $EC_{50} > 50 \mu\text{M}$  in both cases) [113].

Xin and co-workers have carried out a quantitative proteomic analysis of C6/36 cells infected with ZIKV and found upregulated genes from two protein-related pathways: the ubiquitin-proteasome system (UPS) and the unfolded protein response (UPR) [152]. They subsequently tested the dose-dependent effect of bortezomib ( $EC_{50} = 5.525 \text{ nM}$ ) and MG132 ( $EC_{50} = 1.151 \mu\text{M}$ ), 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 [152]. Bortezomib was also detected in one of the previous screens, but it showed moderate toxicity in the hNSC cell line, as expected based on its mechanism and primary use as an anticancer agent [114].

Cyclophilins are cytoplasmic proteins responsible for the isomerization of proline peptide bonds from the trans to cis conformation, thus facilitating protein folding [153]. Their activities were shown to be essential for HIV and HCV replication. Cyclosporine A targets cyclophilins and was found to inhibit HCV [154], WNV, DENV and YFV infections [155]. Barrows *et al.* detected cyclosporine as a potential hit but it displayed controversial results, even enhancing the infection at  $1 \mu\text{M}$  compared with the control [114].

#### Endocytosis and 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. So that these steps can occur, the E protein must undergo conformational changes and expose its fusional loop [156]. Li and co-workers identified that 25-hydroxycholesterol (25HC) is probably 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 (i) reduce viremia in mice and non-human primates and (ii) protect mouse embryos from microcephaly [157].

The antimalarial drug chloroquine is known to exhibit broad-spectrum antiviral activity [158]. It is a weak base ( $pK_1 = 8.1$ ,  $pK_2 = 10.1$ ), and the pH of lysosomes in the presence of chloro-

quine increases from  $\sim 4$  to  $\sim 6$  [159]. 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 ( $9.82 \pm 2.79 \mu\text{M}$ ), human brain microvascular endothelial cells ( $14.20 \pm 0.18 \mu\text{M}$ ) and human neural stem cells ( $12.36 \pm 2.76 \mu\text{M}$ ). 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  $134.54 \mu\text{M}$ ). Moreover, chloroquine partially reversed morphological changes induced by ZIKV in mouse neurospheres [160]. Chloroquine was found to reduce viral burden in the placenta of ZIKV-infected C57BL/6 pregnant mice [161]. However, it also showed controversial results in another study [113]. Quinacrine ( $EC_{50} = 2.27 \pm 0.14 \mu\text{M}$ ), mefloquine ( $EC_{50} = 3.95 \pm 0.21 \mu\text{M}$ ) and GSK369796 ( $EC_{50} = 2.57 \pm 0.09 \mu\text{M}$ ), other antimalarial drugs with a similar mechanism of action, were also recently tested against ZIKV [162].

The anticancer drug obatoclax promotes rapid neutralization of lysosomal pH, showing activity against YFV ( $EC_{50} < 0.125 \mu\text{M}$ ), WNV ( $EC_{50} = 0.10 \pm 0.04 \mu\text{M}$ ) and ZIKV ( $EC_{50} = 0.13 \pm 0.01 \mu\text{M}$ ). The authors suggest that the anticancer and antiviral activities are independent and rely on different mechanisms [163]. Obatoclax was also tested in retinal pigment epithelial (RPE) cells and displayed an  $EC_{50}$  value of  $0.04 \pm 0.01 \mu\text{M}$  in the recovery of infected-cell viability [164]. In addition, it was also detected in a drug screen in Jeg-3 cells with an  $EC_{50}$  value of  $0.08 \mu\text{M}$  [48].

Saliphenylhalamide (Saliphe) is another known viral entry blocker, which acts through the inhibition of the vacuolar ATPase [164,165]. It was tested in Vero 76 cells, yielding an  $EC_{50}$  value of  $0.62 \mu\text{M}$  for MR766 strain and  $0.49 \mu\text{M}$  for the PRVABC59 strain. It was also tested by Kuivanen *et al.* in RPE cells, yielding an  $EC_{50}$  value of  $0.05 \pm 0.02 \mu\text{M}$  [164]. Niclosamide blocks the acidification of endosomes, albeit using a different mechanism that is not yet fully elucidated [166,167]. It was detected in a screen against ZIKV in SNB-19 cells showing an  $EC_{50}$  value of  $0.37 \mu\text{M}$  based on the measurement of intracellular viral RNA [138]. 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 could be promising targets to ZIKV drug discovery (see supplementary material online). Moreover, ZIKV inhibitors with an unclassified or unconfirmed mechanism of action are also discussed (see supplementary material online).

#### 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 [168]. 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 [169]. We also present and discuss the available screening assays for anti-ZIKV hit discovery (see supplementary material online).

## 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 for this flavivirus, nor vaccines to prevent it. In a relatively short time we have gone from having no structures of proteins for this virus to a wealth of data. Owing to the current state of known anti-ZIKV compounds, there is probably some way to go until clinical trials of the discovered candidates are undertaken, especially when considering that some patients, including immunosuppressed and pregnant women, will have underlying medical conditions. In this regard, one strategy to overcome the risk of teratogenesis is the development of new drugs to treat Zika based on two-stage Phase II clinical trials: first-stage focusing on testing the efficacy in nonpregnant patients and the second-stage focusing on pregnant women. These will possibly reduce the overall costs of trials, facilitating their logistical set up and enhancing the safety level for the patients [170].

Many of the compounds are clearly unsuitable for these patients. Moreover, one of the frequent issues we see with many of the published screens is that they used unrealistic concentrations (e.g., 100  $\mu$ M) or compounds (e.g., cytotoxic anticancer compounds) that could never be used in pregnant women. Much of the work described herein would have benefited from having experienced medicinal chemists involved to advise on the utility of compounds selected. This would also help to avoid compounds like PAINS [47], 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 crucial to

advancing the fight against ZIKV but they can also be useful for the next emerging virus outbreak to 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 in future.

## Conflicts of interest

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

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <https://doi.org/10.1016/j.drudis.2018.06.014>.

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