Therapeutic Approaches for Zika Virus Infection of the Nervous System

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Abstract Zika virus has spread rapidly in the Americas and has caused devastation of human populations affected in these regions. The virus causes teratogenic effects involving the nervous system, and in adults and children can cause a neuropathy similar to Guillain-Barré syndrome, an anterior myelitis, or, rarely, an encephalitis. While major efforts have been undertaken to control mosquito populations that spread the virus and to develop a vaccine, drug development that directly targets the virus in an infected individual to prevent or treat the neurological manifestations is necessary. Rational and targeted drug development is possible since the viral life cycle and the structure of the key viral proteins are now well understood. While several groups have identified therapeutic candidates, their approaches differ in the types of screening processes and viral assays used. Animal studies are available for only a few compounds. Here we provide an exhaustive review and compare each of the classes of drugs discovered, the methods used for drug discovery, and their potential use in humans for the prevention or treatment of neurological complications of Zika virus infection.

Keywords Zika virus · Drug discovery · Antiviral agents · Microcephaly · Congenital Zika syndrome · Guillain-Barré syndrome

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Introduction

Zika virus (ZIKV) was discovered incidentally in 1947 during a yellow fever virus study in a Rhesus macaque monkey in the Zika forest of southern Uganda [1]. Historically, human ZIKV infections remained limited to sporadic cases in equatorial Africa and Asia with only 1 small cluster of cases in Indonesia reported in 1977 [2]. Consequently, the virus did not present a major public health concern and remained largely obscure within the medical community.

In 2007, physicians on the island of Yap in Micronesia were confronted with an epidemic form of fever, conjunctivitis, rash, and arthralgia reminiscent of dengue virus (DENV) infection [3]. Additional testing at the Centers for Disease Control and Prevention (Fort Collins, CO, USA) identified ZIKV as the causative agent of the epidemic. In 2014, a second ZIKV outbreak in French Polynesia affected approximately 32,000 patients [4]. During the same period, a potential association between ZIKV infection and Guillain-Barré syndrome (GBS) was reported, challenging the then widely held belief that ZIKV infection was a relatively mild, self-limiting disease [5]. Of even greater concern, ZIKV infection in pregnant women may have caused a 20-fold increase in the incidence of newborns with microcephaly in Brazil from 2014 to 2015 [6]. Despite the lack of definite proof of a causal relationship, the extent of the epidemic and the severity of the associated birth defects prompted the World Health Organization to declare ZIKV a “Public Health Emergency of International Concern” on 1 February 2016 [7]. Subsequently, there has been an explosion in public and scientific interest in understanding ZIKV infection and mitigating its detrimental effects. In this review, we describe the viral life cycle in the context of therapeutic targets and describe the approaches taken for the discovery of compounds capable of preventing or treating the neurological complications associated with ZIKV infection.

Viral Structure

ZIKV is an enveloped, positive-sense (+) single-stranded RNA (ssRNA) virus of the Flaviviridae family [8]. The nearly 11-kilobase pair open reading frame encodes 3 structural proteins—capsid (C), the precursor of membrane (prM)/membrane (M), and envelope (E)—and 7 nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) [9], which are essential for viral replication, assembly, and suppression of the host intracellular immune response (Fig. 1) [10]. The (+) ssRNA genome is protected by an icosahedral capsid [11], surrounded by a glycoprotein envelope [12], and prM/M proteins, which are anchored by a lipid membrane [13]. The relative thermostability of the E, as compared with other flaviviruses such as DENV, is thought to contribute to the virulence of ZIKV by helping the virus survive in the unfavorable conditions of saliva, semen, and urine [14].

Fig. 1 Zika virus genome. Nearly 11 kilobase pairs long, the positive-sense, single-stranded (ss) RNA genome encodes 3 structural and 7 non-structural proteins. Structural proteins include precursor of membrane (prM)/membrane (M), envelope (E), and capsid (C) proteins. The viral genome is encased by prM, E, and C, forming an immature virion. Immature virions mature when pr is cleaved from prM. The remaining M, along with E and C, proteins encompass Zika's genome to comprise a mature virion. The open reading frame also encodes nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5), which form a complex to facilitate viral genome replication

Fig. 2 Zika virus life cycle. The mature virion attaches to the host cell membrane receptor protein, undergoes receptor-mediated endocytosis, followed by endosomal membrane-viral envelope fusion, and viral genome release into the host cytoplasm. Zika's positive-sense (+), single-stranded RNA (ssRNA) genome is then translated by host ribosomes at the endoplasmic reticulum (ER) membrane. A polyprotein is formed and cleaved by host and viral proteases. After polyprotein processing, non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) complex to facilitate viral genome replication at the ER membrane. RNA-dependent RNA polymerase (RdRp) on the C-terminus of NS5 transcribes a complementary negative-sense (−) ssRNA strand from the original (+) ssRNA genome, forming double-stranded RNA (dsRNA). Helicase on the C-terminus of NS3 unwinds and remodels the dsRNA, resulting in 2 separate (−) and (+) ssRNA strands. The (−) ssRNA is used as a template by NS5 RdRp to transcribe new (+) ssRNA, forming another dsRNA strand. This new dsRNA is unwound and remodeled by NS3 helicase, forming separate (−) and (+) ssRNA strands. The resulting (−) ssRNA strand is reused to transcribe multiple (+) ssRNA products. Alternately, the newly separated (+) ssRNA strand is capped by methyltransferase on the N-terminus of NS5. The methylated (+) ssRNA assembles at the ER membrane with capsid (C), envelope (E), and precursor to membrane (prM) proteins to form an immature virion, which buds out of the ER into the Golgi apparatus. The immature virion matures in the trans-Golgi network, where furin cleaves pr from prM, resulting in membrane (M) protein. The M, E, and C proteins encase the methylated (+) ssRNA, forming a mature virion, which undergoes exocytosis and egress

Additionally, modification of E glycosylation sites may influence host cell tropism and consequently ZIKV transmission [13].

Flavivirus Life Cycle

Host cell membrane receptors bind the E protein of the mature Zika virion, triggering endocytosis (Fig. 2) [15]. The acidic environment of the endosome induces conformational changes of the E protein, fusion of the host endosome membrane with the viral envelope, and, ultimately, the release of the RNA genome [16]. The ssRNA is translated on the endoplasmic reticulum (ER) membrane into a polyprotein complex, which is cleaved by host and viral proteases in the ER lumen and cytoplasm, respectively (Fig. 3) [17]. The viral protease is comprised of nonstructural proteins NS2B and NS3. Properties of the NS2B–NS3 protease are highly conserved over ZIKV strains, making it a prime therapeutic target [18].

Following post-translational polyprotein processing, the original viral RNA genome is replicated at the ER membrane [19]. Newly processed nonstructural proteins—NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5—form the replication complex to support the following replication course. First, RNA-dependent RNA polymerase (RdRp) on the C-terminus of NS5 transcribes the (+) ssRNA into double-stranded RNA (dsRNA) [20, 21]. Second, the helicase on the C-terminus of NS3 unwinds and remodels dsRNA into separate (+) and negative (−)-sense ssRNA strands [22]. The

(-) ssRNA is shorter than the original (+) ssRNA template, as it lacks a methylated cap; this (-) ssRNA strand is then transcribed into dsRNA by NS5's C-terminus RdRp [20]. The second round of dsRNA is then unwound [21] and remodeled by NS3's C-terminus helicase, producing individual (-) and (+) ssRNA strands [22]. The resulting (-) ssRNA may then be reused to transcribe multiple (+) ssRNA strands. The newly

synthesized (+) ssRNA is subsequently methylated by the methyltransferase on the N-terminus of NS5, in preparation for virion assembly [20].

