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Broad-spectrum agents for flaviviral infections: Dengue, Zika and beyond

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Abstract

Infections with flaviviruses, such as dengue, West Nile virus, and the recently re-emerging Zika virus are an increasing and probably lasting global risk. This review summarizes and comments on the opportunities for broad-spectrum agents that are active against a range of flaviviruses. Broad-spectrum activity would be particularly desirable as preparatory measure for the next flaviviral epidemic that could emerge from as-yet-unknown or neglected viruses. Potential target sites for broad-spectrum anti-flaviviral compounds include viral proteins and host mechanisms that are exploited by these viruses during entry and replication. A variety of compounds with broad-spectrum antiviral activity have already been identified by target-specific or phenotypic assays. For some other compound classes, broad-spectrum activity can be anticipated because of their mode of action and molecular target(s).

Introduction

Three global megatrends – uncontrolled urbanization, climate change, and increased intercontinental travel – promote the spread of flaviviruses from their habitats in tropical forests. A reversal of these megatrends is highly improbable, and it is therefore worthwhile to evaluate the potential of antiviral treatments against known (and unknown) flaviviral pathogens. We will focus here on pharmacological interventions, but also touch on alternative strategies such as vaccination.

Within the *Flaviviridae* family, the genera *flavivirus* and *hepacivirus* encompass single-stranded, positive-sense RNA viruses with pathogenic effects in humans. Medicinal chemistry was highly successful[1] in addressing hepatitis C virus (HCV), the only significant *hepacivirus*. In contrast, drug discovery against members of the genus *flavivirus* (here

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denoted as ‘flaviviruses’) received, thus far, less attention. This can be attributed to the following factors: effective vaccines exist against some flaviviruses, such as yellow fever virus (YFV)[2] and Japanese encephalitis virus (JEV); the prevalent spread of most flaviviruses in tropical countries; and the variability of the pathological effects of flaviviral infections, ranging from asymptomatic infection to severe and fatal disease[3,4]. Flaviviruses are mostly transmitted by insect (arthropod) vectors, in particular ticks and mosquitos, of the genera *Aedes* and *Culex*, and are therefore also classified as arboviruses (arbo: **ar**thropod-**bo**rne).

Many flaviviral infections, especially dengue, tend to appear as epidemics, causing millions of cases[5]. In this respect, the recent Zika pandemic[6] is not an exception, and highlights the risk potential of the flaviviruses as a group, even though the mortality of many flaviviral infections is relatively low. The following risk factors are demonstrated by the Zika epidemic:

- Globalization and travel distribute previously obscure viruses into populations with no previous exposure or immunity, and therefore a high penetrability for the infection[7].
- Transmission occurs mainly via arthropod vectors, but also by other routes that were previously not considered relevant for flaviviruses, and viruses can persist in some tissues for several months after the viremic period[8].
- Infections with flaviviruses can lead to unexpected pathologies, such as the congenital and neurologic damages caused by Zika (microcephaly and Guillain-Barré syndrome)[9].
- Virus-naïve populations have often previously been exposed to other, closely related viruses and may have developed immunity against these. There is evidence *in vitro* which suggests that antibodies from this previous infections (or possibly vaccination) can exacerbate the course of the disease[10]. However, the clinical relevance of these *in vitro* observations is uncertain.

Fortunately, much has been learned about flaviviruses in the recent past, and numerous methods were devised to characterize potential drug candidates in a variety of assay systems that range from isolated targets to mouse and non-human primate models. Particularly inspiring and promising are also the successes achieved for HCV, which can to a large part be attributed to the availability of cell-based assay systems for viral replication[11]. In our opinion, these systems bridged the highly critical gap between biochemical target-oriented assays and animal models. For flaviviruses, cell-based systems are well established and were recently also used to screen antiviral agents against Zika, with a particular focus on drug repurposing[12,13].

The present review will cover compound classes, targets, and assay methods that currently appear most promising with respect to broad-spectrum activity. The most important criterion for inclusion of a compound or target is the proven activity against more than one flavivirus in a cell-based assay, or ideally in an animal model. In some instances, we also include compounds and targets where either this proof is still lacking, but broad-spectrum activity

appears likely, or where a promising activity has been reported against viruses of considerable current interest like Zika and dengue.

We excluded compounds that do not appear promising as starting points for medicinal chemistry efforts owing to issues such as an “activity” at levels at or above 50 μM; blatant deviation from commonly accepted medicinal chemistry criteria (e.g. polyphenolic compounds with molecular masses in the 1,000 Da range); and evidence for high cytotoxicity, either for the compounds themselves, or if this is not provided, for close analogs.

Following a short overview of flavivirus biology, we will cover targets that appear particularly promising with respect to broad-spectrum anti-flaviviral activity. A focus will be laid on targets with at least some initial medicinal chemistry exploration, or where it appears highly probable that a drug intervention can be successful. A variety of host targets have been proposed on the basis of RNAi screening results, and we restrict our discussion to those which have been validated by follow-up medicinal chemistry or other methods. Results from phenotypic compound screens are included if they fulfill the criteria outlined above. Biochemical assay methods for viral targets that appear most promising with respect to high-throughput capability and transferability, but also cellular and animal models will be outlined. Ancillary topics such as history and epidemiology, phylogeny and antigenic relationships, as well as flavivirus vectors and their control are provided in the Supplementary information.

Biology and Replication

The replication cycle of flaviviruses in cells is outlined in Figure 1 along with the most relevant sites for pharmacological interaction. A variety of receptors have been suggested to mediate the binding of flaviviruses to host cells and subsequent endocytosis. After release of the flaviviral genome from the endosome, the (+)-single-strand RNA is translated at ribosomes to form the viral polyprotein, which is cleaved by host proteases and the viral protease to form structural and non-structural proteins of the virus. This process, and the replication of the viral genome, occurs in a multi-molecular assembly located at the endoplasmic reticulum, which is denoted as “replication complex” and contains membranes, viral RNA, lipid droplets, and viral and host proteins. Most viral and many host targets are localized in or related to the replication complex, such as the viral protease, polymerase, helicase, host kinases, glycosidases *etc*. As further discussed in the assay section in the Box 2, the microenvironment of the replication complex is likely a major factor that influences the biophysical properties of the targets and the ligand binding behavior.

An important step in virion maturation is the sequential trimming of glucose residues on the surface of glycoproteins with participation of endoplasmic reticulum (ER) glucosidases. This process is a prerequisite for the proper folding of the glycoproteins by the ER chaperones calnexin and/or calreticulin. On the incompletely folded proteins, the reglycosylation process is launched by UDP-glucosyltransferase 1, which acts as a sensor of correct protein folding[14]. After budding into the ER, the assembled progeny viruses are processed further in the trans-Golgi network. Key factors for this final maturation are a drop

in pH that induces the conformational reorganization of the E and pre-membrane (prM) glycoproteins, as well as the proteolytic cleavage of the latter by the host protease furin. Finally, mature virions egress from the infected cell via exocytosis modulated by the exocyst complex[15].

Like other RNA viruses, flaviviruses rapidly accumulate mutations because of the low fidelity of the viral RNA polymerase[16]. This leads to the formation of intragenic variation or "quasispecies" in the infected host, which has implications for anti-flaviviral drug and vaccine development. Formation of quasispecies is the main reason for the relatively fast development of drug resistance in RNA viruses. To avoid resistance development, classes of compounds active against different viral and host targets should be developed and used in combination antiviral treatment[17]. Vaccines developed against specific strains might be ineffective against diverse viral populations and vaccine-resistance can develop. The long-time efficiency of the YFV 17D-204 live vaccine strain can be explained by the genetic stability of the YFV wild-strain population[18]. In any case, formation of flaviviral quasispecies should be taken into consideration in broad-spectrum antiviral drug design and polyvalent vaccine development.

Severe cases of flaviviral infections are frequently detected only after the viremia has peaked. Therefore, antiviral compounds that target the viral life cycle have their largest potential in prophylaxis and treatment of early-stage or persistent sub-clinical infections. Alternative treatment options – which may be identified by drug repurposing – could be directed against the pathological immune response which frequently plays an important role in severe cases of acute disease.

Pathology and tissue tropism

The incubation period of flaviviral infections usually ranges from 3 days to 2 weeks, with periods of up to 4 weeks reported for Murray valley encephalitis virus (MVEV). Flaviviral infections often remain asymptomatic, and for some viruses, only a very minor percentage of infected persons develop symptoms. Initial symptoms such as fever, rash, headache, nausea, and fatigue are often non-specific. Symptomatic cases are often self-limiting and resolve in about a week. Potentially lethal or permanently damaging pathologies develop in up to 25–30% of the symptomatic cases, depending on virus type, age, immune status, co-morbidity, and previous heterologous infections of patients.

Flaviviruses infect different cells and tissues with variable preference. The tissue tropism of flaviviruses determines the pathology of severe cases and human-to-human viral transmission patterns. Neurotropic flaviviruses (WNV, TBEV, JEV, MVEV, ZIKV, *etc.*) cause different neurological pathologies from myelitis and encephalitis to seizures, permanent brain damage, and paralysis, as in MVEV infection[19]. Some neurological pathologies, like Guillain-Barré syndrome, are caused by the immune response of the organism as a reply to the viral pervasion, and may occur in infections by neurotropic viruses, like ZIKV[20,21], or severe cases of non-neurotropic viral infections, like dengue and yellow fever[22,23]. CNS entry mechanisms vary and are not well studied for all flaviviruses, but two main routes are suggested: hematogenous and axonal transport[4]. The viral envelope

(E) glycoprotein is the main neurovirulence determinant, and mutation of a single amino acid in its structure can lead to loss of neuroinvasiveness[24]. The viral loads of some neurotropic viruses, like JEV and WNV, have been shown to persist in the CNS, especially in immunocompromised patients[25,26]. Flaviviral neurotropism directly influences the design of anti-flaviviral compounds, making penetration of blood-brain barrier a crucial pharmacokinetic property especially in the cases of persistent neuroinfection.

The hemorrhagic fever observed in other flaviviral infections, like DENV, YFV, Omsk hemorrhagic fever virus (OHFV), Kyasanur forest disease virus (KFDV), Alkhurma hemorrhagic fever virus (AHFV), is linked to the host immune response[27], which makes immunomodulatory and antihemorrhagic properties important for the development of symptomatic agents, while antiviral agents would be effective only if designed to prevent the onset of fever. However, specific viral determinants for the development of hemorrhagic fever have not been determined yet.

