REVIEW ARTICLE

Review of Current Non-Vaccine Based Approaches for the Prevention and Treatment of Flaviviral Diseases

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Abstract

Although there are nearly 70 medically important Flaviviruses including Dengue and Zika, which cause over 100 million new infections each year with significant morbidity and mortality, vaccines are available for only a few of these viruses. Furthermore, some of these vaccines require lengthy immunization courses or multiple boosters subjecting recipients to potential gaps in protection. Therefore, non-vaccine based prophylactics and therapeutics could play a significant role in filling these gaps. Unfortunately, despite over a decade of research only a few antiviral drug candidates have been advanced to clinical trials and for most of these the results have been disappointing. Also, funding for antiviral research has been relatively scant in recent years. Nevertheless, there are reasons for optimism. There has been significant progress made in our understanding of Flaviviral infections, in the identification of promising drug targets and transmission abatement strategies, and in the establishment of protocols for drug discovery and for conducting human clinical safety and efficacy trials. Multipronged efforts are currently ongoing to evaluate highly diverse approaches including (i) small molecule antivirals, (ii) virus-neutralizing and infection-blocking antibodies, (iii) viral receptor antagonists, (iv) infection-blocking oligonucleotides, and (v) strategies targeting the arthropod vectors. A few areas of research that deserve special mention are phosphorodiamidate morpholino oligomers and peptide-linked phosphorodiamidate morpholino oligomers (PMOs/PPMOs), a class of infection-inhibiting, antisense oligonucleotides, which already have shown promising clinical safety and efficacy data, and the novel strategies being developed aimed at reducing viral transmission in endemic areas by targeting the arthropod vector-hosts, which have also shown promise. The hope is that renewed commitment by government, academic, and private institutions will lead to licensed antiviral therapeutics, prophylactics, and other strategies aimed at preventing Flaviviral diseases in the not too distant future.

Keywords: Flaviviruses, Dengue, Zika, Diseases, Prevention, Treatment, Non-Vaccine

1. Introduction

The socioeconomic impact of acute viral diseases is enormous. For example, the common cold is estimated to cost more than 40-billion dollars ($40B) per year in the US alone[1] due to lost work time, physician visits, and over-the-counter medications. Nevertheless, for the many viruses implicated in acute human infection and disease there are little more than a dozen FDA licensed vaccines[2] and some of these, e.g., the inactivated influenza vaccine, are known to be minimally effective, especially in the elderly[3]. Considering that there are now almost 70 arthropod borne flaviviruses circulating worldwide, licensed vaccines exist for only a handful - Yellow fever virus, YFV (reviewed in [4]), Japanese encephalitis virus, JEV (reviewed in [5]), Tick-borne encephalitis virus, TBEV (reviewed in [6]), and most recently, the four Dengue virus, DENV, serotypes (reviewed in[7,8]), which alone are estimated to be responsible for nearly 400 million primary and secondary infections per year. Furthermore, some concerns have been raised about the overall effectiveness of the sole, recently licensed tetravalent Dengue vaccine, CYD-TDV (Dengvaxia™) developed by Sanofi Pasteur, which is based on the DENV structural antigen genes expressed from a live, attenuated YFV 17D vaccine backbone. After nearly half a decade of use in the field this vaccine is estimated to be about 60% effective overall at protecting against disease after primary vaccination, although the efficacy increases after 12 months following the full three-dose immunization schedule. The protection rate appears to be somewhat lower for DENV type-2 (DENV 2), and analysis of emerging data suggests that the vaccine is more effective at preventing hospitalizations in individuals who have had at least one DENV infection before vaccination but less effective in seronegative individuals including children and possibly travelers[9]. The recent emergence of Zika virus (ZIKV), another member of the Flavivirus family, has further challenged vaccine developers and public health authorities because of its potentially devastating reproductive and neurological side effects[10].