The methylated (+) ssRNA, C, E, and prM proteins assemble to form immature virions in the ER [23]. Immature virions have a diameter of approximately 60 nm with the C protein on the internal side of the lipid membrane and a spiky surface

Fig. 3 Therapeutic targets in the viral life cycle. (1) NS2B–NS3 protease inhibitors prevent cleavage of the viral polyprotein. (2) NS5 RNA-dependent RNA polymerase inhibitors prevent transcription of the positive-sense (+) single-stranded RNA (ssRNA) into double-stranded

RNA (dsRNA). (3) NS3 helicase inhibitors prevent the unwinding of dsRNA and thus the release of negative-sense ssRNA. (4) NS5 methyltransferase inhibitors prevent methylation of the (+) ssRNA necessary for virion assembly. C = capsid; pr = precursor; M = membrane; E = envelope

with 60 trimetric projections of E and prM heterodimers [8]. The immature virions bud out of the ER, into the Golgi apparatus, and then mature in the trans-Golgi network. The low pH of the trans-Golgi network induces a reorganization of the spiky E–prM heterodimer on the immature virion into a

herringbone-like array of E homodimers parallel to the viral surface [24, 25]. This structural reorganization induces cleavage of the prM by the host protease, furin, maturing the virus, reducing its diameter to approximately 50 nm, and inducing exocytosis [26].

Pathogenesis and Clinical Presentations

ZIKV is primarily transmitted to human hosts by the *Aedes* genus mosquito vector during blood feeds [27]. In addition to blood, ZIKV has been isolated from human urine [28], saliva [29], breast milk [30], cervical mucus [31], and semen [32]. Both male-to-female and female-to-male sexual ZIKV transmission has been reported [33, 34]. Transmission may also occur vertically as ZIKV infection during pregnancy may cross the placenta and cause symptoms in the developing fetus [35]. Finally, ZIKV may also be transmitted through blood transfusion [36].

Most cases (75–80%) of ZIKV infection are subclinical [37]. Of the symptomatic cases, the typical clinical presentation is a nonspecific, self-limiting flu-like syndrome with myalgias, arthralgias, fever, headache, rash, conjunctivitis, and fatigue [38, 39]. Symptoms are similar to other viral infections, such as DENV and West Nile virus (WNV) fever. These viruses are spread by the same vectors, in the same endemic areas as ZIKV, rendering ZIKV diagnosis based on clinical grounds alone challenging [40, 41].

Growing evidence indicates that ZIKV infection may be linked to severe neurological complications, such as encephalitis [42], myelitis [43], macular atrophy [44], GBS [5], and microcephaly [45, 46]. ZIKV-related GBS, first described in the 2013 French Polynesian outbreak, is an acute polyradiculoneuropathy with transient or permanent flaccid paralysis and loss of motor function [5]. Symptoms begin distally with variable proximal progression [5]. Death secondary to GBS may ensue as a result of respiratory failure, pneumonia, sepsis, or (less frequently) autonomic dysfunction and pulmonary embolism [47].

In the 2015 Brazilian outbreak, cases of ZIKV infection of pregnant women were linked to microcephaly in the developing fetus. ZIKV has since been confirmed as the causal agent. In addition to microcephaly, additional fetal neural anomalies caused by ZIKV have been characterized, and the overall phenotype is now termed congenital Zika syndrome [48]. Despite the severe neuropathology associated with ZIKV infection, no effective vaccines or therapeutic interventions are currently available for clinical use [49, 50].

Drug Discovery: Viral Targets

Attachment, Endocytosis, and Fusion

Although antigenic variability between different strains, and even within the same strain, of ZIKV may pose as a drug design hurdle [8, 13], some noteworthy inhibitors of viral attachment, entry, and fusion have surfaced. Compounds, such as duramycin (Fig. 4a), may block viral entry by preventing viral attachment to host cell receptors [51, 52]. T-

cell immunoglobulin and mucin receptors are transmembrane proteins that mediate flavivirus entry into host cells by binding phosphatidylethanolamine on enveloped virions [51, 53]. Duramycin is a 19-amino acid cyclic peptide, which specifically binds phosphatidylethanolamine, prevents T-cell immunoglobulin and mucin interactions, and blocks infection of DENV [51]. Duramycin used at concentrations $\geq 0.2 \mu\text{M}$ blocked ZIKV (MR766/Uganda 1947)-mediated infection of placental trophoblast progenitor cells, human placental fibroblasts, and human umbilical vein endothelial cells [52]. In chorionic villus explants from first-trimester placentas, $1 \mu\text{M}$ duramycin blocked infection. No *in vivo* studies have been published to date.

Another potential ZIKV entry inhibitor, nanchangmycin (Fig. 4b), was suggested to act by blocking clathrin-mediated endocytosis. Nanchangmycin, a polyether and natural product of *Streptomyces nanchangensis*, was identified from a library of approximately 2000 compounds using an infection assay in U2OS human osteosarcoma cells [54]. Nanchangmycin yielded an IC_{50} value of $0.1 \mu\text{M}$. The assay was repeated in human brain microvascular endothelial cells, which were used as a model for human blood–brain barrier (BBB) cells; nanchangmycin demonstrated an IC_{50} value of $0.4 \mu\text{M}$. No *in vivo* studies evaluating nanchangmycin's antiviral activity against ZIKV have been published.

Once ZIKV is endocytosed, viral E-glycoproteins interact with the acidic environment of the endosome, initiating fusion of the viral envelope with the host endosomal membrane [8]. The E-glycoprotein hinge domain has a hydrophobic η -octyl- β -D-glucoside (β -OG) pocket, which is thought to be conserved across several flaviviruses, including DENV, TBEV, and WNV [55–57], and could serve as a potential ZIKV target. The DENV β -OG was used for *in silico* docking screens [25]. After a 2-tiered screening approach of the 135,000 compounds in the Maybridge database, approximately 67 compounds were selected that demonstrated a 30% greater affinity for the β -OG pocket than β -OG itself. Of the hits, 5 (compounds A1–A5) were chosen for subsequent *in vitro* studies based on structural class (all 5 have chains of aromatic rings), commercial availability, and predicted toxicity, solubility, and ability to be synthetically modified. Compound A5 (Fig. 4c), a phenyl hydrazone derivative, demonstrated the greatest antiviral activity by a plaque assay in Vero cells against multiple flaviviruses: DENV ($\text{IC}_{50} 1.2 \pm 0.7 \mu\text{M}$); WNV ($\text{IC}_{50} 3.8 \pm 2.9 \mu\text{M}$); and yellow fever virus ($\text{IC}_{50} 1.6 \pm 1.5 \mu\text{M}$). Generic virucidal screens using respiratory syncytial virus resulted in IC_{50} values $> 100 \mu\text{M}$ for these compounds, suggesting compound A5's antiviral activity is flavivirus specific.

Drug screens of DENV E-glycoprotein inhibitors identified compound 6, a thiophene–quinazoline derivative [58].

Fig. 4 Structures of attachment, endocytosis, and fusion inhibitors: (a) cyclic peptide duramycin; (b) natural product nanchangmycin; (c) phenyl hydrazone derivative compound A5; (d) thiophene-quinazoline derivative compound 6; (e) oxysterol 25-hydroxycholesterol; and (f) antiparasitic suramin

Treatment with compound 6 at $\geq 2.5 \mu\text{M}$ completely inhibited viral growth in A549 cells. However, while ZIKV and DENV E-glycoproteins are similar, their hinge domain angles are slightly different [14]. Despite positive findings with DENV, antiviral activity of compounds 6 and A5 have yet to be examined for ZIKV.

A synthetic peptide inhibitor, Z2, was developed from ZIKV E protein (residues 421 – 453) [59]. Z2 inhibited ZIKV infection in BHK21 and Vero cells with IC_{50} values of 1.75 and $3.69 \mu\text{M}$, respectively. Z2 (10 mg/kg i.p. injections) inhibited vertical transmission of ZIKV in pregnant mice. Intraperitoneal Z2 injections also protected type I and type I/II interferon (IFN)-deficient mice against lethal ZIKV challenge. While *in vivo* experiments demonstrated no production of endogenous antibody; the necessity of intraperitoneal injections and its relatively short half-life (2.8 h) may pose challenges for development [59].

Antiviral activity against ZIKV was explored *in vitro* and *in vivo* for natural oxysterol, 25-hydroxycholesterol (25HC) [60]. Cholesterol-25-hydroxylase is an IFN-stimulated gene that encodes the enzyme responsible for the oxidation of cholesterol to 25HC (Fig. 4e). 25HC is thought to inhibit ZIKV entry into host cells *in vitro*. Pretreatment of Vero cells with 25HC protected them from infection with ZIKV (GZ01/2016 strain). The

inhibitory effect on cells treated postinfection was less profound, suggesting that 25HC affects ZIKV early in the viral life cycle. Cell-binding assays revealed that 25HC did not block ZIKV from binding the host cell, but internalization assays demonstrated that 25HC inhibits ZIKV uptake. Cells were transfected with ZIKV RNA to perform replicon assays; no inhibition was observed when 25HC was added 6 h post-transfection. Treatment of human cortical organoids with $2.5 \mu\text{M}$ 25HC prior to infection with ZIKV (PRVABC59/2015 strain) reduced ZIKV genomic RNA by 90% [60].