For most flaviviruses, there are contradictory or no data on their ability to cross the placental barrier. ZIKV, however, causes teratogenic effects in developing fetuses, and was associated with microcephaly, CNS lesions, and fetal death, with first reports appearing during the recent outbreaks in Brazil[28,29] and, retrospectively, for an outbreak in French Polynesia[30]. Teratogenic effects of ZIKV have also been observed in animal models[31,32]. From these points, design of antivirals that prevent penetration of ZIKV through placental barrier would be a possible direction of antiviral research, while development of an effective vaccine that would prevent infections in mothers seems to be the best option.

A high viral load in semen and testes was found for JEV and ZIKV[33–37]. Whereas evidence for JEV is so far restricted to animals[33], ZIKV reaches high loads of infectious viral particles (10^4–10^5 times the blood or urine viral loads) both in human semen[34,35] and in animal testes[36,37]. Presence and persistence of ZIKV RNA in cervical mucus has also been reported, increasing the probability of sexual and vertical transmission of the virus[38]. Multiple cases of sexual transmission of ZIKV have been reported recently[39–41], and a long-time persistence of ZIKV in semen and cervical mucus could make sexual transmission the main ZIKV distribution route in vector-free regions. High loads of viral RNA have also been detected in breast milk (DENV, WNV, YFV, ZIKV)[42–45] and saliva (ZIKV)[46], which suggest that these routes can also contribute to vertical and sexual transmission. These factors make it crucial for newly developed antivirals to penetrate and accumulate in the respective tissues and organs, especially in persistent infections (i.e. testes in ZIKV infection), while not affecting their physiological functions.

Viral targets

A viral target protein should ideally combine two attributes: essentiality for the viral cell cycle and a low rate of “allowed” (i.e., non-lethal, but resistance-conferring) mutations. The latter part is of considerable significance for RNA viruses such as HCV and flaviviruses, whose RNA polymerase does not implement a proofreading function and therefore, essentially, acts as a highly efficient mutation machine. The high mutation rate leads to a relatively large fraction of non-functional progeny virions, but this is compensated by other

advantages, such as fast immune evasion and development of drug resistance. With respect to essentiality, the minimal genome of flaviviruses does not allow any duplication of functionalities, or inclusion of non-essential proteins, and therefore this is not an issue to be considered here.

In practical terms, a straightforward biochemical assay procedure with high correlation to a phenotypic effect should exist for the target protein. Enzymatic targets, such as the flaviviral protease, are therefore highly attractive and have extensively – but with variable success – been pursued in high-throughput screening campaigns. Another argument for enzymatic targets are the experiences from related viruses, particularly HIV and HCV, where inhibitors of the protease and RNA-polymerase have gained large clinical significance[47]. In contrast, only very few antiviral agents – which are associated with declining efficacy or other severe drawbacks – target non-enzymatic viral proteins (M2, gp41). A caveat that needs to be considered is that the microenvironment of the replication complex, in which all enzymatic viral targets are localized[48], probably differs significantly from the *in vitro* conditions of biochemical assays on isolated targets. This may, in addition to other confounding factors such as pharmacokinetics, lead to discrepancies between biochemical, cellular and whole-organism assays.

Structural or functional features that have a large barrier towards mutation show a high degree of evolutionary conservation. This allows us to assess the likelihood of resistance-inducing mutations by comparison of viral genomes. At the same time, a high degree of conservation indicates target structures that have the largest potential for broad-spectrum relevance. A multiple sequence alignment of 50 flaviviral polyproteins demonstrates a high degree of conservation for residues with structural functions, such as Gly, Pro, and Cys, with the latter being required for a large number of conserved disulfide bridges in the E protein (cf. Supplementary information). The variability of some non-structural proteins is remarkably high, particularly for the NS2 and parts of the NS4 protein, rendering these proteins less promising as targets for broad-spectrum or resistance-robust antiviral drugs. In contrast, several of the enzymatic motifs involved in protein and RNA processing appear highly conserved and therefore “resistant” towards escape mutants. This is most noteworthy for the catalytic motifs of the NS3 protease, the ATP- and RNA-binding regions of NS3 helicase, and the substrate or metal recognition motifs of NS5. At the same time, these conserved regions offer significant chemical functionality, which may be addressed by drug-like, small-molecular inhibitors.

Allosteric ligands and inhibitors can be identified by high-throughput screening campaigns directed at (enzyme) targets and probably constitute a considerable fraction of initial screening “hits” – albeit a type of hits that is frequently difficult to optimize and may therefore impede further development work. The allosteric binding mode has some advantages, but is also associated with a number of inherent limitations and risks: The orthosteric binding sites for the natural substrates such as RNA or the polyprotein cleavage sites are highly conserved, both during evolution of a single viral species and across flaviviral species. Chemical functionalities and their geometric distribution in the orthosteric – substrate-recognition and catalytic – regions have a high barrier towards mutation, since their substrates remain unchanged. The mutational barrier is lower in most (allosteric)

regions that are not related to substrate binding, and therefore the potential for allosteric ligands to become resistance-robust and broad-spectrum anti-flaviviral agents appears to be relatively low.

In the following sections, we will focus on the targets that hold the largest potential for broad-spectrum activity, considering their genetic variability and experiences from other viruses. The most promising viral targets are the NS3 protease and the NS5 polymerase, and, to a lesser degree, the E-glycoprotein, the capsid protein, NS4B, NS3 helicase and NS5 methyltransferase. Other targets, such as NS5 guanylyltransferase and NS3–NS5 interaction currently appear to have limited potential and are not discussed further in the text, but are still included in the targets-assays Table in SI. The structures of selected compounds that target viral proteins are presented in Figure 2.

Protease inhibitors are highly successful in the treatment of HCV and HIV, where a large number of peptidic and pseudopeptidic inhibitors are currently in clinical use[47]. The substrate binding site in HCV and flaviviral proteases is relatively shallow and therefore not easily amenable to inhibition by small-molecular compounds. The flaviviral proteases have a strong preference for substrates with di- or polybasic recognition sequences[49–51]. Consequently, the recognition motifs in inhibitors also tend to incorporate basic or polar functionalities, which may at least partially explain their frequently lower efficacy in cell-based vs. biochemical assays, because of their low passive membrane permeability[52–56]. The substrate-binding residues and the substrate recognition patterns of the protease are well-conserved across the flaviviruses, and therefore hold promise for the development of inhibitors with broad activity. The extensive experience with inhibitor development for other serine proteases with basic recognition preferences (thrombin, factor Xa), for which arginine mimetics[57] or prodrug strategies[58] were devised, may provide valuable inspiration for the development of clinically effective inhibitors of flaviviral proteases.

The N-terminal domain of NS3 is a trypsin-like serine protease[59] that interacts with the core hydrophilic region of NS2B and processes the viral polyprotein[60,61]. In the catalytically active “closed” form, NS2B contributes to the S_2 and S_3 sub-pockets of the binding site[62–66]. The protease is the only ZIKV target whose structure has been solved to date in complex with a high-affinity inhibitor[67]. Given the importance of protease inhibitors for the treatment of other viral infections, the straightforward and robust enzymatic assay, and the availability of structural data[62,64,68–72], it is not surprising that numerous studies report HTS and virtual screening results for this target[70]. Reported activities, however, are often insignificant, and follow-up hit-to-lead development is frequently missing for these hits. The weak reported activities from HTS studies indicate that the flaviviral protease is – similar to the related HCV protease – not an “easy” target. This can be explained with the factors discussed above: the molecular recognition properties of the protease and its cellular microenvironment. For a detailed discussion that also includes the various constructs and assay conditions used for dengue protease, readers are kindly referred to the review by Nitsche *et al*[70].

Numerous studies focused on the development of protease inhibitors starting from a substrate-mimicking peptide. Strategies included incorporation of a C-terminal

electrophile[52,54,73–75], optimization of the N-terminal capping moiety[53,56,76,77], and modulation of the P_1 and P_2 basic residues through non-natural building blocks[53,78]. Aldehydic inhibitors displayed low micromolar to nanomolar affinity at the DENV-2[74] and WNV[52,75] proteases. An analogue, **cpd. 3a**, reported to be stable in serum and cell-permeant, suppresses WNV replication without detected cytotoxicity, but the antiviral activity against other flaviviruses was not assessed[52]. Another peptide-aldehyde, **cpd. 3b**, inhibits the DENV-2[73] and WNV proteases[62,75] in biochemical assays and was co-crystallized with DENV-3[64] and WNV proteases[62]. A recent study, conducted on this derivative, reported lack of passive permeability in PAMPA and no reduction of DENV-2 titer in cellular assays[55]. However, the *in vitro* target affinities of the two peptide aldehydes differ by three orders of magnitude, which seems to be a more likely explanation for their diverging activity in cell culture[52,55].

An evaluation of tetrapeptides with different C-terminal electrophiles identified a boronic acid analogue with low nanomolar affinity at DENV-2 protease[73]. Replacement of the arginine in the P_2 position by non-natural arginine mimetics[78] generated benzoyl-capped dipeptides with activity against DENV-2, WNV and ZIKV protease in biochemical assays[54]. The two most affine analogues, **cpds. 4** and **7**, reduced DENV-2 and WNV titers in plaque assays[54]. Notably, **cpd. 4** displays high affinity at ZIKV protease and the crystal structure of the complex was recently published[67]. The main limitation of the boronic acid inhibitors appears to be their low selectivity against off-targets such as thrombin and trypsin[54]. This problem may be addressed by extension towards the prime-site or optimization of the N-terminal cap, as shown for other peptide-based compounds[53,56,76,77] and for the aldehyde **cpd. 3a**[52].

While the (pre-)clinical development potential of peptide aldehydes and peptide boronic acids remains uncertain, they are crucially important to understand the molecular recognition and structural transitions of the flaviviral protease[54,62,64,67].

Fortunately, high affinity is not restricted to protease inhibitors that incorporate electrophilic groups. Using a fragment-merging strategy, a class of N-capped tripeptides was developed, incorporating 4-hydroxyphenylglycine benzyl ethers as non-natural C-terminal residues[53]. With variation of the N-terminal moiety and the benzyl ether substituent, these competitive inhibitors reach *in vitro* affinities in the low nanomolar range for DENV-2 and WNV proteases[53]. The compounds display remarkable selectivity against thrombin and trypsin, and representative analogues inhibit DENV-2 and WNV replication in plaque assays at low micromolar concentrations without detected cytotoxicity[53]. A discrepancy between the most active congener in enzymatic assays (**cpd. 83**) and in cellular assays (**cpd. 104**) may be due to the higher metabolic stability and passive permeability (PAMPA) of **cpd. 104** in comparison to **cpd. 83**[53].

