The Race To Find Antivirals for Zika Virus

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ABSTRACT

Zika virus (ZIKV), a flavivirus transmitted by mosquitoes, was an almost neglected pathogen until its introduction in the Americas in 2015 and its subsequent explosive spread throughout the continent, where it has infected millions of people. The virus has caused social and sanitary alarm, mainly due to its association with severe neurological disorders (Guillain-Barré syndrome and microcephaly in fetuses and newborns). Nowadays, no specific antiviral therapy against ZIKV is available. However, during the past months, a great effort has been made to search for antiviral candidates using different approaches and methodologies, ranging from testing specific compounds with known antiviral activity to the screening of libraries with hundreds of bioactive molecules. The identified antiviral candidates include drugs targeting viral components as well as cellular ones. Here, an updated review of what has been done in this line is presented.

KEYWORDS antiviral agents, Zika virus

Zika virus (ZIKV) is a flavivirus (Flaviviridae family) transmitted by mosquitoes, mainly by those of the Aedes genus (1). The virus was first isolated in Uganda in 1947 (2) and has since been confined in Africa until it was detected in Asia in the 1980s. Subsequently, the first large human outbreaks were reported in 2007 in Micronesia and in 2013 in French Polynesia (1). However, ZIKV was an almost neglected pathogen until the virus jumped to the Americas, which most probably occurred by a single introduction of an Asian viral strain during the second half of 2013 (3). In 2015, the association of ZIKV with severe neurological disorders, including a striking increase in the number of cases of microcephaly in fetuses and newborns and an unusual upsurge in Guillain-Barré syndrome (GBS) cases, drove the World Health Organization (WHO) to declare a public health emergency of international concern (4). The neurotropism of ZIKV has been confirmed experimentally by virus isolation from fetal brain tissue from miscarriages, from biopsy specimens from affected children, and from the reproduction of developmental disorders in animal models (5–9). Current data from ZIKV epidemics in the Americas indicates 205,500 confirmed cases, 598,960 suspected, and 2,767 confirmed cases of congenital syndrome associated with ZIKV infection (http://www.paho.org/hq/index.php?option=com_content&view=article&id=12390%3A Zika-cumulative-cases&catid=8424%3Acontents&Itemid=42090&lang=en).

Nowadays, not a single specific antiviral agent against any flavivirus has been approved (10), and treatment, when applied, is generally directed to symptom relief with analgesics and antipyretics. Lately, a great effort has been carried out to assay several drug candidates directed to viral targets (direct-acting antivirals) or against cellular targets (host-targeting antivirals). This vast work has been performed through different approaches that include the screening of different compounds libraries and the repurposing of drugs already used in clinical practice for other diseases, with many of these molecules being broad-spectrum drugs (Table 1). For instance, nucleoside analogs/derivatives, nucleoside synthesis and polymerase inhibitors, immunomodula-
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*aBiological system used to test the drugs. 3D, three-dimensional.*
tors, antibiotics, and anti-inflammatory, antimalaria, and anthelminthic drugs, among others, have been tested.

**DIRECT-ACTING ANTIVIRALS**

**Antivirals targeting the ZIKV polymerase.** The NS5 protein of ZIKV is the RNA-dependent RNA polymerase (RdRp) in charge of viral genome replication. Remarkably, its structure has been recently determined, which will positively contribute to the structure-based design of antiviral compounds against ZIKV (11). In fact, nucleoside analogs/derivatives, which target viral but not cellular polymerases to terminate viral RNA replication after incorporation into the viral nascent RNA chain, are usually safe for use in humans (12), and thus they have been extensively assayed against ZIKV in cell culture and, in some instances, in animal models. Therefore, Eyer and coworkers (13) tested several nucleoside analogues for their ability to inhibit ZIKV replication in Vero cells and found five (7-deaza-2'-C-methyladenosine [7-deaza-2'-CMA], 2'-C-methyladenosine [2'-CMA], 2'-C-methylcytidine [2'-CMC], 2'-C-methylguanosine [2'-CMG], and 2'-C-methyluridine [2'-CMU]) that reduced significantly virus-induced cell death with 50% effective concentration (EC$_{50}$) values ranging from 5.3 to 45.5 μM. Comparable results were previously obtained by testing the effects of different 2'-C-methylated nucleosides on the *in vitro* activity of purified recombinant ZIKV RdRp (14). Similarly, Zmurko and coworkers (15) showed that 7-deaza-2'-CMA exhibits anti-ZIKV activity in Vero cells (EC$_{50}$, 9.6 μM for a selectivity index [SI] of 7) and also delays disease progression and reduces viral RNA loads in the serum of ZIKV-infected AG129 (interferon [IFN]-α/β and IFN-γ receptor knockout) mice treated once daily with 50 mg/kg/day of the drug.