Three- to 4-week-old immunocompetent BALB/c mice were pretreated with 50 mg/kg 25HC for 12 h and then inoculated with 10^5 plaque-forming units of ZIKV (GZ01/2016) intraperitoneally [60]. ZIKV RNA in serum was measured using quantitative reverse transcription polymerase chain reaction (PCR) 1 day post-infection (dpi) and found to be significantly reduced in the treated cohort. Rhesus monkeys infected with 10^3 plaque-forming units of ZIKV and treated with 1.5 mg/kg/day intravenous 25HC were protected against robust viremia as compared with untreated controls. Additionally, *in vivo* studies revealed 25HC is protective against ZIKV-induced symptoms, such as fever in Rhesus monkeys and microcephaly in A129 mice [60];

however, 25HC has not yet been tested in clinical trials. Owing to the need for parenteral administration, its therapeutic potential may be limited.

Suramin (Fig. 4f) is an Food and Drugs Administration (FDA)-approved antiparasitic with antiviral activity against flaviviruses [61–63]. Antiviral activity of suramin against ZIKV shows that relatively high concentrations of 50 to 200 μM are needed to inhibit ZIKV infection in Vero cells. Cell-bound ZIKV was reduced by 50% when pretreated with 25 μM suramin, suggesting suramin inhibits viral attachment. Suramin may also affect ZIKV late in the life cycle, possibly by inhibiting the release of ZIKV progeny. Additionally, *in silico* studies reveal suramin binds NS3 helicase with greater affinity than ZIKV envelope proteins, which may suggest suramin also inhibits ZIKV replication [64]. Although published findings suggest suramin interacts with multiple ZIKV targets and may inhibit viral entry, replication, and release, no *in vivo* data have been published to date. Owing to the high concentrations needed for antiviral activity, suramin might not be a viable therapeutic option.

NS2B–NS3 Protease

Enzymes that catalyze peptide bond cleavage to facilitate protein digestion are present in almost every organism [65]. These proteolytic enzymes, called proteases, utilize different amino acids at their active site, display a preference for specific peptide sequences, and have evolved to function under many different conditions. They are involved in diverse physiological processes ranging from cell death (caspases) to the blood coagulation cascade (thrombin, factor X) [65]. Owing to their specificity and involvement in many vital pathways, proteases have been a highly pursued drug target [66, 67].

Viruses encode their own essential proteases that can be targeted for therapeutic intervention [67]. Protease inhibitors have been a fundamental component of human immunodeficiency virus (HIV) therapy since saquinavir was approved in 1995 [68]. The first FDA-approved, direct-acting antivirals against hepatitis C virus (HCV) were the NS3–NS4A protease inhibitors boceprevir and telaprevir [69]. Viruses within the *Flavivirus* genus encode a serine protease, located at the N-terminal portion of its NS3 domain, which is necessary for the cleavage of the single viral polyprotein into 3 structural proteins and 7 nonstructural proteins (Fig. 3). Like the NS4A co-factor of the HCV protease, without the NS2B co-factor the *Flavivirus* NS3 is inactive [70]. The NS2B–NS3 protease is well conserved across the genus and no mutations have been acquired in the ZIKV protease sequence since 1967, making it a prime viral target [71].

A model system for studying [72] and crystallizing [70] the flavivirus NS2B–NS3 protease utilizes a noncleavable, flexible linker (Gly₄–Ser–Gly₄) between the NS2B and NS3 portions. In general, when using this construct for flavivirus

crystallization, the ligand-free form (apo) is in an *open* conformation [73]. In contrast, when a ligand is bound, the NS2B becomes more ordered and wraps around NS3, in the *closed* conformation [70]. A third, *preopen* conformation, not seen with other flavivirus proteases, was also observed for the ZIKV protease [74]. In this structure, not only is NS2B disordered, but the C-terminus of NS3 is also oriented away from the active site.

Taking advantage of the activity of this construct, an enzymatic assay was used to define the substrate specificity of the ZIKV NS2B–NS3 protease. Gruba et al. [75] screened a library of natural amino acids using a Förster resonance energy transfer-based substrate approach. The tetrapeptide ABZ–Val–Lys–Lys–Arg–ANB–NH₂ was the most efficiently cleaved sequence with a $k_{\text{cat}}/K_{\text{M}}$ of $7.76 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$ in a Tris–CHAPS–glycerol buffer at pH 8.5. The choice of buffer conditions was essential in the design of these biochemical assays; activity was reduced 33-fold when the assay was performed in phosphate-buffered saline at pH 7.4. Rut et al. [76] used a hybrid combinatorial substrate library, which includes unnatural amino acids. Little variability was tolerated at the P1 position: L-Arg and L-Arg (Me) were preferred. The P4 position tolerated large variation and D-amino acids were preferred [76]. The optimal P4–P1 sequence, DArg–Lys–Orn–Arg, was used to develop an activity-based probe with a phosphonate warhead and a biotin detection tag (Fig. 5a). These compounds may aid in the rational design of active site inhibitors and could be used in small-molecule screening campaigns.

Lei et al. [18] solved the first crystal structure of the ZIKV protease. The linked NS2B–NS3 protease was bound to a boronate inhibitor, cn-716 (Fig. 5b), in the *closed* conformation. In this structure, the catalytic serine was covalently linked to the boron atom of the inhibitor. The amino group at the P2 position of the inhibitor formed a salt-bridge with an NS2B aspartic acid residue that is not present in other flavivirus protease sequences. Compared with the WNV protease, the ZIKV protease displayed increased activity towards a pan-flavivirus substrate (Bz–Nle–Lys–Lys–Arg–AMC). The authors suggest the observed hyperactivity may be due to the interaction of the unique aspartic acid residue and the amine at the P2 position in the substrate. The FDA-approved proteasome inhibitor bortezomib [77, 78] contains a boronic acid functional group [79]; however, bortezomib can cause severe side effects [77] and its use is hindered by dose-limiting toxicity [78]. The FDA lists bortezomib as a class D drug that is not recommended for use in pregnant patients [78]. Therefore, while cn-716 may serve as a useful starting point for drug discovery, successful translation to an *in vivo* setting appears unlikely.

Several studies aimed at identifying inhibitors of the ZIKV protease have been initiated with moderate success. Aprotinin (BPTI), a canonical serine protease inhibitor, was an effective

Fig. 5 Structures of NS2B–NS3 protease inhibitors: (a) biotin probe with a phosphonate war-head biotin–Ahx–DArg–Lys–Orn–Arg–PO(OPh)₂; (b) boronate ligand *cn*-716 in PDB 5LC0; (c, d) competitive inhibitors compounds 2 and 3; (e) Food and Drug Administration-class B drug bromocriptine; (f, g) allosteric inhibitors NSC157058 and NSC135618; (h) ligand EN300 from fragment screen in PDB 5H4I; (i) West Nile and Zika virus protease inhibitor compound A

inhibitor of the fusion protein with a K_i of 361 ± 19 nM [73]. Once used in the clinic to mitigate bleeding during surgery, aprotinin has now been replaced owing to toxicity concerns [80]. Nevertheless, it is still frequently used as a positive control for serine protease inhibition assays. A screen of nearly 41,000 compounds led to the identification of 71 compounds active against the NS3–NS4A protease of HCV. These 71 compounds were subsequently screened against the linked NS2B–NS3 protease of ZIKV [74]. Two lead scaffolds, represented by compounds 2 and 3 (Fig. 5c, d), were identified that exhibit IC_{50} values of 5.2 and 4.1 μ M, respectively [74]. No validation of these hits was reported *in vitro* or *in vivo*.