Apart from peptidic inhibitors, **palmatine**, an isoquinoline alkaloid from *Coptis chinensis*, a medicinal plant, was investigated for its antiviral effects against flaviviruses[79]. **Palmatine** reduces the viral titer for WNV, YFV, and DENV-2 (but not vesicular stomatitis virus, *Rhabdoviridae*), without cytotoxicity[79]. Enzymatic assays at WNV protease showed an uncompetitive mechanism of inhibition with relatively low potency compared to the antiviral

effects in cells[79]. The same phenomenon was observed previously for two inhibitors of DENV protease identified from phenotypic assays, BP2109[80] and BP13944[81], in which the discrepancy between cell- and target-based results was explained by the artificiality of the protease construct and the assay conditions[80,81]. The compounds were active against DENV 1–4, but not JEV in viral yield reduction assays[80,81].

A recent study explored the inhibitory potential of HIV and HCV protease inhibitors against DENV-2 and chikungunya virus (CHIKV)[82]. Because of the weak antiviral activity against DENV-2 and CHIKV, all drugs had a much lower selectivity index than for HIV or HCV. At DENV-2, **nelfinavir** showed similar antiviral activity to **cpd. 104** in cellular assays, but displayed much higher cytotoxicity[53,82]. Identifying broad-spectrum protease inhibitors with sufficient activity *via* drug repurposing appears quite challenging.

NS5 polymerase inhibitors are another promising class of compounds. The flaviviral RNA-dependent RNA polymerase (RdRp) is located at the C-terminal part of the NS5 protein[83–85]. The structure of WNV and DENV-3 RdRp shows a typical right-hand orientation with three subdomains: fingers, palm, and thumb[84,85]. The catalytic site is positioned at the intersection of two tunnels; one provides access to the active site for the ssRNA template and the second tunnel allows entry of the NTPs at one end and exit of the nascent dsRNA at the other end[84,85].

The essential role of the RdRp enzyme in the viral replication cycle, its high conservation, and the lack of an eukaryotic homolog renders the flaviviral polymerase an attractive target for drug development. This is further underlined by the clinical success of polymerase inhibitors for HCV and HIV[47].

Nucleoside inhibitors (NIs) are substrate analogues. Following their phosphorylation, they inhibit the RdRp activity by competing with the natural NTPs; their incorporation into the nascent RNA leads either to chain termination, or a lethal accumulation of mutations denoted as “error catastrophe”[86].

NIs for flaviviruses based on the structure of the four natural nucleotides were reported[87–92]. 7-Deaza-2′-C-methyl adenosine (**7DMA**), a potent HCV inhibitor, exerted broad-spectrum antiviral effects against WNV, YFV, DENV-2[87], TBEV[93], and ZIKV in cell-based assays[94,95]. **7DMA** reduced viremia in AG129 mice infected with DENV or ZIKV[94,96]. Another NI, **2′-C-methyladenosine (2′CMA)**, was also reported to inhibit ZIKV[95] and TBEV[93] in titer reduction assays, indicating the potential of 2′-C-methylated nucleosides[93,95].

Replacing the 2′-C-methyl by an ethynyl group provided another potent NI, **NITD008**[88,97,98], which inhibited DENV 1–4 at submicromolar to micromolar concentrations in different assays and cell-lines[88]. The spectrum of activity included also HCV, and other flaviviruses such as WNV, YFV, Powassan virus (POWV), TBEV, KFDV, AHFV, OHFV, and ZIKV[12,88,99]. Despite efficacy in DENV mouse model, and favorable pharmacokinetic properties, the compound was not pursued further due to failure at the preclinical stage during *in vivo* toxicity studies[88,98,100].

A C-nucleoside analogue of adenosine, **BCX4430**, originally developed against filoviruses, was found to exert broad-spectrum activity against numerous viruses, including YFV, DENV-2, and JEV[89]. **BCX4430** displayed a favorable pharmacokinetic profile and efficacy against Ebola virus (EBOV) and YFV in animal models[89,101], and a phase I clinical trial (NCT02319772) for the compound was already completed[102].

Efforts for the development of NIs against the flaviviral RdRp are complicated by a number of challenges. Unfortunately, none of the reported examples could be further developed as a drug due to low efficacy, toxicity, or differences in the cellular tropism exhibited by flaviviruses in the case of repurposed NIs from the HCV or HIV fields. The first critical issue is the conversion of the “prodrug” NI to the biologically active triphosphate by host kinases, a crucial step that is not assessed for the synthesized nucleoside triphosphate (NTP) in the initial biochemical assays. The chemical “freedom to operate” on the nucleoside scaffold are often limited by the restricted substrate specificity of cellular kinase enzymes[103]. Furthermore, the phosphorylation efficiency of kinases is influenced by several factors resulting in variation of the EC_{50}-value of a NI between different cell types used for the assay, and was reported to be higher in immortalized cell lines, such as the frequently used Huh-7 cells, in comparison to primary hepatic cells[104]. Based on these considerations, the tropism exerted by different flaviviruses may also play an important role in the potency of NIs.

In most cases, the rate-limiting step of the intracellular formation of NTPs is the first phosphorylation. It is therefore tempting to employ “partially activated” nucleoside monophosphates as drugs. These, however, are poorly permeable and prone to degradation by phosphatases[100,105]. Prodrug strategies were developed to overcome these limitations[105], and the phosphoramide prodrug approach proved successful for the HCV NI sofosbuvir[106].

The activity of host kinases is influenced by the viral infection. HIV-1 and DENV were reported to increase cytokine levels, causing activation of peripheral blood mononuclear cells (PBMC), which results in lower phosphorylation efficiency of some NIs[107,108]. This effect, in addition to lower potency in DENV-infected hepatocytes, explains the efficacy failure of balapiravir against DENV in clinical trials, despite its established activity *in vitro*[91,107]. Noteworthy, the influence of PBMC activation on phosphorylation appears to be scaffold-specific, since the cytidine-based balapiravir was more pronouncedly affected than the adenosine-based NI, **NITD008**[107]. Both PBMC and hepatocytes are key host cell types targeted by DENV, which highlights the role played by flaviviral tropism in the efficacy of the tested NIs.

The second important issue related to NIs is their insufficient selectivity against off-target polymerases and toxicity, caused in part by inhibition of the mitochondrial DNA polymerase-γ[109]. The difficulty to predict the side effects of NIs during *in vitro* testing appears to a main reason for failures at the clinical stage[110].

To minimize toxicity, a combination strategy may prove useful. Antiviral synergy was observed for a combination of INX-08189 with ribavirin in cell-based assays of DENV[111],

and for **NITD008** with vorinostat (SAHA), a histone deacetylase inhibitor, against WNV infection in C57BL/6 mice[112].

Recently, allosteric inhibitors of DENV NS5 polymerase were reported[113] to be active against DENV 1–4 in biochemical and cell-based assays[113]. The inhibitors target the "N pocket" near the active site, thus interfering with the conformational changes required during transition of the RdRp from initiation to elongation[113]. Residues lining the N pocket are conserved across other flaviviruses[113], which may offer an alternative to NIs, but so far the activity was assessed only in DENV. A potential problem with this strategy is the expected low genetic barrier to resistance, as observed for allosteric inhibitors of HCV polymerase[114].

Apart from the flaviviral protease and polymerase, other viral targets showed varying potential for broad-spectrum antiviral effects. In these cases, the promising activity profile covering more than one flavivirus was mostly observed for few examples within a particular class. **Entry/fusion inhibitors** act by targeting E-protein, interfering with the viral lipid bilayer or the host membrane. Studies aiming at inhibition of flaviviral entry by interference with host receptors did not yield notable results with the exception of Hsp70-ligands[115], which are discussed in the section ***Host targets*.**

With respect to heparan-sulfate proteoglycans as host cell receptors for viral attachment, we kindly refer the reader to the section on cellular assays (Box 2), where the characteristics of these disputable targets are discussed in more detail.

The flaviviral **E-glycoprotein** mediates the first steps of viral infection by attachment to the host cell, entry, and membrane fusion[116,117]. The structure of the E-protein was first elucidated for TBEV[118]. The envelope protein is composed of three ectodomains and the stem anchor that provides a link to the viral membrane[117–119].

A hydrophobic site between the domains I and II of E-glycoprotein was found to bind to n-octyl-β-D-glucoside (β-OG) – which was present in very high concentrations in the crystallization buffer – in one of the crystal structures of DENV-2 E-protein[119], and hence is referred to as the (β-OG) pocket. Compounds binding to this pocket are suggested to interfere with conformational changes of the E-protein required for fusion[119]. However, the validity of the β-OG pocket as target for antiviral drug discovery is doubtful. Several other crystallization experiments with flaviviral E-proteins also included β-OG and similar detergents in high concentrations, but found no occupation of the hydrophobic pocket by these compounds[117,118,120–122]. The conservation of the residues lining this pocket is limited and it does not appear to be a viable target for broad-spectrum agents. Nevertheless, virtual screening at the β-OG pocket identified two drug-like compounds with nanomolar to low micromolar antiviral potency in cell-based assays[123,124]. The supposed interaction with the β-OG pocket could not be confirmed by structural, biochemical, or resistance selection studies. Phenotypic assays for viral entry/fusion and time-of-addition studies are therefore the only confirmation for the assumed mechanism of action of these compounds, and it appears likely that they act by another mechanism. The first compound, a thiophene-quinazoline derivative, (**cpd. 6**) exhibited a broad spectrum of activity against DENV 1–4, YFV, WNV, and JEV[123]. Time-of-addition studies at DENV verified an effect of **cpd. 6** at

an early stage of the viral lifecycle[123], but no further assessment in animal models was performed. The second compound, **cpd. A5**, a phenyl hydrazone derivative, was active in plaque assays against DENV-2, WNV, and YFV[124], but the expected toxic liabilities associated with the phenyl hydrazone moiety could hinder further development of this substance.

Natural products such as griffithsin (GRFT) and squalamine were reported as entry/fusion inhibitors for a number of viruses, including flaviviruses, with efficacy in mouse models[125]. GRFT, a 13 kDa lectin isolated from algae[125], mediates its effects by binding to oligosaccharides at the surface of enveloped viruses[125–128] and was tolerated as systemic antiviral with minimal toxicity following subcutaneous administration in mice[129]. However, being a xenogeneic protein, GRFT may trigger an immune-mediated response of varying severity. Therefore de-immunizing the molecule was recommended before long-term treatment is envisaged[129]. Squalamine, a cationic aminosterol, is proposed to disturb the electrostatic interaction between virus and host membranes during the early steps (entry/fusion) in the viral life cycle or also the late stages of virion assembly/budding[130]. The proposed mechanism was not yet confirmed by time-of-addition studies.