Although safe, effective, and affordable vaccines should be our ultimate goal, other strategies, including anti-viral drugs, surely deserve serious consideration. This is especially true in cases where vaccines do not exist and are unlikely to be developed, e.g., for so-called 'orphan' viruses, and where existing vaccines exhibit low efficacy rates or require prolonged dosing schedules to elicit a protective immune response making them poorly suited to travelers, for example. That non-vaccine based strategies can potentially provide at least an interim solution or backstop in lieu of vaccination is best exemplified by the successful development of licensed antiviral drugs for the treatment of chronic viral infections including from the human immunodeficiency virus (HIV), which causes acquired immune deficiency syndrome (AIDS), Hepatitis C implicated in hepatocellular carcinoma, and the Herpesviruses[11]. Unfortunately, examples of the use of antiviral drugs for prevention or treatment of acute viral infections are more limited but include Tamiflu (oseltamivir), Relenza (zanamivir), Rapivab (peramivir) and related compounds aimed at reducing morbidity and mortality from seasonal influenza[12], although the risk-benefit of some of these drugs has been recently called into question[13]. A few of the important considerations and challenges for development of non-vaccine based, antiviral strategies in general, in addition to economic ones, are (i) how best to establish overall safety and efficacy of a given platform framed in terms of risk-benefit, (ii) the development of safe human challenge/infection models for testing efficacy at relatively early stages, e.g., in Phase 1/Phase 2[14], and (iii) accurate assessment of the risk for viral escape variants, which may be mitigated to some extent by the choice of highly conserved drug targets. Therapeutic drugs, i.e., those administered post-symptom, as well as post-exposure prophylactics would seem to be best suited for acute viral diseases with relatively long incubation periods, which would extend the therapeutic window, but could also be used in other cases to shorten the disease course and reduce disease severity and complications. For example, there may be particularly good indications for their use against neurotropic flaviviruses such as West Nile and Zika, where systemic infection with mild disease is relatively common during outbreaks but is sometimes followed by rare (<1%) but severe

neuroinvasive disease[15], and in the case of Zika where there appears to be the added risk for a Guillain-Barre (G-B) like syndrome after natural infection[16], and possibly even after vaccination. Similarly, drug and other non-vaccine interventions could have utility against secondary Dengue infections where early febrile disease may be followed by dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). These severe diseases are typically preceded by a period of defervescence, which is then followed by a second burst of amplified viral replication thought to be at least partly immunologically mediated[17]. With accurate diagnosis and risk assessment, drugs that target and block this second burst of viral replication may significantly reduce disease severity and deaths by supplementing current supportive treatments.

The Flaviviruses, on which this review will mainly concentrate, comprise a genus of enveloped, single-stranded RNA viruses transmitted to humans by arthropod, mainly mosquito (primarily *Aedes* sp.) vectors, within the family *Flaviviridae* [18]. For some Flaviviruses, including DENV, humans are the only known natural host if one excludes the sylvatic DENVs, which infect non-human primates. DENV infects its host cells by a number of primary and secondary cellular receptors including DC-SIGN[19] and highly sulfated heparin sulfate[20] among others. In addition, antibody complexes of DENVs, and possibly other Flaviviruses as well, are able to infect macrophages, monocytes, and some dendritic cells via an alternate Fc-receptor (FcR)-mediated route[21]. This can result in antibody-dependent enhancement (ADE) of infection believed to contribute, at least in part, to more severe disease, DHF/DSS[22]. Following virus entry three structural proteins (C, prM/M, and E glycoprotein) and seven nonstructural (NS) proteins (NS1 glycoprotein, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) are produced by translation of the message-sense, monocistronic, virion RNA followed by post-translational proteolytic processing by both viral and host proteinases, including NS2B-NS3 and the host enzyme furin[23]. The Envelope (E) glycoprotein, the major virion surface protein, contains three domains and mediates viral entry into host cells (putatively via interaction between E Domain III and cellular receptors), and contains most of the important virus-neutralizing epitopes [24,25]. The NS proteins are involved in virus replication with NS5 acting as a viral RNA dependent RNA polymerase/methyltransferase and NS3 acting as a helicase/protease, while NS1, NS2A, NS2B, NS4A, and NS4B function as essential components of the host cell membrane-associated viral replication complex [26]. RNA synthesis and virion assembly appear to be closely coupled processes [27]. Some viral proteins including NS1, NS4A, NS4B and NS5 appear to have dual roles in modulating viral replication and the host innate immune response [28,29]. Thus, for Dengue and other Flaviviruses there are a number of attractive targets for antiviral drug therapies and other non-vaccine interventions. In the following sections we will briefly review some of the more promising and clinically advanced approaches under investigation. The reader is also encouraged to refer to some recent, very comprehensive reviews of anti-flavivirals [30-33].