Novobiocin was identified following an *in silico* screen of 8227 compounds (DrugBank database) against the Lei et al. [18] ZIKV NS2B–NS3 crystal structure [81]. Novobiocin blocked protease activity in a fluorescence-based assay ($IC_{50} = 14.2$ μ g/ml) and inhibited ZIKV infection in Vero ($IC_{50} = 42.63$ μ M) and Huh-7 cells ($IC_{50} = 62.24$ μ M). Kinetic experiments suggested novobiocin competitively inhibits ZIKV protease. Molecular dynamics simulations predicted a direct interaction between novobiocin and the catalytic HIS51 residue of the NS2B–NS3. Novobiocin significantly reduced ZIKV titers when used within 12 h postinfection (hpi). Dexamethasone-immunosuppressed mice treated with novobiocin (100 mg/kg q12h) from 1 to 13 dpi

exhibited a 100% survival rate, whereas untreated negative controls demonstrated 0% survival. Novobiocin is FDA approved, a pregnancy category C drug (considered for clinical use if benefits outweigh side effects), and has IC_{50} values that are achievable by oral dosage; however, it has not yet been tested in clinical trials to treat ZIKV and penetration into central nervous system (CNS) through noninflamed meninges may be limited [81].

Chan et al. [82] identified bromocriptine (Fig. 5e) as an inhibitor of Vero cell death caused by ZIKV PRVABC59. Antiviral activity of bromocriptine was confirmed by a reduction in plaque-forming units. Molecular modeling predicted interaction of bromocriptine with the Zika protease and was therefore tested in an enzymatic assay. While protease inhibition was observed, it does not appear to be the only mechanism of action. The IC_{50} was higher against the protease (22 μ M) than in the viral assay (13 μ M) [82]. Bromocriptine carries a class B pregnancy label and was first approved by the FDA for hyperprolactinemia-associated dysfunctions, acromegaly, and Parkinson disease; it later gained approval for glycemic control in patients with diabetes [83]. Based on its oral availability and documented use as a fertility treatment (and therefore its use in early pregnancy) [84], further investigation into the mechanism of action and evaluation of efficacy *in vivo* may be warranted. However, the high concentrations

needed for its antiviral activity may be difficult to achieve clinically.

An allosteric inhibitor of the ZIKV protease with an IC_{50} of 0.82 μ M, NSC157058 (Fig. 5f) was identified from several compounds with activity against the WNV protease [85]. Compounds were incubated with the linked protease for 30 min prior to the addition of 20 μ M substrate (Pyr-RTKR-AMC) and fluorescence was monitored continuously. The 3 best hits were tested for their ability to inhibit ZIKV infection in neural progenitor cells (NPCs). Staining for ZIKV envelope was monitored 4 dpi. Treatment of NPCs with NSC157058 inhibited ZIKV infection (FSS13025, multiplicity of infection 0.1) with an IC_{50} of 50 μ M. Administration of 30 mg/kg/day to ZIKV-infected SLJ mice through their drinking water resulted in a 10-fold reduction in plasma viremia by PCR. While these results demonstrate the potential for an orally available protease inhibitor, this compound is limited by its rapid plasma clearance, displaying a half-life of only 20 min. No information was reported on CNS penetration; however, its highly polar nature suggests it may not cross the BBB [85].

A conformational switch assay, based on split luciferase complementation, was developed to identify compounds that inhibit formation of the *closed* conformation [86]. A virtual screen of the NCI diversity set library II was performed to narrow the candidate compounds for *in vitro* testing. The best 29 compounds were tested for inhibitory activity against the DENV protease. Compound NSC135618 (Fig. 5g) also inhibited ZIKV protease with an IC_{50} of 0.38 μ M. Activity was confirmed by inhibition of ZIKV PRVABC59 in A549 cells; NSC135618 reduced immunofluorescence for ZIKV envelope and production of viral transcripts by reverse transcriptase PCR. Furthermore, dose-dependent inhibition of ZIKV was observed in human placental epithelial cells and neural stem cells, with IC_{50} s of 1 μ M and 2 μ M, respectively. A protein thermal shift assay confirmed the direct binding of NSC135618 to the linked and unlinked DENV protease [86]. Further studies evaluating the activity of NSC135618 derivatives, specificity over host proteases, and *in vivo* efficacy are needed. As no *in vivo* data are available, a thorough study of all toxicity and pharmacokinetic properties would be necessary for further development.

The potential for confounding effects introduced by the artificial linker has spurred the study of alternative constructs. By fusing NS2B and NS3 using the native, cleavable amino acid sequence as a link (NS2B_{45–96;126–130}–NS3_{1–177}), a crystal structure was obtained exposing the postproteolysis state [87]. In this structure, the final 4 amino acids (TGKR) of co-factor NS2B are bound at the active site in the closed conformation. In a separate report, unlinked ZIKV protease was prepared using separate promoters for NS2B and NS3 [88]. The crystal structure of this binary complex was in the closed conformation, whether an inhibitor was present (PDB 5H4I;

Fig. 5h) or not (PDB 5GPI), causing the authors to question the biological relevance of the open conformation [88]. Solution nuclear magnetic resonance studies indicated the linker caused an increased preference for the open conformation [89]. Furthermore, in 2 independent studies the unlinked construct demonstrated heightened enzymatic activity compared with the linked construct [87, 90]. When a compound known to inhibit WNV (Fig. 5i, compound A) was tested against both constructs, however, the IC_{50} values were equivalent (~1 μ M) [90].

There is a lack of promising lead compounds published as ZIKV protease inhibitors. Several peptidic inhibitors have been identified against WNV and DENV proteases with activity in the low nanomolar range; however, activity has not translated well to *in vitro* viral inhibition [91]. Identifying a potent *flavivirus* protease inhibitor that is also bioavailable and can cross the cell membrane has been difficult because the active site of the NS2B–NS3 is shallow and highly charged. Additionally, the cleavage preference is for dibasic amino acids, complicating efforts towards the rational design of noncharged inhibitors [91]. Given the previous successes of protease inhibitors as antiviral agents, efforts to overcome these challenges and develop a therapeutically useful protease inhibitor will likely continue.

NS5 RdRp

The C-terminal domain of the flavivirus NS5 protein contains the RdRp, a heavily pursued target for antiviral drug discovery owing to its essential role in the viral life cycle. The design of analogs of the natural nucleoside and nucleotides has been the focus of drug development (Fig. 6a). Even though there is no mammalian homologue for RdRp, toxicity has caused the failure of many polymerase-targeting compounds late in clinical development [92]. Finally, in 2013, a phosphoramidate nucleotide prodrug, sofosbuvir (Fig. 6b), which is a uridine analogue, was approved for clinical use [93]. Sofosbuvir's addition to the market changed the landscape of treatment options for patients with HCV, demonstrating the utility of a successful RdRp inhibitor with limited side effects [94].

Owing to the similarities between the HCV and ZIKV RdRp, sofosbuvir was one of the first compounds tested for anti-ZIKV activity [95, 96]. Sofosbuvir is a class B FDA-approved drug, meaning animal studies have not demonstrated a risk when used in pregnancy; however, controlled human trials are not yet available. Sofosbuvir triphosphate, the biologically active compound formed *in vivo* (Fig. 6b), can inhibit ZIKV RdRp with IC_{50} s < 1 μ M [96]. The efficacy of sofosbuvir was subsequently tested in several cell lines where viral replication was quantified by a plaque forming assay. Sofosbuvir was about 2 times more effective at inhibiting viral infectivity than viral RNA levels. Activity was cell line-dependent, with sofosbuvir displaying the best activity in Huh-7

Fig. 6 Conversion of NS5 polymerase nucleosides to nucleotides *in vivo*. (a) Example of a natural adenosine phosphorylation process. (b) Sofosbuvir does not have to undergo the first, slow step because the phosphate group is built into its structure

cells ($EC_{50} = 0.12 \mu\text{M}$), good activity in BHK-21 and SH-Sy5y cells ($1.7 \mu\text{M}$ and $0.65 \mu\text{M}$, respectively), and no activity in Vero cells ($> 50 \mu\text{M}$). Additionally, sofosbuvir inhibited viral production in NPCs and brain organoids [96]. Reports from other research groups confirm these findings in neuroepithelial stem cells [97], human placental carcinoma cells, and C57BL/6 mice [50].

Collectively, the reported data may suggest that sofosbuvir should be investigated in additional animal models and potentially in a clinical trial; however, the price of sofosbuvir could be prohibitive. In the USA the cost for a 12-week course treatment is around \$65,000. Even with significant discounts given to developing countries, the reduced cost in Brazil is still close to \$7000 [98].