T-cell immunoglobulin and mucin (TIM) proteins are receptors of the apoptotic marker phosphatidylserine (PS), and phosphatidylethanolamine (PE)[131]. TIM receptors can promote viral entry through binding to virion-associated PS and PE through apoptotic mimicry mechanism[132,133]. Duramycin-biotin inhibits TIM-1 mediated entry of DENV-2, WNV, and EBOV at submicromolar concentrations without detected cytotoxicity[131]. Although duramycin-biotin has less hemolytic effects than duramycin, the suitability of the compound for clinical use in the case of hemorrhagic viral infections needs to be assessed. Furthermore, the strategy of interfering with PS or PE to inhibit viral entry should be tested in animal models to evaluate the safety profile considering the potential for interference with other cellular processes that depend on TIM-mediated binding to PS or PE in host cells.

Compared to E-protein, the **capsid protein** received minimal attention in the past years. The flaviviral capsid is a dimeric protein with a high density of positively charged residues at the surface and a hydrophobic core pocket. The monomer unit contains four α-helices and an N-terminal disordered region as elucidated for WNV[134] and DENV[135] capsid structures. The N-terminal region was implicated in interactions with lipid droplets[136] and VLDL[137]. A single small-molecule inhibitor, **ST-148**, has been identified by phenotypic HTS screening followed by resistance selection[138]. The spectrum of antiviral effects in cell-based assays covered DENV 1–4, Modoc virus (MODV), YFV, and HCV (but not JEV) with favorable CC_{50}-values[138]. Despite its poor oral bioavailability, **ST-148** displayed efficacy in the AG129 mouse model for DENV infection. **ST-148** interfered with both assembly/release and entry of DENV infectious particles probably by stabilization of the capsid protein structure and enhancing capsid self-interaction[139].

Numerous studies were directed at inhibitors of the flaviviral **NS4B** protein[140,141], a highly hydrophobic protein with integral membrane topology[142]. **NS4B** mediates several interactions with other non-structural viral proteins and host proteins to modulate viral replication[143,144]. Compounds targeting **NS4B** lacked broad-spectrum antiviral

activity[140,141]. When assessed against multiple flaviviruses, inhibitory effects were limited to a single virus, or even specific serotypes[145,146]. The single exception is **lycorine**, which could reduce viral titers for WNV, DENV-2, and YFV[147]. Resistance to **lycorine** at WNV was conferred by V9M mutations in the 2K peptide located between NS4A and NS4B[147]. A modification of the structure to **1-acetyl-2-oxo-lycorine** provided a slightly enhanced potency at WNV with remarkable improvement in cytotoxicity[147,148].

In contrast to the flaviviral capsid protein and NS4B, targeting NS3 helicase/ATPase and NS5 methyltransferase/guanylyltransferase is often complicated by the need to achieve selectivity against host enzymes with similar functions.

The flaviviral **helicase** belongs to the helicase superfamily 2 (SF2), and is located at the C-terminal domain of **NS3**[149,150]. It is responsible for unwinding of viral RNA during replication, and the activity is driven by an intrinsic nucleoside triphosphatase activity[149,150]. The structure of NS3 helicase has been elucidated for many flaviviruses[151], and recently for ZIKV[152]. NS3 helicase comprises three subdomains with the well-conserved ATP binding pocket being located between subdomains 1 and 2. A long tunnel runs across the protein and is expected to accommodate the viral RNA[151,152].

A benzoxazole analogue, **ST-610**, was reported as inhibitor of helicase activity[153]. The compound displayed low cytotoxicity, inhibited viral replication of DENV 1–4 and YFV (but not WNV or JEV) in different cell types, and reduced viral load in DENV infection mouse model[153]. A pyrrolone derivative (**cpd. 25**) inhibited viral replication in cell-culture by targeting helicase-catalyzed ATP hydrolysis, without any effect on HCV helicase[154]. The compound acted at WNV and DENV, albeit with low SI[154]. Another compound that has demonstrated inhibitory activity against YFV, DENV-2, and WNV helicase in the upper nanomolar range[155], as well as weak inhibition of DENV protease[156] is **ivermectin**, which is discussed in detail in the section ***Compounds with other and unknown mechanisms of action***.

The N-terminal domain of the flaviviral **NS5** protein functions as **guanylyltransferase**[157,158] and **methyltransferase**[159,160]. The latter catalyzes N7 and 2′-O methylation reactions using SAM as methyl donor[159,160], and is inhibited by the nonselective competitive inhibitors SAH and sinefungin[161]. In addition to the SAM pocket, the crystal structure of DENV-3 and WNV methyltransferase revealed a conserved hydrophobic cavity next to the SAM binding site, which could be used to design specific inhibitors against flaviviruses[162,163], but structural evidence for the binding of compounds to this pocket is missing. Introduction of a silyl group at the 5′-position of azidothymidine-based triazoles, a class with potent antiviral activity against HIV-1, resulted in inhibitors of DENV and WNV methyltransferases[164]. The compounds showed antiviral effects in DENV and WNV replicon assays, and DENV plaque assay, but relatively high cytotoxicity. Docking studies suggested the positioning of the bulky 5′-silyl group in the hydrophobic cavity, located near the SAM binding site[164]. Using virtual screening at WNV methyltransferase, a compound (**NSC 12155**) was identified with MTase inhibitory activity at WNV, DENV-2,3, and YFV in enzymatic assays. **NSC 12155** reduced viral titers for WNV, DENV-2, JEV, and Saint Louis encephalitis virus (SLEV)[165].

Host targets

Flaviviruses interfere with the host cell in numerous ways. Some cellular pathways may be upregulated to promote replication, while other functions of the cells, particularly those related to cellular immune response, are suppressed by the virus. Interference with processes exploited or controlled by the virus has therefore long been considered a conceptually promising route towards antiviral treatment, albeit with limited success so far.

Since certain host factors are usurped by a large number of viruses, one would expect these targets to convey a broader spectrum of antiviral activity. In addition, host factors are expected to be less prone to resistance development, even though some cases of resistance have already been reported (see below). As it will be shown here for several cases, broad-spectrum activity must be carefully confirmed on a case-by-case basis for each antiviral agent to avoid activation of other viral co-infections and coverage of all co-circulating flaviviruses. A further, and very obvious, complication is that the interaction with host factors involved in the normal physiological function of the cell has a higher potential for side effects, and it does not appear easy to strike a balance between antiviral activity and toxicity. In addition, resistance development has also been described for a number of experimental compounds that interfere with host factors.

A general, cautionary remark must be made with respect to the “re-purposing” of host targets between different viruses. For example, CCR5 antagonists were developed for anti-HIV-1 therapy and suggested for treatment of DENV infection[166]. However, there is conflicting evidence for the role of CCR5 in JEV[167] and WNV[168] infections, and anti-HIV CCR5 ligands could be totally inefficient or even aggravating in these infections. This demonstrates that even approved therapies targeting host factors should be cautiously evaluated for each group of viruses to be covered by them, or that may be present as (unapparent) co-infections in the patient.

Some of the most promising or interesting classes of compounds acting against host factors will be discussed here, based on their mechanism of action. The structures of selected compounds are presented in Figure 3. Other classes, such as SAH hydrolase, and autophagy inhibitors are not discussed in the text due to limited broad-spectrum activity or high toxicity of known compounds that does not even allow their tests *in vivo*. Still, these targets are mentioned in the targets-assays table in the SI. Nuclear transport inhibition is discussed as one of the mechanisms of action of **fenretinide** and **ivermectin** in the section ***Compounds with other and unknown mechanisms of action***.

α-Glucosidase is a host enzyme that removes glucose units from N-linked glycans and thereby participates in the maturation and folding of flaviviral glycoproteins[169]. Glucosidase inhibitors have broad-spectrum antiviral activity *in vitro* and *in vivo* against a multitude of enveloped viruses[170,171], including flaviviruses, and *in vivo* confirmed high genetic barrier to escape mutations[172]. The most promising glucosidase inhibitors are the iminosugars, such as castanospermine (CST) and 1-deoxynojirimycin (DNJ). Their main disadvantages are high dosages, relative toxicity, and weak activity during the post-infection period. Previous studies of iminosugars *in vivo* and in clinical treatment of infections produced by other

enveloped viruses (HIV, HCV, influenza)[171,173,174] have demonstrated that most of their disadvantages can be overcome by derivatization into prodrugs, association with other antivirals, and/or by early (possibly: prophylactic) treatment.

CST is a potent antiviral compound *in vitro* and *in vivo* against all DENV serotypes, but it has much lower activity against YFV and no effect on WNV[169,175]. Its 6-*O*-butanoyl derivative, **celgosivir**, is an oral prodrug that is 100-fold more active *in vitro* and 2 times more active *in vivo*[176,177]. **Celgosivir** failed in a proof-of-concept clinical trial in patients with dengue fever[178], and is probably more efficient if treatment starts on the day of infection[177]. Increased doses of **celgosivir** initiated on the 2nd or 3rd day post infection significantly reduce viremia, and a phase II clinical trial (NCT02569827) with an optimized **celgosivir** regimen was recently approved in Singapore[177].

A N-nonyl-derivative of DNJ inhibits *in vitro* replication of JEV and DENV-2[179], and further structural modifications afforded derivatives with lower toxicity and higher activity. The introduction of a cyclohexyl group in the N-alkyl chain resulted in enhanced potency against DENV, WNV, and bovine viral diarrhea virus (BVDV) and an improved safety profile[180]. Modification by oxygen-containing functionalities in the N-alkyl side chain results in higher activity against DENV-2, and to a lesser extent against WNV and BVDV[181]. Increased cellular uptake appears to be the main reason for improved activity in this compound class. Further optimization of the pharmacokinetic profile led to derivatives with low toxicity and good oral bioavailability, but a narrow therapeutic window in AG129 mice, limited to the first 48 hours post infection[182–184].