Despite much work, the majority of antiviral drug candidates developed thus far for Flaviviruses have shown only limited success, mainly due to toxicity at therapeutic levels in small animal models, which usually halts further development. Therefore, relatively few candidates have advanced to human clinical trials with mainly disappointing results. The lack of good animal disease models, especially for the non-neurotropic Flaviviruses such as DENV, should also be mentioned as a significant impediment to drug development. The AG129 mouse, in which both alpha- and gamma-interferon genes are silenced, is the most widely used small animal model although there are others [34]. Although imperfect, since they may not mimic human disease, some of these small animal models at least allow for morbidity and mortality (which is typically due to encephalitis) endpoints, and can to some degree replicate the increased risk for DHF like syndromes including capillary leak, which may occur in the presence of infection-enhancing antibody [35]. Of note, a Dengue human infection model (DHIM) such as developed by researchers at the Walter Reed Army Institute of Research (WRAIR) [14] may serve to facilitate testing of future candidate dengue vaccines and

antivirals under highly controlled conditions and at relatively early stages in clinical development (reviewed by [36,37]). (See Table 1 for or an overview of current non-vaccine based approaches for the prevention and treatment of flaviviral diseases).

Finally, public funding for research on Dengue and other arthropod-borne viral diseases is relatively low, at least by current US standards, and this could slow the development of antivirals. However, given the potential market size for antiviral drugs for Dengue, and now Zika, the private sector may be induced to pick up the slack, especially if provided some incentives.

Table 1. Overview of Current Non-Vaccine Based Approaches for the Prevention and Treatment of Flaviviral Diseases

Approaches	Leading Candidate(s)	Status
Small molecule antivirals		
-Synthetics	Celgosivir (host alpha-glucosidase inhibitor)	Modest efficacy vs. DENV in clinical trials
		DENV clinical trial planned
	Celgosivir + Modipafant (anti-inflammatory)	Effective in cell culture vs DENV, no efficacy shown in clinical trial
	Balapiravir (nucleoside analogue pro-drug)	Blocked ZIKV and WNV infection of human placental and amniotic sac-derived cells
	Duramycin (antibiotic)	Inhibited ZIKV replication and cytopathic effect in human astrocyte and glial cell cultures
-Plant-derived bioflavonoids, alkaloids, terpenoids, and polycyclic quinones	Azithromycin (antibiotic)	Inhibited DENV replication in cell culture; reduced viral load in a non-human primate infection model
	Melaleuca alternifolia (Tea Tree) oil (anti-oxidant)	
Virus-Neutralizing and Infection-Blocking Antibodies		
-Polyclonal antibodies	Immune serum globulin (ISG) and Intravenous immunoglobulin (IVIG)	Possibly effective at treating WNV encephalitis in a clinical setting; ADE is a concern for some flaviviruses
-Monoclonal antibodies	Anti-WNV monoclonal antibodies Mab11 and Mab11/mutFc	Effective against DENV 2 in a non-human primate infection model
Host Cell Receptor Antagonists and Soluble Virus Receptors	Soluble, highly-sulfated heparin sulfates to block early viral attachment	Anti-viral activity demonstrated in cell culture
Ribo- and Deoxyribo-oligonucleotides		
-Antisense PMO/PPMOs	AVI-4658 (muscular dystrophy); AVI-7288 (Marburg virus)	Successful in early phase clinical safety and efficacy trials
	PPMOs vs. DENV 5′ terminal stem-loop (5′SL) and 3′-cyclization sequence (3'CS)	Blocked DENV infection in AG129 mouse model
-small-interfering RNA (siRNA) and micro-interfering RNA (miRNA)	21-mer anti-DENV 1 prM siRNA	Reduced DENV 1 infection in cell culture by > 80%
Arthropod Vector-Targeted Strategies		
-Lethally mutated arthropod vectors	Release of insects carrying a dominant lethal mutation (RIDL technology) using genetically modified male mosquitoes	Pre-deployment proof-of-concept stages
-Wolbachia-infected mosquitoes	Release of *Wolbachia pipientis*-colonized *Aedes aegypti*	Successful releases in Northern Australia, Vietnam, and Indonesia aimed at reducing DENV transmission
-Host anti-vector immunity	Host immunization against mosquito C-type lectins (mosGCTLs)	Early pre-clinical proof-of-concept stages