Likewise, the nucleoside analog BCX4430 also inhibited ZIKV multiplication in Vero cells with EC$_{50}$s of 3.8 to 11.7 and SIs of 5.5 to 11.6, depending on the viral strain tested (16). Furthermore, 7 of 8 ZIKV-infected AG129 mice treated with 300 mg/kg/day showed significant reductions in viremia and were protected compared with vehicle-treated animals (100% mortality). BCX4430 treatment also protected AG129 mice even when administered after infection, although in this case, RNA viral loads in serum were similar to those of vehicle-treated animals.

Sofosbuvir is a nucleotide analog that is an RdRp inhibitor approved by the U.S. Food and Drug Administration (FDA) for the treatment of hepatitis C virus (HCV) infection. This drug was proposed as a ZIKV antiviral after showing that it reduced viral NS1 staining in human neuroepithelial stem cells (17). Another study demonstrated that sofosbuvir efficiently inhibits the replication and infection of ZIKV in cell lines of different origins, such as hepatoma (Huh-7) and human placental choriocarcinoma (Jar) cells (EC$_{50}$, 1 to 5 μM; SI, ≥40), as well as in hindbrain and cerebral cortex-derived neural stem cells (NSCs) (EC$_{50}$, ~32 μM) (18). Moreover, the same study showed that when sofosbuvir was orally administered (33 mg/kg/day) for 7 days to ZIKV-infected mice, a greater overall survival rate against ZIKV-induced death compared with that of vehicle-treated mice was recorded (50% versus 20%, respectively) (18). Remarkably, these experiments were performed using a recently developed model, wild type (WT) C57BL/6 mice treated with an anti-IFN-α receptor 1 (IFN-αR1) blocking antibody (19).

Another study also reported that sofosbuvir inhibited ZIKV replication in Huh-7 hepatoma cells (EC$_{50}$, 0.4 μM; SI, 1,191), as well as in SH-Sy5y neuroblastoma cells (EC$_{50}$, 1.1 μM; SI, 384) and, to a lesser extent, in baby hamster kidney (BHK) cells (EC$_{50}$, 1.9 μM; SI, 184) (20). However, it did not exhibit anti-ZIKV inhibitory activity in Vero cells, indicating that its inhibitory efficiency varied among different cell types. Moreover, the same study also reported the reduction of viral replication in treated human induced pluripotent stem (iPS) cell-derived NSCs by inducing cell death and impairing ZIKV-mediated neuropathogenesis (20), as was also found in brain organoids, which have been used to address brain development and microcephaly (21). Additionally, further analysis of ZIKV sequences from infected cells treated with sofosbuvir showed an increase in the frequency of transition mutations compared with untreated cells (20), suggesting that besides its direct inhibitory effect, the drug also increases the incor-
poration of mutations in the viral genome, thereby increasing error-prone replication (22).

Adcock and coworkers (23) used a cell-based assay for the high-throughput screening of broad-spectrum antiviral compounds as ZIKV inhibitors. They reported that drugs that inhibit purine synthesis (ribavirin and mycophenolic acid [MPA]) were toxic or did not reduce ZIKV multiplication in Vero cells. On the contrary, the pyrimidine synthesis inhibitors tested (NITD008, CID 91632869, finasteride, brequinar, and 6-azauridine) were able to reduce viral multiplication to different levels, with EC50s ranging from submicromolar (brequinar) to 3.2 μM (6-azauridine). Remarkably, 6-azauridine, an anticancer drug and viral inhibitor, was also identified (EC50, 2.3 μM; SI, >33.3) in another screening (24). Regarding NITD008, this compound also exhibited antiviral activity in A129 mice deficient in type I interferon receptor treated with 50 mg/kg/day of the drug, as all vehicle-treated mice died within 12 days after ZIKV infection, whereas 50% of the NITD008-treated animals survived without developing any neurological signs (25). In this line, it has also been reported that gemcitabine, a nucleoside that interferes with de novo pyrimidine biosynthesis, inhibited ZIKV multiplication (EC50, 1 μM; SI, >1,000) by interfering with the transcription of viral RNA (26). Similarly, another screening also identified the thymidylate synthase inhibitor 5-fluorouracil, an anticancer drug, as a potent inhibitor of ZIKV multiplication (EC50, 14.3 μM; SI, >2.5) (24), further supporting pyrimidine synthesis inhibitors as potential antiviral candidates against ZIKV.