α-Glucosidase inhibitors can therefore be considered as broad-spectrum, drug-like antivirals. Their main drawback – the necessity to initiate treatment very soon after infection – will likely also apply to other treatments that interfere with flaviviral replication, such as helicase and protease inhibitors. In this respect, the exploration of iminosugar antivirals may have yielded a generally applicable conclusion: emergency prophylaxis under epidemic conditions may be more promising than post-infection treatment. If the treatment is started after symptomatic diagnosis of the infection, a more aggressive dosing and treatment regimen, as in the example of **celgosivir**, appears to be necessary to reduce viremia. Considering that α-glucosidase is a host factor, and that high doses of iminosugars are not well tolerated in humans, a low-dose emergency prophylaxis regimen could be more promising both with respect to efficacy and safety than the treatment of verified infections.

Despite the high potential of **nucleoside biosynthesis inhibitors** for broad-spectrum antiviral activity and the considerable knowledge on this class of compounds, the results obtained so far are modest and remain largely restricted to one compound in clinical use: Ribavirin was one of the first broad-spectrum antivirals on the market and is commonly used to treat HCV infections[185,186]. The antiviral activity of ribavirin has been explained with inhibition of the host inosine monophosphate dehydrogenase (IMPDH), inhibition of viral polymerase and RNA capping, a mutagenic effect on viral RNA and/or immunomodulation[187]. It has been shown to be active against flaviviruses *in vitro* only in high concentrations[188], while *in vivo* studies or clinical application often gave negative results[189,190] or showed activity only in early phases of the infection[191,192].

Development of more active IMDPH inhibitors – with the intention to enhance antiviral potency – resulted in compounds with higher cytotoxic or immunosuppressive activity such as 5-ethynyl-1-beta-D-ribofuranosylimidazole-4-carboxamide (EICAR; 5-ethynylribavirin)[193], which demonstrated significantly higher activity against MODV, YFV, and DENV than ribavirin. Cytostatic effects comparable to those of 5-fluoruacil[194] and a narrow therapeutic window prohibited their clinical use as antivirals A non-nucleoside mycophenolic acid (MPA)[193] has been reported to be highly active against DENV[193], YFV[193], JEV[195], and ZIKV[12], but its immunosuppressive activity limits its potential as antiviral compound[196].

Brequinar is an inhibitor of dihydroorotate dehydrogenase (DHODH), a host enzyme responsible for pyrimidine nucleoside biosynthesis. Brequinar has potent antiviral activity *in vitro* against DENV, WNV, YFV, and POWV[197], but was not approved for clinical use due to a low therapeutic index[198]. Other promising non-nucleoside DHODH inhibitors are the indole derivative **cpd. A3**[199] and 2-(4-benzyl-3-ethoxy-5-methyl-1H-pyrazol-1-yl)pyrimidine[200]. The latter compound has been described as low-nanomolar DHODH inhibitor[200], whose activity against flaviviruses is as-yet unknown. **Cpd. A3** demonstrated broad-spectrum antiviral activity against multiple viruses *in vitro* in the submicromolar range and no resistance development in influenza virus[199,201] but its *in vivo* activity or toxicity data are not available[197].

The main obstacles for the development of nucleoside biosynthesis inhibitors as antiviral agents are a narrow therapeutic window and the potential for immunosuppressive effects, properties that are incompatible with expected co-infections, pregnancy, and extended (prophylactic) dosage regimens. Another problem is the possibility of resistance development, which can appear via different mechanisms depending on the target and virus. The latter issue could be addressed by compounds that inhibit multiple biosynthetic steps or by combination therapy with antivirals acting via other mechanisms.

Immunosuppression is also a key pharmacological property of the **cyclophilin** inhibitors. Cyclophilins are peptidyl-prolyl isomerases that facilitate protein folding and play an important role in viral replication. Inhibition of cyclophilin A (CyP A) by **cyclosporine** has been demonstrated to reduce interaction with flaviviral NS5 and produce antiviral effect against DENV-2, WNV, and YFV in cells[202]. **Cyclosporine** was more effective against DENV-2 and YFV, and less effective against WNV[202]. Recently, cyclosporine has been shown to be effective against ZIKV in some *in vitro* tests[12]. Since the immunosuppressive activity of **cyclosporine** is attributed to the inhibition of the protein phosphatase calcineurin[203] and the binding domains for CyP A and calcineurin are located at different sites of **cyclosporine** molecule[204], it is possible to design non-immunosuppressive CyP A inhibitors. The non-immunosuppressive (non-calcineurin inhibiting) CyP A inhibitor alisporivir (Debio 025)[205] was developed as anti-HCV agent, but its efficacy against flaviviruses remains unknown.

Lipid biosynthesis, signaling, and metabolism[206] have long been a subject of research with respect to other diseases, such as atherosclerosis, resulting in numerous well-characterized drugs and drug candidates that can potentially be re-purposed as broad-

spectrum antiviral drugs. For example, inhibition of acetyl-CoA carboxylase (ACC) and FASN[207,208] has been linked to an antiviral effect. Dose-dependent inhibition of DENV-2, YFV, and WNV replication has been demonstrated for the FASN inhibitor 4-methylene-2-octyl-5-oxotetrahydrofuran-3-carboxylic acid (C75)[207], which has also been reported to suppress the formation of intracellular lipid droplets that occurs in cell infected with DENV[136,207]. However, FASN inhibitors have been described to cause severe anorexia and weight loss[209], and inhibition of ACC appears to have a higher clinical potential for the treatment of viral infections[209]. From the recently studied ACC inhibitors, “TOFA” (5-(tetradecyloxy)-2-furoic acid) and “MEDICA 16” (3,3,14,14-tetramethylhexadecanedioic acid) have been reported to induce dose-dependent reduction of WNV and Usutu virus (USUV) replication[210].

Pharmacological interference with the biosynthesis of host sphingomyelin (SM) can lead to different reactions, depending on the type of virus studied, including the flaviviruses[211,212]: the tricyclic antidepressants amitriptyline and imipramine, inhibitors of acid sphingomyelinase, which hydrolyzes SM to ceramide, have been reported to decrease infectivity of pseudotype JEV in pretreated Huh-7 cells[213]. At the same time, inhibition of neutral sphingomyelinase by GW4869 suppressed the release of WNV viral particles from HeLa, Vero, and C6/36 cells, as well as of USUV from HeLa cells, but had the opposite effect for Sindbis virus[214] from the alphavirus family. On the other hand, inhibition of SM biosynthesis by the sphingomyelin synthase inhibitors SPK-601 and MS-209 reduced the production of infectious viral particles in WNV-infected Vero cells[214]. **Fenretinide** (4-HPR), an inhibitor of ceramide synthase and dihydroceramide desaturase, is discussed in the section ***Compounds with other and unknown mechanisms of action***.

Conflicting results have also been obtained for inhibitors of cholesterol intracellular transport and biosynthesis, which, however, could be explained by differences in the modelling of the experiments[215,216]. Another obstacle, similar to development of inhibitors against other targets is lower efficiency *in vivo* in post-infection treatment. The HMG-CoA reductase inhibitor **lovastatin** has demonstrated increased survival rates for all treatment regimens *in vivo* against DENV-2 infection[217], but a reduction of viremia could be observed only in pre-treated animals. Considering the broad usage and good tolerability of the statins, these appear to be candidates for an (emergency) prophylactic antiviral regimen.

Flaviviruses, as well as the majority of other viruses, usurp a large number of **host kinases** at various steps in their cellular lifecycle. Flaviviral proteins from the RNA replication complex, such as JEV NS3[218] and DENV NS5[219] were shown to be phosphorylated with participation of host kinases. Kinase inhibitors, extensively explored for applications in oncology, therefore offer opportunities for antiviral repurposing. However, several challenges and caveats must be considered.

First, resistance development – which is usually considered less probable for antivirals acting at host targets – can occur via mutations in the viral proteins that act as kinase substrates. For example, the DENV-2 NS4B-T108I mutation confers resistance against RNAi mediated depletion of the Fyn kinase or its inhibition by **dasatinib** and AZD0530[220]. Even though this type of resistance is unlikely to be developed during normal transmission

of DENV between human and mosquito hosts, it nevertheless demonstrates the potential of resistance development against kinase inhibitors.

Second, significant unwanted effects of the repurposed cancer-related kinase inhibitors (**dasatinib** and its analogues) can arise from their "original" pharmacodynamic profile, but also from the reactivation of latent, silent viral infections, like HBV[221,222]. Therefore, this does not appear to have potential as a first choice treatment option for persons with chronic and latent viral infections, in long-time prophylaxis treatment, and during pregnancy. Moreover, inhibition of lymphocyte-specific protein tyrosine kinase by **dasatinib** and AZD0530 can have a detrimental effect on the cellular immune response[223].

Third, kinases have varying expression levels and functions, depending on the cell and virus type, and some of them participate in the cellular immune response. Certain kinases play an important role in mediation of antiviral IFN-dependent cell protection, like JAK-STAT signalling. IFN-induced κB kinase ε signalling, via STAT1 phosphorylation and induction of IFIT2 expression, restricts WNV infection and pathogenesis[224]. Therefore, each potential antiviral kinase inhibitor should be checked against inhibition of immunologically important kinases.

The outlook for kinase inhibitors as broad-spectrum anti-flavivirals is mixed: resistance development is not ruled out; repurposing of anticancer kinase inhibitors appears risky; and multi-target kinase inhibitors may lead to activation of dormant, unrelated viral infections. An alternative approach could involve the development of inhibitors with increased selectivity or dual action on viral and host targets, possibly at the expense of broad-spectrum activity, or the development of non-antiviral kinase inhibitors which modulate the host response immune and therefore the severity of symptoms. One may also envisage the use of small-molecular activators acting at pro-immunogenic kinases, to counteract the viral suppression of the cellular immune response.

Another broad-spectrum antiviral strategy is inhibition of **polyamine biosynthesis**[225]. Polyamines play an important role in both translation and transcription of ZIKV and CHIKV[226]. A variety of viruses, including flaviviruses, are sensitive to compounds altering polyamine levels: **eflornithine**, an ornithine decarboxylase inhibitor, and diethylnorspermine, an activator of the spermidine/spermine N1-acetyltransferase[225]. Eflornithine has shown efficacy in animal models against CHIKV and coxsackievirus B3, also following post-infection administration. Eflornithine has low toxicity, good stability, was approved for the treatment of African trypanosomiasis, and proposed for chemoprevention[227]. Its main disadvantage is the requirement of high doses. It has been suggested that development of more potent derivatives or a combination with other therapeutics may enhance its antiviral activity[225].