2. Small Molecule Antivirals

Dengue antiviral drug discovery has generally followed four main strategic pathways, which, while outside the scope of this review, are discussed in a comprehensive review of the ten-plus years of experience at the Novartis Institute of Tropical Diseases (NITD) [38]. The common drug discovery pathways can be summarized as: (i) High throughput screening (HTS) using viral replication assays in cell culture, (ii) HTS in viral enzyme assays, (iii) structure-base *in silico* docking and rational drug design, and (iv) repurposing of existing antiviral compounds, e.g., drugs effective against Hepatitis C, which is in the same genus as the Flaviviruses and has a similar genome organization and protein structural-functional relationships. Following the discovery of candidate compounds drug target sites are typically identified by isolation and genetic sequencing of resistant mutants/variants. This information can then be used to make chemical modifications aimed at improving drug sensitivity/selectivity profiles.

2.1 Clinical Trials

Of the compounds advanced to testing in the AG129 mouse disease model at Novartis only a few showed efficacy but most were eliminated due to toxicity or lack of potency (reviewed by [38]). Only one compound, Celgosivir, a host alpha-glucosidase inhibitor shown to inhibit virus morphogenesis in vitro was advanced to human clinical trials [39]. In a phase 1b, randomized, double-blind, placebo-controlled, proof-of-concept trial, volunteers aged 21–65 years who had a fever (≥38°C) for less than 48 hrs and Dengue infection demonstrated by RT-PCR assay were randomly assigned (1:1; random permuted block length four) to receive Celgosivir at an initial oral dose of 400 mg, followed by 200 mg every 12 h for a total of nine doses (24 patients), or a matched placebo (26 patients). Celgosivir was safe and well tolerated and resulted in a modest reduction in viremia and fever burden compared to placebo but this was not statistically significant [39]. Although the initial results were somewhat disappointing it might be possible to increase the pharmacological effect by earlier treatment, higher doses, or by combination with other drugs such as ribavirin [40]. Therefore, further testing would seem to be warranted, and a future clinical trial is being planned in Singapore to look at Celgosivir and Modipafant (an anti-inflammatory drug that blocks platelet activation) as possible treatments for adult patients with uncomplicated Dengue fever (DF) (see ClinicalTrials.gov).

Another randomized, double-blind, placebo-controlled trial was carried out in Vietnam in early acute phase DF patients to look at Balapiravir, a prodrug of the nucleoside analogue 4′-azidocytidine, which was developed for the treatment of chronic hepatitis C Virus (HCV) infection as R1479 by Hoffmann-La Roche but deemed too toxic for long-term use. Although the HCV and Dengue viral RNA polymerases have similar protein structures, and Balapiravir was moderately effective at reducing DENV replication in mammalian cell culture at pharmacologically attainable concentrations, it did not reduce virus burden in the patients or lead to an earlier resolution of fever compared to placebo [41]. The disparate in vitro and in vivo results were further examined in another study, which demonstrated a marked inhibition of prodrug conversion to the active drug form in DENV infected, immunologically-activated monocytes [42]. These results provide a possible explanation for failure in the clinical efficacy trial and also suggest that blocking infection of monocytic cells in the human host may be important for the prevention and treatment of Dengue disease.