To characterize the selectivity of the ZIKV RdRp, a nonradioactive primer extension assay was developed. Recombinant RdRp was expressed, purified, and used to determine the efficiency of incorporation and chain termination potential for various 5'-triphosphate analogs [99]. 2'-C-methyl and 2'-C-ethynyl substituted nucleotides were efficient inhibitors while adenosine analogs performed the best. Additionally, when testing nucleotide analogs against both DENV and ZIKV RdRp, the discrimination pattern was 2'-O-Me $>$ 2'-C-Me-2'-F $>$ 2'-C-Me (Fig. 7a) [100]. This discrimination preference differed from that of the HCV RdRp, where the 2'-C-Me-2'-F (that of sofosbuvir) was preferred.

The 2'-C-Me analog, 7-deaza-2'-C-methyladenosine (7DMA/MK-608; Fig. 7b) failed as an HCV drug but has anti-ZIKV activity. Using triphosphate nucleotide analogs to test anti-HCV compounds for their ability to inhibit recombinant ZIKV RdRp, several compounds, including 7DMA, had IC_{50} s in the single-digit micromolar range [101]. Separately, a

variety of nucleoside analogs were tested for their ability to inhibit ZIKV (MR766) titer in Vero cells. 2'-C-methylated nucleosides, including 7DMA, were the most potent inhibitors [102]. In another report, the authors screened known RNA polymerase inhibitors for a reduction in ZIKV-induced (MR766) cytopathic effects in Vero cells. 7DMA reduced cytopathic effects with an EC_{50} of $20 \mu\text{M}$. In AG129 mice (mice lacking IFN- α/β and IFN- γ receptors) infected with ZIKV, 7DMA reduced viremia, morbidity, and mortality [103]. However, these results must be interpreted with caution, since 7DMA also showed promising results in chimpanzees against HCV but failed in humans for unknown reasons [104].

Antivirals are being repurposed from other diseases, in addition to those from HCV. Owing to its activity as an anti-Ebola compound, BCX4430 (galidesivir; Fig. 7c) [105] underwent a phase I dosing and toxicity clinical trial (NCT02319772) [106]. The toxicity results have not yet been disclosed from this trial; however, the drug was administered by intramuscular injection, which could make at-home administration difficult. Nevertheless, the potential application of BCX4430 against the ZIKV was tested. BCX4430 inhibited cytopathic effects of different strains of ZIKV (MR766, P6-740, and PRVABC-59) in RD, Huh-7, and Vero cells [107]. Additionally, treatment of AG129 mice infected with ZIKV (P6-740) with BCX4430 at 300 mg/kg/day increased survival and body weight and reduced viral RNA in serum.

Another adenosine analog, NITD008 (Fig. 7d), was developed by Novartis as an inhibitor of DENV [108]. Monitoring viral production by a plaque-forming assay and intracellular RNA, NITD008 was shown to inhibit ZIKV infectivity in Vero and BHK-21 cells [109]. Treatment of AG129 mice infected with ZIKV (GZ01/2016) with NITD008 at 50 mg/kg/

Fig. 7 Structures of NS5 polymerase inhibitors: (a) nucleotide analogs 2'-O-Me-adenosine, 2'-C-Me-2'-F-adenosine, and 2'-C-Me-adenosine; (b-d) nucleoside analogs 7-deaza-2'-C-methyladenosine, BCX4430 (galidesivir), and NITD008

day improved survival and decreased peak viremia. While NITD008 can serve as a useful laboratory tool or starting point for drug design, toxicity in other animal models prevented further clinical development [108].

Further investment in identifying RdRp inhibitors for the treatment of ZIKV should consider the lessons learned from the late and costly failures of HCV and DENV antivirals. Compounds that have limited side effects and can be delivered orally should be prioritized. While many nucleoside analogs display potent anti-ZIKV activity *in vitro* and in mice, it is important to test these compounds in additional animals early in development. The prephosphorylated prodrug approach taken with sofosbuvir has proven effective; thus, the development of additional phosphate-protecting groups could be a fruitful avenue to explore.

The rational design of RdRp active site and allosteric inhibitors is possible now that several crystal structures of the ZIKV NS5 protein have been reported. Using the construct NS5_{306–903} from the MR-766 strain, the RdRp was crystallized at 1.9 Å resolution [110]. In this study, it was noted that while the overall structure of the RdRp for all flaviviruses is quite similar, the priming loop, a main target of allosteric inhibitor development, has structural elements that distinguish it from that of DENV. The ZIKV RdRp priming loop is in a closed conformation, more closely resembling those of Japanese encephalitis virus (JEV) and WNV. Three additional published crystal structures that encompass the entire NS5 protein provide relevant information on how the methyltransferase affects the polymerase conformation and activity [111–113].

NS3 Helicase

After the newly synthesized dsRNA is released from the RdRp it must be unwound for translation and further replication [114, 115]. This unwinding is achieved by the helicase enzymes, making them an essential component of nucleic acid metabolism across all lifeforms. Helicases use the energy derived from their nucleotide triphosphatase (NTPase) activity to power the unwinding or remodeling of double-stranded

nucleic acids [116]. The helicase enzyme could be targeted for therapeutic intervention at either the NTP or RNA binding site. In addition to its NTPase and RNA unwinding activity, the ZIKV NS3 also has a third function—preparing the newly synthesized (+) ssRNA for capping by removing 1 phosphate group [114].

Though several crystal structures of the C-terminal NS3 ZIKV helicase are available, limited investment into drug discovery is apparent [117–120]. The NS3 crystal structure was used to computationally identify potential drug binding sites, but no experimental data were provided [119]. Another study solved 2 crystal structures of NS3, 1 bound to ATP–Mn²⁺ and the other bound to RNA. The authors also determined the kinetics of ATP hydrolysis and RNA unwinding but did not investigate potential inhibitors [118]. Resveratrol (150 μM) partially inhibited the ZIKV helicase ATPase activity (Fig. 8a) [121], but no further studies were performed. Lack of continued interest in resveratrol is likely a result of its poor bioavailability and the termination of a phase II clinical trial of a water-soluble formulation owing to toxicity with > grade 3 adverse events in > 50% patients who developed anemia, thrombocytopenia, renal failure, and gastrointestinal manifestations [122].

Ivermectin (Fig. 8b), an FDA-approved antiparasitic, was identified by an *in silico* screen of commercially available compounds against the WNV NS3 helicase [123]. Ivermectin inhibited the helicase unwinding activity of several flaviviruses *in vitro*. As this study was completed prior to the recent ZIKV outbreak, ivermectin was not tested against the ZIKV helicase; however, ivermectin was later identified from a screen of FDA-approved compounds tested for their ability to block ZIKV infection in Huh-7 cells. Furthermore, ivermectin inhibited ZIKV infectivity in human neural stem cells and primary amnion epithelial cells [78]. Despite these promising results, investigation into the ability of ivermectin to inhibit the ZIKV helicase or its activity in an *in vivo* ZIKV model have yet to be reported. It is unclear if ivermectin is safe for use in pregnancy owing to the limited amount of data available. The FDA has applied a class C label owing to possible teratogenic effects in mice; however, studies in sheep show it

Fig. 8 Structures of NS3 helicase inhibitors. (a) ATPase activity inhibitor resveratrol; (b) Food and Drug Administration-approved antiparasitic ivermectin (stromectol); (c) benzoxazole Dengue virus (DENV) helicase inhibitor ST-610; (d, e) DENV ATPase activity inhibitors CID 45382104 and CID 49849289; (f) pritelivir, herpes simplex virus helicase inhibitor in clinical trials

does not accumulate within the fetal compartment [124]. Furthermore, treatment of pregnant women for helminth infection during the second trimester was not correlated with adverse effects [125].

The most potent *flavivirus* helicase inhibitor, the benzoxazole ST-610 (Fig. 8c), was not identified using an assay for helicase activity [126]. Instead, a cell-based screen for inhibitors of DENV infection led to its discovery. It is effective against all serotypes of DENV at an IC_{50} of 0.2 to 0.3 μ M. Sequencing of drug-resistant strains identified the helicase as the target. A single point mutation, A283T, can confer resistance. ST-610 inhibited RNA unwinding activity, but not NTPase activity. Additionally, ST-610 was nontoxic and reduced viremia in a DENV mouse model; however, it showed limited oral bioavailability and rapid clearance [126]. Anti-ZIKV activity has not yet been published.