Several groups of compounds reported to target pleiotropic host targets such as **ribosomes** (lactimidomycin and its derivatives), **proteasome** (bortezomib and its derivatives), **DDX3**, and **Hsp70**, have been recently found to possess broad-spectrum anti-flaviviral activity *in vitro*[12,15,115,228,229]. However, most of these compounds show side effects that significantly limit their potential use as (prophylactic) antiviral. The 60S ribosome blocker

lactimidomycin is highly cytotoxic[230,231], and bortezomib, a covalent-reversible inhibitor of the 26S proteasome, has been labeled as class D for potential teratogenic effects[232]. Inhibitors of DDX3, the proteasome, and Hsp70, targets contested between virus and the immune system, have been shown to present potential for reactivation of chronic co-infections like HCV in the case of DDX3 inhibition[233] and varicella zoster virus and HBV in proteasome inhibition by bortezomib[144,234]. Moreover, inhibition of Hsp70 could reduce protection against other infections[235–237] and tumors[238], and inhibition of the proteasome could even enhance some infections[239–241]. While these difficulties prohibit the use of these compounds in long-time treatment of persistent infections, prophylaxis, and pregnancy, they may be tolerable for the short-term treatment of flaviviral infections.

Compounds with other and unknown mechanisms of action

A number of compounds with antiviral activity against flaviviruses were identified from phenotypic assays. Although the molecular target of these compounds has not been identified in many cases, the spectrum of activity of these examples is considered a promising starting point for further investigation and drug development efforts. Of particular interest are repurposed drugs, due to their established safety and pharmacokinetic profile. The structures of selected compounds are provided in Figure 4.

Nitazoxanide, an antiparasitic ester prodrug used for the treatment of diarrhea caused by *Cryptosporidium parvum* and *Giardia intestinalis* infections[242], was found to exert antiviral effects against a broad range of RNA and DNA viruses[243–246]. **Nitazoxanide** (or its active metabolite **tizoxanide**) inhibit flavivirus replication in cell-culture for JEV[245], DENV-2 and YFV[246], and provide protection against JEV in mouse model[245]. The antiviral efficacy and lack of adverse effects of **nitazoxanide** treatment, either alone or in combination with other antiviral agents, was demonstrated in patients infected with HCV[247], rotavirus[248], norovirus[248], and influenza[249]. The drug is currently under investigation in phase III clinical trial (NCT02612922) for treatment of influenza. The mechanism of action of **nitazoxanide** at flaviviruses was not investigated, but for other viruses the drug was found to interfere with glycosylation of viral proteins and production of mature viral particles[250,251], in addition to inducing antiviral innate immunity[252,253]. The removal of the nitro group in **RM-5038** (or its active metabolite **RM-4848)** is considered an attractive modification for this class, considering the toxicological liability of this moiety[242]. Another antiparasitic drug, niclosamide, was identified from a phenotypic screening[254] as potent inhibitor of ZIKV in different cell types, although it displayed some degree of cytotoxicity[254].

Bromocriptine, an agonist of dopamine receptors 2 and 3, was identified from a screening of pharmacologically active compounds against DENV in focus reduction assays[255]. The spectrum of activity covered DENV 1–4, and to a lesser extent TBEV[255]. The antiviral effects were not observed with other dopamine agonists, quinpirole and rotigotine. A mutation in the NS3 helicase domain was identified in escape mutants, but could only confer minimal resistance to the drug effect, suggesting the involvement of other viral or host proteins in the mechanism of action. Unfortunately, **bromocriptine** lacked efficacy in the AG129 mouse model[255].

The antiviral activity of the dihydrodibenzothiepines can probably not be explained by a single mechanism of action. A representative of this group – SKI-417616 – has been shown to display inhibitory activity against DENV 1–4, WNV, as well as SINV, via antagonism at dopamine receptor d4 with subsequent inhibition of downstream phosphorylation of epidermal growth factor receptor-related kinase (ERK)[256]. However, other cellular signaling pathways besides ERK also seem to be involved, and further investigations in this direction are necessary.

A number of antimalarial drugs, containing a quinoline scaffold, were evaluated for their antiviral activity against DENV[257–259] or WNV[257]. Amodiaquine reduced DENV-2 replication assay, and inhibited DENV-2, DENV-4, and WNV replication but with rather poor selectivity[257]. Interestingly, other antimalarial drugs of the same class were reported to interfere with different steps of the DENV life cycle: Amodiaquine is proposed to affect the initial steps of RNA replication and to a lesser extent entry[257], hydroxychloroquine activity is mediated through activation of the host immune system[259], while **chloroquine** is suggested to act during entry or assembly, as it lacked activity in the replicon assay[257]. Despite the efficacy of **chloroquine** against DENV-2 in monkeys[258], the compound failed to reduce viremia in dengue patients[260,261]. A positive effect on acute dengue symptoms was observed, which could be related to **chloroquine**'s anti-inflammatory effect, a pharmacological property of the drug that forms the basis for its medical use in rheumatic diseases[262]. **Chloroquine** may be a candidate for prophylactic use, considering the previous, extensive clinical experience with this drug in the context of malaria.

A cardiac glycoside, lanatoside C, was reported to exert potent antiviral effects against DENV 1–4, KUNV, and other RNA viruses[263]. Analysis of the mechanism of action suggests possible targeting of the viral RNA synthesis[263]. Digoxin, another cardiac glycoside, was recently evaluated against ZIKV and displayed antiviral effects[12]. However, considering the narrow therapeutic index of digoxin, the authors suggested that the concentrations needed for anti-Zika effects may reach toxic levels[12].

Screening of a compound library in a DENV-2 replicon assay resulted in the identification of a lead compound (cpd. 15a) with antiviral activity against DENV-2 and YFV in the low micromolar range[264]. Systematic optimization of the aromatic rings in the original imidazole 4,5-dicarboxamide (I45DC) scaffold of cpd. 15a allowed modulation of the inhibitory potency and cytotoxicity of the obtained analogues[264,265]. The most promising antiviral profile against DENV-2 and YFV was observed for cpd. 7g[265].

A group of hydroxyquinoline derivatives has been reported to activate IRF3 through mitochondrial antiviral signaling and drive antiviral gene expression in cells. This upregulation of the **innate immune response** leads to an antiviral effect against multiple viruses, including DENV-2, WNV, HCV, EBOV, and Lassa virus[266,267]. However, the molecular target of the compounds has not been identified. Moreover, *in vivo* studies are necessary to exclude that activation of the immune response produces undesirable side effects.

Multiple mechanisms of antiviral activity have been suggested for **ivermectin**, shown to be active *in vitro* against multiple flaviviruses, including DENV 1–4[268] and ZIKV[12]. The compound has been shown to block interaction of DENV 1–4 NS5 with importin α/β1 (IMPα/β1), a nuclear protein import receptor[268], making it the main mechanism of its antiviral activity. It was active in a cell-based flavivirus immunodetection assay for DENV 1–4 in the low-micromolar range[268]. As demonstrated previously, DENV NS5 contains a nuclear localization sequence (NLS) that confers interaction with the IMPα/β1 dimer and exportin receptor CRM1[269,270]. The NLS is highly conserved in the flavivirus genus, and consequently a very attractive target for broad-spectrum antiviral development[270]. Besides, it has been demonstrated that inhibition of CRM1 by leptomycin B caused increase in nuclear accumulation of NS5, suppression of IL-8 induction, and augmentation of DENV-2 production in cells[270]. **Ivermectin** also inhibits helicase unwinding activity for YFV, DENV-2, and WNV in the upper nanomolar range[155], and shows weak inhibition of DENV protease[156]. Even though these are probably not the main mechanisms of antiviral activity of **ivermectin**, their contribution should be considered.

Another compound with multiple mechanisms of antiviral activity is **fenretinide** (N-(4-hydroxyphenyl)retinamide, 4-HPR), a retinoic acid derivative, which protects cells against DENV 1–4[271]. In addition, the compound has demonstrated anti-WNV[271,272] and anti-HCV[272] activity in cells and anti-DENV-2 activity in AG129 mice[271,272]. The main mechanism of **fenretinide** activity appears to be inhibition of interaction of viral proteins with the IMPα/β1 importin receptor. Besides, the compound has been demonstrated to induce phosphorylation of eukaryotic translation initiation factor 2α, controlling translation attenuation and thus promoting an antiviral state[273]. Moreover, **fenretinide** influences the ceramide homeostasis by inhibiting ceramide synthase and dihydroceramide desaturase. However, the latter two activities do not appear to contribute to the antiviral effect of the compound[272].

Phosphorodiamidate morpholino oligomers (PMOs) are uncharged, water-soluble compounds that contain nucleobases attached to a backbone of morpholine rings connected via phosphorodiamidate linkages and block the interaction of viral RNA with ribosomes. Arg-rich peptidic conjugates of PMOs (PPMOs) were reported to have a higher permeability across cell membranes[274], and positively charged PMOs (PMO*plus*) were more efficient due to improved binding kinetics[275]. PMOs, PPMOs, and PMO*plus* have several important advantages as compared to siRNA, while being similar in structure and mechanism of action: resistance to enzymatic degradation and a good level of safety as shown in clinical trials, i.e. for eteplirsen (AVI-4658) and AVI-7288[276–278]. Their mechanism of action is steric blockade of complementary RNA that suppresses the formation of the 43S preinitiation complex and ribosome scanning during viral translation or blocks RNA replication[279,280]. PMOs complementary to the viral UTR and their peptide conjugates are relatively specific in their antiviral activity and, with respect to the antiviral spectrum, best cover closely related viruses[279,281]. While structural features of the viral UTR are relatively well-conserved, the sequences are divergent, which limits the broad-spectrum potential of compounds acting at this target. The therapeutic windows in mice have been shown to be very small for some compounds, with the toxicity and activity of PPMOs being generally

higher, due to better pharmacokinetics[280]. The treatment with PMOs and PPMOs is effective if started as early as possible, might be totally ineffective at later stages, and requires parenteral administration[279–281]. Nonetheless, phase I clinical trials in nonhuman primates and humans of a PMO*plus*, AVI-7288, specifically targeting the mRNA sequence of a filovirus – Marburg virus (MARV) – nucleoprotein, demonstrated that it is possible to achieve survival rates of 83–100% when the drug is administered up to 4 days after animals infection with MARV, while maintaining a good pharmacokinetics and toxicity profile[277,278]. This observation demonstrates a good level of activity and low toxicity for this group of compounds.