Compared to Dengue, the development of antiviral drugs for Zika is very early in pre-clinical development. However, a few lines of investigation in particular deserve mention. In one study the FDA approved antibiotic, Duramycin, was found to inhibit infection of human placental and amniotic sac-derived cells by both Zika and WN viruses [43]. These cells express a protein called TIM1 which has been implicated in viral attachment so the drug may act extracellularly. A second study [44] evaluated several FDA approved drugs that are considered safe in pregnancy including Azithromycin, Daptomycin and Sofosbuvir (a viral RNA polymerase inhibitor) for their ability to inhibit ZIKV replication and

cytopathic effects in human astrocyte and glial cell cultures, potentially important host cell targets in the fetal brain. Both Zithromycin and Sofosbuvir showed efficacy whereas Daptomycin exhibited a less favorable dose-response curve. These positive *in vitro* results need to be confirmed in an animal model and if promising results are obtained then future clinical development is a possibility.

2.2 Natural compounds

Finally, brief mention should also be made of the use of natural (e.g., plant derived) products for the prevention and treatment of Flaviviral diseases. Herbal compound classes under pre-clinical investigation include plant bioflavonoids, alkaloids, terpenoids, and polycyclic quinones (reviewed by [45]). One such alternative drug under investigation is the oil extract from Melaleuca alternifolia (Tea Tree). It has been shown to have antiviral activity in cell culture and when added to purified DENV preparations, and also modest activity at reducing DENV 2 viremia and post-challenge antibody responses in a rhesus macaque infection model, when administered orally, compared with placebo (JRP, unpublished data). There are also anecdotal reports of its efficacy against Dengue disease in non-case-controlled clinical studies in endemic areas. For the natural drugs under investigation for their antiviral activities, the active ingredients have not been isolated in stable form with the possible exception of licorice-derived Gylcyrrhetinic acid derivative Carbenoxolone disodium [46]. As with all drugs and vaccines, batch-to-batch potency of naturally derived compounds remains an issue to be addressed; nevertheless, herbal and other natural medications surely have a place in antiviral drug research and development.

In summary, although some promising leads have been uncovered in the selection of suitable drug targets, and precedents and protocols have been established for conducting human clinical trials, much work remains before any small molecule antiviral can be successfully brought to market.

3. Virus-Neutralizing and Infection-Blocking Antibodies including Polyclonal and Human/Humanized Monoclonal Antibodies

Antibodies, both virus-neutralizing and non-neutralizing, are thought to be important correlates of antiviral immunity including to Dengue and other Flaviviruses [47], although their mechanisms of action are incompletely understood and absolute protective titers are established only for some viruses. In the case of JE, for example, in a mouse encephalitis model passively transferred human anti-JEV neutralizing antibody (from vaccine recipients) at a titer of around 1:10 correlates with immunity and protection against challenge [48]. The US FDA will often consider data from such relevant animal disease models under its "Animal Rule" for approval of new drugs or vaccines when placebo-controlled human efficacy trials are either not feasible (e.g., due to low infection rates) or unethical due to existing effective prophylaxis/therapy. Since immunity to Flaviviruses appears to be primarily mediated by antibodies rather than being cell-mediated, the use of therapeutic antibodies, either polyclonal or monoclonal, may be feasible as long as issues with ADE can be dealt with.

3.1 Utility of Immune Serum Globulin (ISG)

The use of immune serum globulin (ISG) for post-exposure disease prophylaxis, alone and in conjunction with vaccination, has long historical precedents, e.g., for prevention of hepatitis A [49]. In a mouse WNV encephalitis model, human or mouse derived WNV ISG administered before or after WNV challenge resulted in reduced morbidity and mortality even after CNS dissemination although the antibodies did not eliminate all virus tissue reservoirs [50]. In addition, case reports from Israel and from the US [51] suggest that high-titered intravenous immune globulin (IVIG) may be useful for the treatment of West Nile encephalitis, provided that therapy is initiated soon enough after symptom onset and diagnosis. Presumably, it could also have utility in treating infections with other neurotropic Flaviviruses. Furthermore, IVIG has been used prophylactically in organ transplant patients to prevent complications from infections