**Methyltransferase and protease inhibitors.** The NS5 protein of ZIKV exhibits not only RdRp activity, but also displays a methyltransferase domain responsible for capping the 5' end of viral genomic RNA. This enzymatic activity has just started to be explored as a potential antiviral target to combat ZIKV (27–29). ZIKV proteins other than NS5 also constitute potential druggable antiviral targets. This is the case with the N2B-NS3 trypsin-like serine protease, which plays a key role in virus replication by contributing to viral polyprotein processing (30), or with NS3 helicase activity (31). Due to the relevance of NS2B-NS3 function in the ZIKV life cycle, the search for inhibitors of the enzymatic activity of this complex is at the front line of antiviral discovery against ZIKV (30,32–34). For instance, taking advantage of a previous work that identified inhibitors of the HCV protease by high-throughput screening of over 40,000 compounds (35), the same group have recently analyzed 71 of these nonpeptidic small molecules against ZIKV and found that 10 of them showed 50% inhibitory concentrations (IC50s) lower than 50 μM, with IC50s of 5.2 μM and 4.1 μM for compounds 2 and 3, respectively (30). Additionally, the structure of the NS2B-NS3 complex has been resolved under different circumstances, including in complex with a peptidomimetic boronic acid inhibitor (36).

**Therapeutic antibodies and virucidal compounds.** The administration of specific potent neutralizing antibodies appears as a potential strategy for the treatment of flavivirus infections (37). In the case of ZIKV, it has been recently shown that passive transfer of human neutralizing antibodies through the intraperitoneal inoculation of pregnant mice suppressed ZIKV replication, inhibited cell death, reduced the number of infected neural progenitor cells (NPC) in fetal brains, and prevented microcephaly (38, 39). Furthermore, the treatment of mice with a monoclonal antibody against domain III of the envelope protein of ZIKV is sufficient to protect mice from lethal ZIKV infection (40). Overall, these studies support the potential of anti-ZIKV strategies based on the usage of therapeutic antibodies.

In addition to therapeutic antibodies, other compounds targeting the viral particle, such as epigallocatechin gallate (EGCG), a polyphenol present in many natural products, exhibit anti-ZIKV activity (EC50, 21.4 μM), probably due to a virucidal effect (41). Similar results have been obtained with EGCG and delphinidin, a different polyphenol (A. Vázquez-Calvo, N. Jiménez de Oya, M.A. Martin-Acebes, E. García-Moruno, J.-C. Saiz, submitted for publication).
HOST-TARGETING ANTIVIRALS

Apart from drugs targeting viral components, those targeting cellular factors directly involved in the viral life cycle may also be useful, since their effect is less prone to evasion by mutations in the viral genome that frequently appear in RNA viruses. In this line, 2,000 compounds from a library of FDA-approved drugs, as well as molecules known to be bioactives, have been tested by means of a microscopy-based assay to uncover inhibitors of ZIKV infection (42). By using human osteosarcoma cells (U2OS), it was shown that up to 38 molecules blocked flavivirus infection, including nanchangmycin (IC50 0.1 μM), a natural product of *Streptomyces nanchangensis* that was shown to have insecticidal activity against silkworms and anti-bacterial activity in vitro, tenovin-1 (IC50 0.7 μM), which protects against MDM2-mediated p53 degradation, MPA (IC50 0.4 μM), and gemcitabine (IC50 0.3 μM). MPA and gemcitabine have also been reported to be ZIKV inhibitors by others (26, 43). A less potent effect was also observed in human brain microvascular endothelial cells (hBMECs), an immortalized model of the human blood-brain barrier microvasculature that may be involved in ZIKV access to fetal brain. Furthermore, some of the semolecules were also active on Jeg 3 human placental cells.