Conclusion and Outlook

The current upsurge of interest in anti-flaviviral drug discovery and flavivirus biology, triggered to a large part by the Zika epidemic, will certainly lead to an increased understanding of these important pathogens. As in other “hot” areas of drug discovery, some of the currently proposed targets, pathways and compounds may later turn out to lack sufficient maturity for further development. The present review attempts to provide some indication as to which approaches currently appear, or have already been shown, to be most promising.

The design and development of new anti-flaviviral compounds must take their activity spectrum into consideration, and a strong preference must be given to drug candidates that are active against the largest number of co-circulating viruses. This is particularly important for compounds that target host factors, since even closely related flaviviruses interact differently with cellular components. Somewhat unexpectedly, drugs that target host factors are not exempt from resistance development. Furthermore, the targeting of host factors with multiple functions – besides their involvement in viral replication – may be associated with severe side effects.

Antiviral treatments should generally be initiated as soon as possible after infection, or as prophylactic measures. This is particularly true for acute flaviviral infections, where the most severe sequelae occur after the peak viremia has passed. Antiviral agents that target the viral non-structural proteins or other replication-relevant factors will probably be most efficient in prophylaxis and treatment of early-stage or persistent sub-clinical infections, and less promising for advanced stages of acute disease. Even more effective for the prophylactic purposes could be compounds that target the entry of the virus in the cell. Unfortunately, developments in the latter direction have not yielded any tangible results so far.

In case of persisting flaviviral infections, it is important to adjust the pharmacokinetic parameters of the antiviral compound in order to ensure its penetration – or even accumulation – in the most affected organs or tissues. In the case of severe disease, which is frequently caused by a pathologic immune response, a pharmacological interference with this ill-directed host reaction appears promising. For this particular approach, the repurposing of established immunomodulatory drugs could be envisaged.

Taking together the current situation, we expect to see a two-tiered approach: in the near-to-midterm, drug-repurposing in connection with phenotypic screens has the potential to yield “emergency” antivirals, for which a higher incidence of side effects and limited broad-spectrum activity can be tolerated. In the longer term, and in consideration of the recent developments within related antiviral fields such as HCV and HIV, it can be anticipated that novel compounds acting at viral targets, and in particular at the evolutionary well-conserved enzymatic functions localized within NS3 and NS5, will allow us to counter the persistent public health risk that is posed by the already prevalent, as well as the still clandestine flaviviruses.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Box 1

Vaccination

Vaccines are available against a number of flaviviral infections: YFV; JEV; DENV (a tetravalent vaccine, approved in seven countries as of December 2016)[282,283]; TBEV; Louping ill virus (vaccination of sheep to prevent transmission to humans)[284]; and Kyasanur Forest disease virus (KFDV)[285]. The efficacy, safety and durability of anti-flaviviral vaccines vary widely, with the long-established YFV vaccine having relatively favorable properties, such as long-lasting effect and low level of resistance development. Several important lessons (and caveats) can be learned from the development of anti-flaviviral vaccines in the past:

- Antibody-dependent enhancement (ADE): prM antibodies cause mostly ADE and induce less viral neutralization, as was demonstrated in DENV[286]. While antibodies to the fusion-loop epitope of glycoprotein E are mostly involved in development of ADE, the antibodies targeting the quaternary site of the E glycoprotein, conserved in DENV and ZIKV[287], possess broadly neutralizing activity[288,289].
- Cross-protection: For some viruses like TBEV and JEV, new vaccines produce reliable cross-protection for all viral subtypes[290,291]. The only available tetravalent DENV vaccine has lower efficiency against some serotypes due to multiple genotypes within serotypes of the virus[292,293]. Moreover, it has been demonstrated that the vaccine's efficiency is higher in seropositive than in seronegative individuals, with the latter usually found in the youngest age group (2-5 years) and particularly this group showed more cases of dengue shock syndrome upon infection[10]. As a consequence the vaccine's minimum licensed age is 9 years. Recent evidence *in vitro* suggests that the presence of antibodies to DENV, either from previous infection or from vaccination, has the potential to induce ADE of ZIKV infection[294]. Therefore, broad protection against all four DENV strains and ZIKV could be a crucial safety requirement especially in the Zika naïve population. Additional *in vivo* studies are required to further study this issue.
- The durability of immune protection varies widely, ranging from 3–5 years for TBEV[295] to (frequently) lifetime immunity for JEV and YFV[296].
- More side effects for live-attenuated vaccines vs. inactivated vaccines, partially related to adjuvants, like gelatin- and/or chicken egg derived proteins, which could cause severe allergic reactions[293].
- Chimeric vaccines against other flaviviral infections[293] can be developed from the widely employed, safe and well-characterized YFV-17D live-attenuated vaccine.

Box 2

In vitro assays for anti-flaviviral compounds

A number of **cell-based phenotypic assays** have been developed to screen antiviral compounds against flaviviruses. These can be classified into 3 main groups (see Supplementary information, Table SI-1): (i) assays using live viruses (LV); (ii) assays that employ subgenomic viral replicons (VRPs) containing a subset of viral genes that are required for replication; and (iii) assays using virus-like particles (VLPs) containing viral E and prM glycoproteins and no viral RNA[297]. The first group, and in particular the cytopathic effect (CPE) and plaque assays, are relatively time- and resource-intensive, but represent the reference standard for antiviral screening. Modifications of the CPE assay were devised to allow a screening of compounds in medium-throughput format (see Table SI-1), which has proven particularly valuable in target-independent drug repurposing approaches, where the number of screened compounds is limited[298]. Replication-competent viruses are also used to evaluate candidate antivirals that have been identified by other – usually target-oriented – means. The main disadvantage of the LV assays is the obvious necessity of high-level biosafety containment, high labor intensity and cost. VRP and VLP assays overcome safety concerns. However, cases of replication competent VRPs are known[299], and VRP and VLP assay results must be validated carefully in order to avoid false-positive hits resulting from cytotoxicity or interaction with the luciferase readout. An advantage of VLP as compared to VRP is their capacity to identify entry inhibitors in addition to replication inhibitors.

A potentially highly problematic issue that concerns the replication competent virus assays for antiviral compounds, as well as fundamental biological studies – in particular, with respect to host factors and entry receptors – are the inevitable adaptations of viruses that occur during extended cell culture and formation of intragenic variation[300]. Virus strains kept in cell culture may differ to a variable extent from the wild-type, leading to artifacts that cannot be extrapolated towards clinically encountered viruses. A noteworthy example are mutations in the viral E glycoproteins that increase the cellular attachment of viruses to the heparan-sulfate proteoglycans on the outer host cell membrane, an effect that has been described for a variety of viruses from several genera[301–304]. Obviously, then, the clinical relevance of heparan-sulfate proteoglycans as mediators of viral attachment and entry must be questioned, and thereby also the antiviral “drug” discovery work that was aimed at these targets. This consideration, along with other – particularly the questionable drug-likeness of the compounds, and the scarcity of broad-spectrum antiviral data – motivated us to exclude mimics of heparan-sulfate from the present review. Such compounds were repeatedly put forward as ligands of the viral E protein, intended to interfere with viral attachment[305,306].

Biochemical **viral target assays** aim at inhibitor screening against viral structural and nonstructural proteins (Table SI-1). The feasibility, ease, and robustness of enzymatic assays have contributed significantly to the discovery of compound that inhibit the enzymatic functions of the viral NS3 and NS5 proteins, especially the flaviviral NS2B–NS3 protease[70] and the NS5 polymerase[307]. Furthermore, linking a process, such as

membrane fusion, to an enzymatic reaction has allowed the development of biochemical assays to identify fusion inhibitors[308].

High-throughput approaches that are capable of interrogating a multitude of gene interactions have accelerated the discovery of **host factors** relevant for viral replication. Subsequent enzymatic or binding assays can then be used to perform compound screens in analogy to viral targets, against host factors involved in the viral life cycle from entry to egress, and resulted in the discovery of kinase, inosine monophosphate dehydrogenase, proteasome and other inhibitors and ligands for Hsp70, importin, *etc.* (cf. Table SI-1). Finally, metabolomic studies led to the discovery of pyrimidine biosynthesis and glucosidase inhibitors.

Box 3

Animal models

Many flaviviruses do not cause symptomatic disease in non-human primates (NHPs) and immunocompetent mice. However, many[309,310] NHPs develop viremia and neutralizing antibodies, although the levels of viremia may be low in some instances [311]. Upon (intracranial) infection with DENV, immunocompetent mice die from paralysis, but do not develop the hemorrhagic complications that are fatal in human disease[312]. Immunocompetent mice are therefore not well suited to serve as model organisms for drug discovery, and, as a first step, immunodeficient mouse models for DENV have been developed to mimic the course of human disease more closely[313,314].

The A129 mouse model, lacking type I interferon receptors, and the AG129 mouse model, lacking type I and type II interferon receptors, both develop DHF/DSS-like symptoms when infected with adapted DENV[313]. AG129 is more often used in antiviral testing because it has been characterized to a larger extent and develops human-like symptoms at a lower viral challenge dose. However, due to absence of the interferon-ɣ pathway, DENV replicates uncontrollably in the AG129 CNS, causing paralytic death about 10 days post infection. Besides, the effector function of T cells in AG129 cannot be measured. The A129 mouse is better suited for the investigation of immune mechanisms and can be thus used for testing antivirals and vaccines[314]. Both mouse models have also been found suitable for antiviral screening and vaccine testing against ZIKV[36]. In addition, AG129 was reported to be useful for testing antivirals against YFV and JEV[315–317].

To further improve the understanding of flaviviral pathogenesis and immunology, immunocompromised mice have been transplanted with human stem cells that allowed the development of a functional human–like immune system. Upon infection with DENV-2 strain, the humanized NOD-*scid IL2rγ* mice show typical symptoms of dengue disease, along with increased cytokines and chemokine levels[318,319]. However, the infection could not be detected in the liver and the production of antibodies was low or absent[318,319].

To improve T cell functionality, immunocompromised NOD-*scid* mice have been transplanted with small pieces of autologous fetal liver and thymus. These are implanted under the kidney capsule and then injected with stem cells, resulting in so-called “bone-marrow/liver/thymus” (BLT) mice. Infection of these mice with DENV-2 resulted not only in increased viremia and cytokine levels, but also caused production of DENV-2 neutralizing human IgM antibodies[320].

For some neuroinvasive flaviviruses (like OHFV), BALB/c and C57BL/6 mouse models have been shown to efficiently reproduce the pathology of infection in humans with mild meningoencephalitis, little cerebral and significant cerebellar involvement[321,322]. For persistent CNS and renal infection with WNV, Syrian golden hamsters (*Mesocricetus auratus*) were described as suitable hosts[323–325]. Moreover, it has been shown that WNV

infection in hamsters better reproduces the course of infection in humans and horses than the mouse model[323].