To bypass the problem of low hit rate frequently encountered in helicase unwinding assays, Sweeney et al. [127] designed an ATP hydrolysis high-throughput screening assay. Screening a focused library of helicase inhibitors led to the identification of 2 classes of compounds. The first, a set of pyrrolones, including CID 45382104 (Fig. 8d), inhibited ATP hydrolysis, but not RNA unwinding. Nevertheless, CID 45382104 inhibited DENV in a subgenomic reporter replicon assay with an EC_{50} of 36 μ M and WNV replication in a plaque-forming assay with an EC_{50} of 22 μ M. The second class included a derivative of the benzothiazole HCV helicase probe ML283, CID 49849289 (Fig. 8e), which inhibited DENV ATPase activity with an IC_{50} of 500 nM. This class of compounds displayed activity in a secondary screen measuring RNA duplex separation inhibition (CID 49849289 had an IC_{50} of 1.5 μ M). CID 49849289 showed inhibition in the DENV replicon assay, but the insolubility of the benzothiazoles above 50 μ M prevented testing in a full virus cell-based assay. In addition to bioavailability concerns, like many other

helicase inhibitors identified, these compounds may be limited by nonspecific binding interactions [128]. No reports on these compounds against ZIKV are available.

The lack of a successful model for the development of helicase inhibitors is likely contributing to the scarcity of reports identifying an inhibitor of the ZIKV helicase. Screens for helicase inhibitors using unwinding assays or an ATPase activity assay have yielded few promising results [116]. The only viral helicase inhibitor in clinical development, pritelivir (Fig. 8f), is directed against herpes simplex virus. Pritelivir was successful in a phase I clinical trial (NCT01047540) and a phase II trial showed pritelivir reduced viral shedding; however, the phase II trial was terminated early owing to concurrent negative results from a nonclinical toxicity study (NCT01658826) [129, 130]. Two additional phase II trials are actively recruiting subjects; 1 trial will evaluate the efficacy of a topical ointment, whereas the other will examine pritelivir in an aciclovir-resistant cohort. Further results are eagerly awaited as they would provide the confidence necessary to establish the helicase as a robust and feasible target.

NS5 Methyltransferase

The addition of a cap structure to the 5' end of most ssRNA is essential for its stability, splicing, transport, and translation [131]. While viruses that replicate in the nucleus can utilize host capping enzymes, many RNA viruses, such as ZIKV, must encode their own. This provides an opportunity for selective therapeutic intervention. The N-terminal portion of the NS5 protein, the methyltransferase, performs two sequential capping events. Methylation at the N-7 position is essential for viral replication and methylation at the 2'-O position helps the RNA evade the host immune response [131].

Prior to the publication of crystal structures of the ZIKV methyltransferase domain, an effort to identify inhibitors was undertaken using molecular modeling [132]. A homology

model was generated using the methyltransferase from Modoc virus as a template and a virtual screen of > 28,000 compounds was performed. The 10 best compounds were tested for their ability to inhibit ZIKV (KF993678.1) production, as determined by plaque formation in Vero cells. Four compounds were found to have EC_{50} s < 25 μ M, with the best 2 compounds having EC_{50} s around 5 μ M and 12 μ M, respectively (Fig. 9a, b) [132].

In addition to the full-length ZIKV NS5 crystal structures available [111–113], there are now many structures of just the N-terminal methyltransferase. Structures representing different stages of the methylation process are available, including the methyltransferase bound to the methyl donor S-adenosylmethionine (SAM; Fig. 9c) (5KQR [133], 5WZ1 [134]); 1 chain of the methyltransferase bound to SAM and the other free (5M5B [135]); and a third with the methyltransferase bound to the product of the methylation reaction, S-adenosylhomocysteine (SAH; Fig. 9d) (5WXB [136]). Together these structures provide insight into the methylation process and guide the design of SAM/SAH analogs.

A ZIKV methyltransferase crystal structure was reported with a SAM analog inhibitor bound to it. Sinefungin (Fig. 9e) was first isolated from *Streptomyces griseolus* and investigated as an antifungal antibiotic [137]. It is a broad inhibitor of both DNA and RNA methyltransferases and capable of inhibiting viral replication [137]. A crystal structure of the

ZIKV methyltransferase complexed to sinefungin was obtained (5MRK [138]). Sinefungin's activity is too broad to be useful as a therapeutic agent but could be used as a starting point for further development.

Other structures have been determined with a nucleotide or nucleotide analog in the RNA binding site. These structures include 1 with SAM and 7-methyl guanosine diphosphate (7-MeGpp; Fig. 9f) bound (5KQS [133]), another with SAM and RNA analog 7-MeGpppA (Fig. 9g) bound (5WZ2 [134]), and a final with SAH and either guanosine triphosphate (Fig. 9h, 5GO2) or 7-MeGpppA bound (5GP1 [139]). Based on the reported crystal structures, a SAM analog (MS2042; Fig. 9i) was designed to occupy both the SAM and 7-MeGpp binding sites [119]. MS2042 was synthesized and incubated with denatured ZIKV methyltransferase (to remove the natively bound SAM), resulting in a high-resolution crystal complex (5ULP). The K_D for SAM binding to the methyltransferase is about 2.6 μ M, whereas MS2042 had about a 10-fold lower affinity (24 μ M). Based on the observed binding interactions and a comparison with a human methyltransferase, the authors suggest additional structural modifications to increase affinity.

Much research remains to develop selective viral methyltransferase inhibitors [140, 141]. Many clinical trials have been conducted investigating the use of methyltransferase inhibitors, and a cytidine derivative was approved for use as an anti-cancer agent in 2004 [142]. However, these drugs must be

Fig. 9 Structures of NS5 methyltransferase ligands. (a, b) Inhibitors F3043-0013 and F0922-0796 identified by virtual screen; (c) methyltransferase native substrate S-adenosylmethionine; (d) native product S-adenosylhomocysteine; (e) methyltransferase inhibitor sinefungin; (f) capped nucleotide 7-methyl guanosine diphosphate (7-MeGpp); (g) capped nucleotide analog 7-MeGpppA; (h) guanosine triphosphate; (i) methyltransferase inhibitor MS2042

administered intravenously, have significant side effects, and cause birth defects in animal models (FDA class D) [143].

Drug Discovery: Compound Repurposing

Owing to the urgent need for therapies that can be quickly translated to the clinic, several laboratories have investigated repurposing existing drugs or drug candidates. Using a compound that already has safety and pharmacokinetic data in humans can reduce the time and cost of drug development [144]. An example of successful drug repurposing in antiviral research was azidothymidine (Zidovudine) [145]. It was originally investigated as an anticancer agent in the 1960s [146, 147], then in 1974 it was shown to suppress Friend murine leukemia virus [148]. From the first *in vitro* test of azidothymidine against HIV in 1985 [149] it quickly progressed through clinical trials and went on to become the first drug approved to treat HIV/AIDS in 1987 [150, 151].

To identify inhibitors of ZIKV, Barrows et al [78] screened 774 compounds from the FDA-approved therapeutics library. Huh-7 cells were treated for 1 h prior to infection with ZIKV isolate MEX_I_7 (KX247632). Cells were fixed at 24 hpi and stained for the ZIKV envelope. Hits were confirmed in HeLa, NPC, JEG3 (human placental choriocarcinoma), and human amniotic epithelial cells. Bortezomib, a host proteasome inhibitor, potently inhibited ZIKV infection; however, it is also cytotoxic. Furthermore, it is an FDA class D drug that is not recommended for use during pregnancy. Mycophenolic acid, an inosine-5'-monophosphate dehydrogenase inhibitor, displayed an IC_{50} of 0.1 to 1 μ M in all cell lines tested. The fact that mycophenolic acid is a class D immunosuppressant greatly limits interest in its use against ZIKV. Ivermectin is an antiparasitic, class C drug with an EC_{50} against Zika infection of 1 to 10 μ M. It was previously identified as a flavivirus helicase inhibitor [123] (see section on helicase inhibitors for details).

A compound with no previously reported antiviral activity, daptomycin (Fig. 10a), was a promising hit from this screen [78]. The EC_{50} value was between 0.1 and 1 μ M and no cytotoxicity was observed. Daptomycin is a FDA class B antimicrobial agent that displays no evidence of fetal harm. It exerts its antibiotic activity by insertion into cell membranes, suggesting its effect on ZIKV may be due to insertion into the late endosomal membranes critical for viral entry. A limitation to daptomycin's potential use is that it must be administered intravenously [152, 153]. Additionally, daptomycin displayed low cerebrospinal fluid penetration in patients with neurological infections following surgery [152].