Chloroquine is an anti-inflammatory FDA-approved 4-aminoquinoline widely used as an antimalarial drug and administered to pregnant women at risk of exposure to *Plasmodium* parasites, and has also shown antiviral activity against several viruses through the inhibition of pH-dependent steps of viral replication. This drug has been described to exhibit anti-ZIKV activity in Vero cells (44). The agent also affected ZIKV-infected hBMECs, as well as human NSCs, for which the depletion of is one of the main mechanisms responsible for primary microcephaly (45). Chloroquine reduced the number of ZIKV-infected cells, thereby inhibiting virus production (including of defective viral particles), and reduced cell death promoted by ZIKV infection, thus interfering with the early stages of the ZIKV replication cycle, possibly during the fusion of the envelope protein to the endosomal membrane (44). The reported EC50s were 9.8 μM, 14.2 μM, and 12.3 μM (for therapeutic indexes [TIs] of 13.7, 8.2, and 7.7) for Vero, hBMECs, and NSCs, respectively. Saliphenylhalamide (SaliPhe), which targets vacuolar ATPase and blocks the acidification of endosomes, also inhibits ZIKV replication in human retinal pigment epithelial (RPE) cells, which are natural targets for ZIKV infection (46), with an EC50 of 1 μM and an SI of >200 (26). Interestingly, SaliPhe, chloroquine, and other compounds interfering with the endocytic pathway, such as dynasore and monensin, were also identified as potential anti-ZIKV compounds in a different screening (23). In this line, it has also been reported that obatoclax, also known as GX15-070 (an inhibitor of the Bcl-2 family of proteins that targets cellular Mcl-1 and inhibits endocytosis, thereby inducing apoptosis), presented an EC50 of 0.3 μM and an SI of 65 and could impair ZIKV endocytic uptake, as well as several agents used for the treatment of cancers and with an already known capacity to inhibit multiplication of other viruses (26).

Barrows and coworkers, after assaying an FDA-approved library of compounds, showed that over 20 of them reduced ZIKV infection in hepatoma-derived HuH-7 cells, including bortezomib (a selective inhibitor of proteasome activity used in patients with multiple myeloma), daptomycin (a lipopeptide antibiotic), MPA, sertraline, pyrimethamine, cyclosporine, azathioprine, and mefloquine (43). Next, selected drugs were tested in human cervical cells (as ZIKV can be sexually transmitted, probably when infected semen comes in contact with the vaginal mucosa or the cervix [47]), placental cells, neural stem cell lines, and primary human amnion cells, showing EC50s of 1 μM (daptomycin), 0.1 μM (MPA), and 1 to 10 μM (ivermectin).

Another recent study of drug repurposing, based on the expression of the viral NS1 protein as a readout of anti-ZIKV activity for primary screening, tested over 5,000 compounds from the LOPAC (library of pharmacologically active compounds), the NCATS (National Center for Advancing Translational Sciences) pharmaceutical collection, and a collection of clinical candidates (48). The study was conducted in ZIKV-
infected glioblastoma SNB-19 cells, as well as in human NPCs and astrocytes, both of which are target cells for ZIKV infection in the fetal brain (49, 50). Emricasan, a pan-caspase inhibitor currently in phase 2 clinical trials in chronic HCV patients, inhibited increases in caspase-3 activity induced by ZIKV infection and protected human NPCs in both monolayer and three-dimensional organoid cultures, showing neuroprotective activity but not suppressing viral replication. Emricasan also showed anti-cell death activity in SNB-19 cells (IC_{50} 0.1 to 0.9 μM) and astrocytes (IC_{50} 0.2 μM). Likewise, niclosamide, a category B (and thus considered to be without risk to fetuses) anthelmintic drug approved by the FDA with a broad antiviral activity due to its ability to neutralize endolysosomal pH and interfere with pH-dependent membrane fusion (51), also inhibited ZIKV replication in a submicromolar range, as did PHA-690509, an investigational compound that functions as a cyclin-dependent kinase (CDK) inhibitor. This finding, as no CDKs encoded by flavivirus have been described, suggests that host cellular CDKs might be involved in ZIKV replication; however, these compounds are unlikely to be suitable for use in pregnant women because of their potentially hazardous effects on the fetus. As these two compounds (niclosamide and PHA-690509) were effective in ZIKV inhibition when added 1 h before or 4 h after viral inoculation, they probably affected a postviral entry step during viral replication (48). In addition, emricasan and PHA-690509, with different mechanisms of action (neuroprotective and antiviral), when administered in combination, showed an additive effect in inhibiting caspase-3 activity in SNB-19 cells and astrocytes.