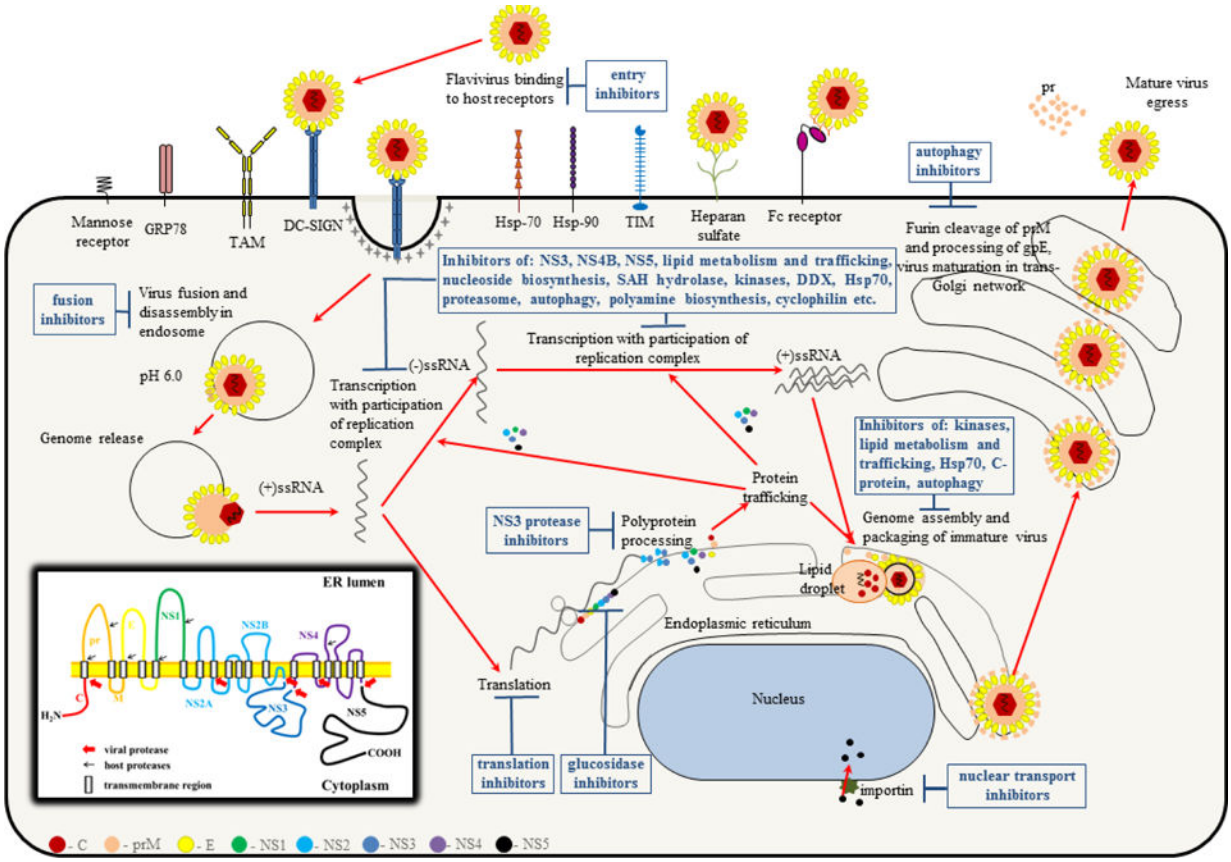

Figure 1.

Replication cycle and polyprotein organization of flaviviruses. A number of putative host cell receptors for flaviviruses is indicated at the cellular membrane, with significant evidence indicating the importance of DC-SIGN. The insert in the lower left corner shows the sequential and structural organization of the flaviviral polyprotein at the endoplasmic reticulum membrane, with the cleavage sites of the host and viral proteases. Note the color coding of the viral proteins, indicated at the bottom. C – capsid protein, prM – membrane protein, E – envelope protein, NS1, NS2, NS3, NS4, NS5 – nonstructural proteins 1–5.

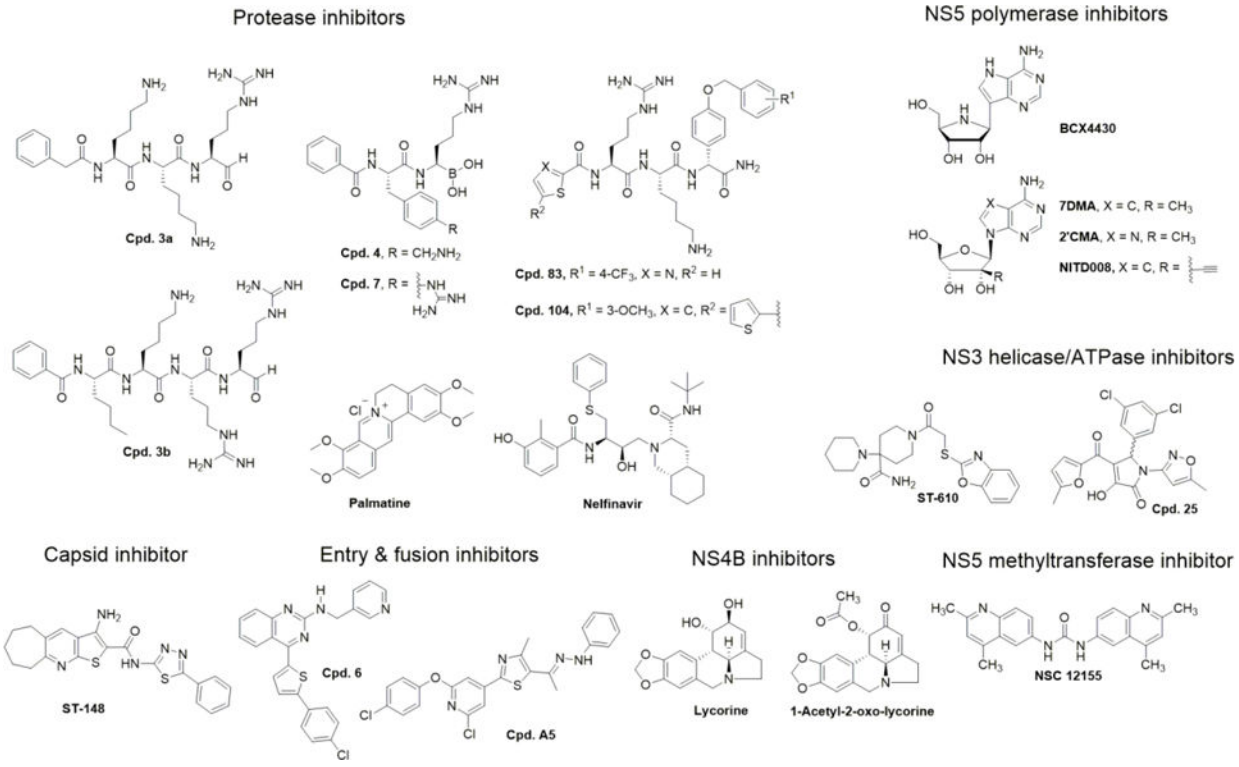

Figure 2.
Compounds acting at viral targets.

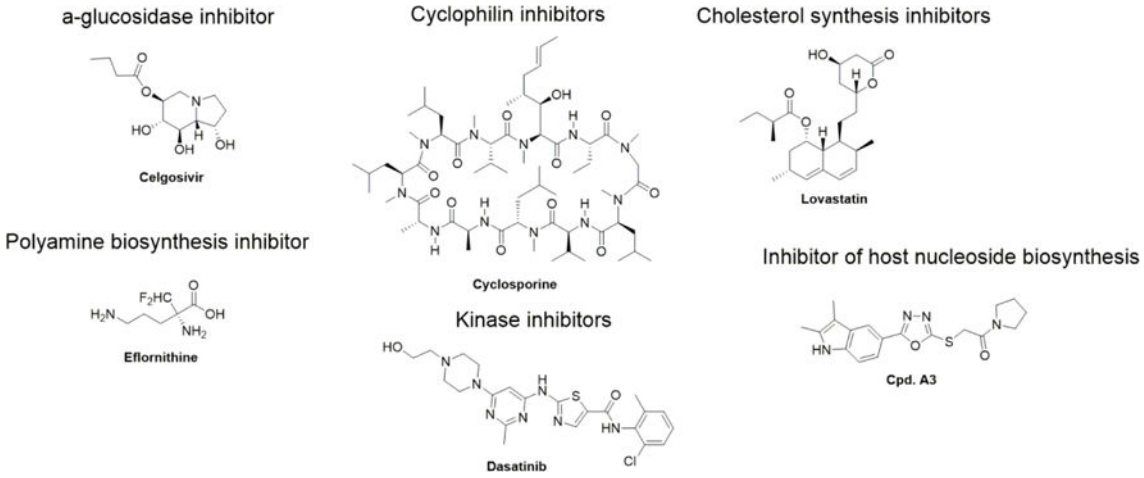

Figure 3.
Compounds acting at host targets.

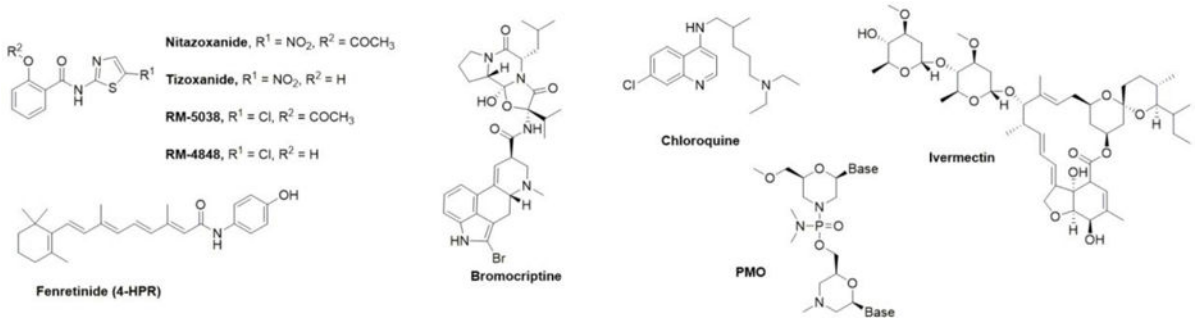

Figure 4.
Compounds with other and unknown mechanisms of action.