with adventitious agents including Echo, Parvo and Cytomegaloviruses [52,53]. Since some human IgG subclasses have *in vivo* half-lives greater than 30 days, passively administered antibodies could theoretically confer antiviral immunity for several months, but the main concern for Dengue, and other Flaviviruses including Zika, is that passively administered antibodies may lead to enhanced infection and more severe disease via the ADE-monocyte infection pathway as protective titers wane. Given that ADE seems to occur mainly with sub-neutralizing or sub-protective levels of antibody, this risk might be mitigated by using higher initial antibody doses or repeat administration to maintain protective levels. Also, since ADE is mediated through the antibody Fc portion, the use of IgG Fab or Fab(2) fragments should prevent it from occurring, although their half-life, avidity and efficacy may be less than intact IgG. Alternatively, antibodies with genetically modified Fc chains (see below) could also be used to prevent attachment to FcR-bearing host cells including monocytes and macrophages.

3.2 Polyclonal and Monoclonal Antibody Prophylaxis for Flaviviruses

The feasibility of antibody prophylaxis/therapy for Flaviviruses was demonstrated by several earlies studies using animal models where passively administered monoclonal and polyclonal antibodies directed against viral proteins, most notably the virion structural antigens E and prM/M, and the nonstructural antigen NS1 were shown to reduce viral burden and disease [54-58]. Antibodies against E and prM/M may reduce viral infection by several mechanisms, dependent upon the state of virus maturity reflected in the completeness of cleavage of prM to M protein [59], the specific epitopes recognized [60], the antibody subclass and Fc-type [61], and the particular host target cell(s). These mechanisms include the neutralization of free virus, blocking virus attachment to cellular receptors, or blocking subsequent steps in viral entry (For reviews see [62,63]). However, under certain conditions antibodies against both of these structural antigens, E and prM, may also enhance viral replication and infection, and the line between neutralization and enhancement is not always well-demarcated [64]. The mechanism of action for non-neutralizing antibodies, including ones against NS1 and other nonstructural antigens, are not yet fully understood but may involve antibody Fc-mediated effector activities [65] including killing of infected cells, e.g., via antibody-dependent complement-mediated immune cytolysis (ADCC), or down-regulation of infection via signaling pathways. However, like any disease abatement strategy, before antibody prophylactics and therapeutics can be taken to the clinic they must be shown not only effective but safe; more specifically, there should be no risk for ADE of infection or worsened disease.

A recent study in the authors' laboratories sought to establish early proof-of-concept for antibody prophylaxis of DENV infection using a non-human primate rhesus macaque infection model to evaluate several DENV neutralizing antibodies including a human polyclonal anti-DENV ISG preparation, and a human monoclonal DENV-neutralizing antibody (Mab11/wt) and its Fc-mutated version (Mab11/mutFc) for ability to protect against viremia following live DENV 2 challenge. At relatively low antibody doses (3-10 mg/Kg) only animals receiving the polyclonal ISG showed reduced viremia compared to saline placebo controls after challenge on day 10. However, when the antibodies were administered at a higher dose of 25 mg/Kg there was significant reduction in viremia not only in the ISG group (100%) but also in the groups that received Mab11 and Mab11/mutFc (> 80%). An *in vitro* ADE assay in Fc-gamma receptor-bearing K562 cells performed on sera collected immediately prior to challenge showed increased DENV-2 infection levels in the presence of both ISG and Mab11/wt, which peaked at a serum dilution of 1:90, but not in Mab11/mutFc containing sera. These results demonstrate the potential feasibility of at least short term antibody prophylaxis for Dengue and suggest that blocking FcR interactions through genetic engineering may be one way to mitigate the risk for ADE. However, the use of monoclonal antibodies still risks selection of antibody escape variants *in vivo* as was demonstrated in another virus challenge study in non-human primates [66],

although the use of antibody 'cocktails' should reduce this risk.