Anti-ZIKV activity was detected in a drug screen using the Prestwick library, which holds > 1000 FDA-approved compounds and drug candidates known for safety and

bioavailability [154]. Human NPCs (hNPCs) were pretreated with the compounds and then infected with ZIKV MR766. Hippelastine hydrobromide (HH) demonstrated the greatest inhibition of ZIKV with an IC_{50} of 1.95 μ M. HH also blocked ZIKV infection when used 2 hpi (IC_{50} = 3.62 μ M). Notably, HH eliminated ZIKV RNA without affecting hNPC identity and differentiation abilities. These antiviral properties of HH were further confirmed in a human forebrain model of ZIKV infection and in a severe combined immunodeficiency mouse model [154].

A primary screen for compounds able to inhibit apoptosis caused by ZIKV was monitored by caspase activation [155]. About 6000 compounds, including the Library of Pharmacologically Active Compounds, the National Center of Advancing Translational Sciences Pharmaceutical Collection, and a collection of clinical candidates, were tested in SNB-19 (human glioblastoma) cells. Compounds were added 30 min prior to the addition of ZIKV isolate MR766. At 6 hpi, activation of caspases was detected using a luminescent caspase substrate. Primary hits were confirmed by inhibition of cytotoxicity and caspase activation in SNB-19 cells, hNPCs, and astrocytes. Further validation was performed using isolates from the current outbreak—FSS13025 and PRVABC59 [155].

The most potent inhibitor of ZIKV-induced cell death was the pan-caspase inhibitor emricasan (Fig. 10b), with IC_{50} values of 0.13 to 0.9 μ M [155]. Emricasan is orally available, has shown favorable profiles in human trials [156], and is in a phase II trial for the treatment of hepatic injury and liver fibrosis in patients with HCV (NCT02686762). No information is currently available on its use during pregnancy or its ability to cross the BBB. Emricasan's activity against ZIKV was corroborated by inhibition of cytotoxicity in a 3-dimensional organoid culture of hNPCs. Emricasan's activity against Zika appears to stem exclusively from its ability to inhibit cell death; it had no effect on the presence of ZIKV NS1 protein. A combination of emricasan with a direct-acting antiviral provided an additive effect and may prove a beneficial treatment protocol to test in an animal model [155].

Two compounds from the primary caspase activity screen that caused a reduction in NS1 staining were the cyclin-dependent kinase (CDK) inhibitor PHA-690509 (Fig. 10c) and the FDA-approved anthelmintic drug niclosamide (Fig. 10d). Both compounds caused a reduction in intracellular ZIKV RNA levels and infectious viral particles. Niclosamide was more potent with IC_{50} values of 0.37 μ M and 0.28 μ M, respectively, whereas PHA-690509 had IC_{50} values of 0.9 μ M and 1.72 μ M, respectively. Both compounds were effective when added either 1 h before or 4 h after infection and restored hNPC proliferation in brain organoids [155].

Fig. 10 Structures of promising compounds from repurposing screens. (a) Food and Drug Administration (FDA) class B antimicrobial agent daptomycin; (b) pan-caspase inhibitor emricasan; (c) cyclin-dependent kinase inhibitor PHA-690509; (d) FDA class B anthelmintic agent niclosamide; (e) FDA class B antibiotic azithromycin; (f) antiparasitic bithionol; (g) antimalarial agent mefloquine; (h) conversion of antiviral agent nitazoxanide to its biologically active form tizoxanide

Owing to the activity of CDK inhibitor PHA-690509, many other CDK inhibitors were subsequently tested. Nine additional compounds in this class had $IC_{50}s < 1 \mu M$ [155]. Flaviviruses are not known to encode any CDKs, meaning the CDK inhibitors may act on host functions necessary for the ZIKV lifecycle. While many CDK inhibitors have been evaluated in clinical trials, they are generally used in cancer treatment [157]. The ability of CDK inhibitors to cross the BBB vary depending on the structure [158]. The most common side effect of the FDA-approved CDK inhibitor palbociclib is neutropenia, which could limit the ability of the patient's immune system to fight infection [159]. A pregnancy category has yet to be assigned to palbociclib, but the FDA prescribing information states that animal data have shown clear fetal damage and, owing to the mechanism of action of CDK inhibitors, they are expected to cause adverse effects in human pregnancy.

The most promising compound identified from this screen, for potential use in pregnant patients, was niclosamide [155]. Niclosamide is known to inhibit several viruses *in vitro*, including JEV, also a flavivirus [160]. Previous studies identified that its antiviral activity stems from its ability to neutralize endolysosomal pH and interfere with membrane fusion [161]. The fact that niclosamide was effective when added 4 hpi suggests it has additional activity against ZIKV after the entry step. Further investigation into the mechanism of action and efficacy in a Zika animal model appears warranted; niclosamide displayed no risk to fetuses in animal studies, is orally available, causes minimal side effects in humans, and is recommended by the World Health Organization for

treatment of pregnant women with intestinal tapeworm infections [155, 162].

A third major screening effort was validated using known antiviral compounds, then expanded to include the 727 compounds in the National Institutes of Health Clinical Compound Collection [163]. Test compounds were added 2 h before infection with ZIKV isolates MR766 and PRVABC59. After 3 or 4 days, cell viability was determined using a luminescence-based readout. Direct antiviral activity of hits was confirmed by PCR, Western blot, and plaque-forming assays. Although similar compound libraries were used, no common hits were identified from this screen as the 2 discussed above.

Antiviral compounds identified included the known RdRp inhibitor NITD008 and the pyrimidine synthesis inhibitors brequinar and CID 91632869 [163]. EC_{50} values for brequinar and CID 91632869 were $0.08 \mu M$ and 1 to $2 \mu M$, respectively. An additional pyrimidine synthesis inhibitor, 6-azauridine with an EC_{50} of $\sim 3.5 \mu M$, was identified from the full library screen. Owing to the identification of 3 structurally diverse pyrimidine synthesis inhibitors, an additional compound of this class, teriflunomide, was also examined. While brequinar and CID 91632869 displayed potent activity by PCR and plaque-forming assays, 6-azauridine was only active in the plaque forming assay (no effect observed by PCR), and teriflunomide displayed no activity. As it has previously been suggested that this class of compounds exert their antiviral effect by inducing the expression of antiviral genes [164], the authors suggest that pyrimidine synthesis inhibition may not be the only mechanism contributing to ZIKV inhibition [163]. Two additional compounds were identified in the cell

viability screen. Mevastatin, an early lead statin compound that never advanced past clinical trials, and finasteride, a testosterone analog, both displayed promising activity in the primary screen, but failed to produce a dose–response curve, possibly owing to poor solubility [163].

Over 2000 compounds from the University of California, San Francisco Small Molecule Discovery Center’s library of FDA-approved compounds were screened for activity against ZIKV infection [165]. Vero cells were treated with 2 μM of each compound for 2 h prior to ZIKV (SPH2015, Brazil) infection. After 72 h, cell viability was quantified. The safe and commonly prescribed macrolide antibiotic, azithromycin (Fig. 10e), restored viability of Vero cells and was chosen for further investigation. An increase in cell viability (EC_{50} 7.1 μM) and decrease in viral production were observed in the glial cell line U87. Azithromycin also caused inhibition of immunostaining for flavivirus envelope in U87 cells and hPSC-derived astrocytes with EC_{50} s of 3 μM and 15 μM , respectively [165].

Azithromycin has many beneficial features that could make it a worthwhile pursuit for the treatment of ZIKV infection. No increased risk of fetal abnormalities has been observed when taken in human pregnancy [166, 167]. Furthermore, this is not because azithromycin does not reach the fetal compartment; high placental concentrations were reached within 6 h of oral administration and maintained for up to 72 h [168]. Azithromycin’s distribution throughout the CNS was examined in human patients undergoing brain surgery [169]. A significantly higher concentration was present in the brain than in patient cerebrospinal fluid, aqueous humor of the eye, or serum. Additionally, the concentration in the brain was maintained for > 48 h [169]. Validation of azithromycin’s activity against ZIKV *in vivo* will be necessary to provide the rationale for further study.