By means of a cell-based high-content screening assay, different FDA-approved molecules included in the National Institutes of Health (NIH) clinical collection compound library were reported to present moderate activity against ZIKV infection in Huh-7 cells (24). Among these were palonosetron (EC_{50} 16.3 μM; SI, >3.1), an antiemetic, kitasamycin (EC_{50} 41.7 μM; SI, >12), a broad-spectrum antimicrobial, and lovastatin (EC_{50} 20.7 μM; SI, >2.5), a hypolipidemic agent that inhibits cholesterol biosynthesis. Interestingly, other hypolipidemic agents targeting sterol-responsive element binding protein (SREBP) activity, such as nordihydroguaiaretic acid and its derivative, tetra-O-methyl nordihydroguaiaretic acid (M_{4}N), an anticancer drug in clinical trials, PF-429242, and fatostatin, also reduced ZIKV infection in cultured Vero cells (T. Merino-Ramos, N. Jiménez de Oya, J.-C. Saiz, and M. A. Martin-Acebes, submitted for publication). Along this line, anti-ZIKV activity has also been reported for 25-hydroxycholesterol, which also regulates SREBP activity and is involved in the innate immune response to toll-like receptor ligands and interferon (52). Importantly, 25-hydroxycholesterol inhibited ZIKV infection in vitro (IC_{50} 0.2 μM) by blocking viral entry, reduced viremia and conferred protection against ZIKV in mice and rhesus macaques, and reduced tissue damage in human cortical organoids and the embryonic brains of infected mice (52). Finally, an additional study has identified three antimarialar compounds that inhibited ZIKV in Vero cells, namely, quinacrine (EC_{50} 2.3 μM), mefloquine (EC_{50} 3.9 μM), and GSK369796 (EC_{50} 2.8 μM) (53).

CONCLUSIONS

During the past months, the scientific community has made an enormous effort in the search for antiviral candidates to fight ZIKV infection. To do so, different approaches and methodologies have been used, from testing specific compounds with known antiviral activity in other virus models, to libraries composed of hundreds of bioactive molecules, many of them already approved for human use. These molecules target viral and cellular components and included nucleosides analogues, nucleoside synthesis inhibitors, drugs targeting viral enzymes, anticancer and anti-inflammatory molecules, antibiotics, antiparasitics, and so on. In any case, care should be taken as, in many instances, the described in vitro antiviral activities are difficult to extrapolate to their possible use in humans, as only a few have been assayed in immunodeficient mouse models and only one has been assayed in nonhuman primates. Taking into account that the main target populations for antiviral therapy will be people with underlying
and thus, careful evaluation should be conducted before using them in clinical practice.

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REFERENCES


Juan-Carlos Saiz received his PhD in biochemistry and molecular biology from Universidad Autónoma de Madrid (UAM), Spain, in 1987. He was then trained in virology (working with foot-and-mouth disease virus) as a postdoctoral researcher at the PIADC/ARS in New York (1987 to 1991), at the Instituto Nacional de Investigación Agraria y Alimentaria, INIA, in Madrid (1991 to 1992), and at the Centro de Biología Molecular “Severo Ochoa,” CBMSO (1992 to 1996), also in Madrid. From 1996 to 2002 he was in charge of the laboratory of viral hepatitis at the Hospital Clinic (Barcelona), working mainly on hepatitis C virus. Since 2002, Professor Saiz has been the research leader of the “ZOOVIR” laboratory at INIA, where he was director of the Department of Biotechnology (2011 to 2016). Saiz’s group studies different aspects of flavivirus (West Nile, Dengue, Zika, and Usutu viruses) biology and their interactions with the host, focusing on the pathogenesis, prevention, and control of these worldwide threatening infectious pathogens.

Miguel A. Martín-Acebes received his PhD in biological sciences (molecular biology) from Universidad Autónoma de Madrid (UAM) and his postdoctoral training in virology from the Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA) and the Centro de Biología Molecular “Severo Ochoa” (CBMSO). He is currently principal investigator at the Department of Biotechnology of INIA. Dr. Martin-Acebes studies virus host-interactions to identify novel antiviral targets and improve vaccine strategies. His work has contributed to the identification of key roles of cellular components and processes involved in flavivirus replication (i.e., lipids), opening new research lines for therapeutic interventions.