To identify compounds that block the pathogenicity of 3 different types of bacterial toxins, a clinical compound library was screened for inhibition of caspase activation and cell death [170]. The anthelmintic agent bithionol (Fig. 10f) was identified as a potent inhibitor [170]. Bithionol was successfully used to treat parasitic infections in humans [171, 172] and animals [173] until more fast-acting anthelmintic agents became available [174]. Additionally, bithionol was widely used as a topical anti-infective, until it was suspended owing to photosensitizing effects in some users [175].

Because bithionol displayed potent activity in the primary screen against bacterial toxins, it was subsequently tested for an ability to inhibit pathogenicity of several agents, including ZIKV [170]. Bithionol was added to Vero cells and astrocytes for 2 h prior to ZIKV infection (DAK AR D 41525 or PRVABC59). At 48 hpi cells were fixed and stained for ZIKV envelope. Inhibition of both ZIKV isolates was observed with EC_{50} values of 5 to 6 μM [170]. Despite the

removal of bithionol from the US market, a wealth of knowledge is available on its metabolism and effect in humans from the National Library of Medicine’s website and the MAK Collection for Occupational Health and Safety [176]. Bithionol is orally available and crosses the BBB [177] and the placental barrier [178]. Considering these potentially beneficial features, bithionol may be an old drug worth bringing out of retirement for further investigation against Zika infection, both *in vitro* and *in vivo*.

Using the structures of several antimalarial compounds with known activity against DENV, a guideline for stereoelectronic properties necessary for activity was determined [179]. This guideline was used to select 3 additional compounds with antimalarial activity to test against DENV and ZIKV. ZIKV MR766 was used to infect Vero cells; drugs were added 1.5 hpi, and virus production was quantified using a plaque-forming assay. Quinacrine, an old agent no longer in clinical use owing to toxicity, and GSK369796, which was discontinued after an unsuccessful phase I clinical trial (NCT00675064) inhibited ZIKV with EC_{50} values around 3 μM . Mefloquine (Fig. 10g) displayed an EC_{50} of 4 μM , is in current clinical use, crosses the BBB [180], and is recommended by the Centers for Disease Control for the treatment of malaria in pregnant patients [181].

Derivatives of mefloquine and chloroquine were synthesized to compare their anti-Zika activity with that of the parent compounds [182, 183]. Vero cells were infected with ZIKV isolated from Brazil for 1 h. Cells were subsequently washed and compounds were added. At 24 hpi, RNA was extracted from the supernatant. Mefloquine displayed an EC_{50} of 3.6 μM , like that reported in the above study, whereas chloroquine was less active, with an EC_{50} of 12 μM . Three of the compounds designed based on the mefloquine and chloroquine scaffolds displayed significantly improved ability to inhibit ZIKV, all with EC_{50} s of 0.8 μM . Given the use of mefloquine in pregnant women and its ability to cross the BBB, further investigation into it and its derivatives anti-Zika activity appear to be warranted. However, the FDA has issued an updated warning that in some patients mefloquine can cause serious and sometimes permanent mental conditions, so caution must be used. The use of lower amounts of mefloquine as a prophylaxis (*vs* treatment) is associated with less severe psychiatric symptoms in patients with malaria [184]. Therefore, use of a lower dosing regimen of mefloquine in combination with other anti-Zika drugs may reduce the risk of psychiatric side effects.

An FDA-approved drug whose scaffold was designed based on niclosamide—nitazoxanide (Fig. 10h)—has also shown inhibitory effects against ZIKV *in vitro* [185].

Nitazoxanide was approved by the FDA in 2004 for the treatment of diarrhea caused by *Giardia lamblia* and *Cryptosporidium parvum* in children and adults > 12 months of age [186]. Broad-spectrum antiviral activity was displayed against a range of pathogens, including influenza, rotavirus, norovirus, hepatitis B and C, dengue-2, yellow fever, and JEV. A phase III clinical trial was completed for its use in uncomplicated influenza (NCT01610245). In mice infected with JEV, treatment with 100 mg/kg/day increased survival from 0% to 90% [187]. Several mechanisms of action have been proposed, and vary depending on the pathogen it is being used against.

Nitazoxanide is metabolized *in vivo* to its bioactive form, tizoxanide (Fig. 10h), by the removal of an acetate group. Both compounds were tested for an ability to inhibit ZIKV (SZ-WIV01) [185]. Compounds were added to Vero cells concurrently with ZIKV. At 72 hpi, both compounds inhibited viral particle production, immunofluorescence for Zika envelope, ZIKV RNA production, and Western blot detection of the Zika capsid. Activity was confirmed in human A549 cells. Time of addition experiments show the drug to be effective at the postentry phase of the virus life cycle. No effect was observed when cells pretreated with compounds were washed prior to inoculation. In contrast, addition of compounds up to 24 hpi resulted in robust virus inhibition [185]. This suggests the ability to start treatment after symptom onset or viral detection may be possible.

Nitazoxanide is a class B drug, having no documented detrimental effects to fetal development when taken during

pregnancy. It is orally available and side effects are very mild. Data documenting its distribution throughout the CNS were not available; however, *in vivo* studies suggest it reaches the brain. After direct delivery to the stomach, viral load was significantly reduced in the brains of mice infected with JEV [187]. These data indicate study of nitazoxanide in a Zika animal model hold strong potential.

Conclusions

Tremendous progress has been made at an unprecedented pace in identifying compounds that target every step of the ZIKV viral life cycle by interacting with the viral or host proteins that are critical for viral replication. Several candidate drugs have been identified that are worthy of further preclinical studies (Table 1). Some drugs for which there is prior FDA approval for other conditions, such as antiparasitic agents, could be quickly moved to human studies. It is also likely that some of these drugs, although investigated for effects against ZIKV, may display broad effects against other flaviviruses. This could be particularly useful, since often these classes of viruses exist in the same geographical region and the clinical manifestations may overlap, making it difficult to differentiate one from another. It is anticipated that to treat conditions caused by ZIKV an arsenal of drugs would be needed. For example, drugs with a low adverse effect profile might be needed for short-term use for individuals travelling to high endemic regions. Whereas individuals infected with the virus might need prolonged combination therapy to

Table 1. Selection of promising therapeutic approaches for the treatment of Zika virus infection

Compound name (proposed target)	Zika activity: <i>in vitro</i>	Zika activity: <i>in vivo</i>	FDA approval/ pregnancy/ administration*	BBB
25-Hydroxycholesterol (entry inhibitor)	IC ₉₀ 2.5 μ M cortical organoids	BALB/c or A129 mice; Rhesus macaques	Not FDA-approved/NA/ parenteral	Data suggest favorable brain penetration
Bromocriptine (protease inhibitor)	IC ₅₀ 13 μ M Vero cell death	None	Approved/class B/oral	Poor brain penetration
Sofosbuvir (polymerase inhibitor)	IC ₅₀ < 1 μ M viral titration Huh-7 and SH-Sy5y	C57BL/6 mice	Approved/class B/oral	Not enough available data
Ivermectin (helicase inhibitor)	EC ₅₀ 1–10 μ M IF Huh-7, HeLa, NPC, JEG3, HAEC	None	Approved/class C/oral	Poor brain penetration
Niclosamide (undefined)	IC ₅₀ 0.4 μ M RNA in SNB-19 cells; IC ₅₀ 0.2 μ M in astrocytes	None	Approved/class B/oral	Few data; suggests poor brain penetration
Azithromycin (undefined)	EC ₅₀ 7.1 μ M cell viability U87; EC ₅₀ 15 μ M IF astrocytes	None	Approved/class B/oral	Good brain penetration in humans
Nitazoxanide (undefined)	IC ₅₀ ~1 μ M, Vero cell death, viral particle, IF, RNA	None	Approved/class B/oral	Not enough available data

FDA = Food and Drug Administration; BBB = blood–brain barrier; NA = Not applicable; NPC = neural progenitor cells; HAEC = human amnion epithelial cells; IF = immunofluorescence

*Pregnancy class: class B indicates animal studies have found no risk to fetal development; however, controlled human trials are not available. Class C indicates animal studies have shown a detrimental effect on fetuses but there have been no controlled studies in humans; however, use during pregnancy may be warranted depending on the condition

adequately treat the infection and prevent resistance to the drugs. For these drugs, their ability to cross the placental barrier and the BBB might be critical.

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