4. Host Cell Receptor Antagonists and Soluble Virus Receptors

Drugs that block attachment of Flaviviruses to host cells, an essential first step in infection, could, in theory, be used to significantly reduce disease burden in the human population. However, there are numerous obstacles and knowledge gaps that need to be overcome before such an approach is feasible. Firstly, the nature of receptors expressed on critical host target cells, which can be used by the virus to gain entry, is not completely understood and likely to be a large number. Secondly, DENV attachment and entry is a complex, sequential process [67] involving interactions with multiple ligands – (i) low affinity (non-specific) interactions, (ii) high affinity (specific) attachment to a primary receptor, and (iii) subsequent interactions with secondary and tertiary receptors as a requirement for endocytosis, membrane fusion, and un-coating. Furthermore, drug discovery screening may be further complicated if the virus receptor molecules expressed by cultured cells in vitro are different from those expressed in vivo, i.e., in the human host where there are likely to be multiple target cells/tissues depending on virus tropism (e.g., CNS, non-CNS, hematopoietic, and non-hematopoietic). Finally, the success of such strategies may depend upon a high degree of conservation of the receptor binding region on the virion (putatively E Domain III); otherwise, attachment and infection by drug resistant variants could still occur and such selection may also impact viral virulence, e.g., by biasing toward FcR-mediated infection and more severe disease. Unfortunately, attempts to block DENV replication *in vivo* using various ligands including soluble, highly sulfated heparans have for the most part been unsuccessful, and more research is needed in this area.

5. Antisense Oligonucleotides and RNA Interference (RNAi)

5.1 Small Interfering RNA

RNA interference (RNAi) via small-interfering RNA (siRNA) and micro-interfering RNA (miRNA) is an important antiviral defense mechanism for plants and invertebrates, and possibly mammalian cells, which could be exploited as an antiviral strategy (reviewed in [68,69]). That it may play a role in DENV infection is suggested by the finding that the NS4B protein of all four dengue virus serotypes is a potent RNAi suppressor in cell culture [70]. In another study to evaluate whether RNAi might suppress Dengue infection a 21 nucleotide-long siRNA targeting the prM gene of DENV 1 was synthesized and transfected into mosquito C6/36 cells followed by virus challenge. The results showed that the siRNA persisted in the cells for at least 7 days and that this was accompanied by a reduction in cytopathic effect (~80%), increased cell survival, and a significant reduction in DENV 1 viral RNA burden [71]. Based on these results the authors suggest that siRNA may offer a potential new strategy for prevention and treatment of Dengue.

5.2 Antisense Oligonucleotides

Similarly, antisense oligonucleotides were originally developed as a class of antiviral compounds to inhibit intracellular replication of viruses by interfering with transcription and/or translation, which showed initial promise at fighting DENV infection [72]. However, problems with their stability, solubility, bioavailability and specificity/affinity prevented this approach from reaching fruition. A new compound class, the phosphorodiamidate morpholino oligomers (PMOs), was then developed to achieve higher solubility, bioavailability and resistance to nucleases. PMOs are uncharged, water-soluble macromolecules with nucleic acid bases attached to a backbone of morpholine rings connected via phosphorodiamidate linkages. They act at multiple levels including by sterically blocking the interaction of viral mRNA with ribosomes, thereby suppressing the formation of the preinitiation

complex and ribosome scanning to inhibit translation (review in [73]). Conjugates of PMOs with arginine-rich peptides to produce peptide-linked phosphorodiamidate morpholino oligomers (PPMOs) may have the advantage of higher permeability across cell membranes and better binding kinetics although at the expense of marginally increased toxicity. PMOs and PPMOs may also have some important advantages over siRNAs including improved resistance to enzymatic degradation, and have demonstrated safety in clinical trials as was shown for test compounds AVI-4658 for muscular dystrophy [74] and AVI-7288 for Marburg virus [75]. In an experiment in the AG129 mouse model animal survival after DENV2 infection was increased significantly by early administration of PPMOs targeting the 5′ terminal stem-loop (5′SL) or the 3′-cyclization sequence (3'CS) regions of the DENV genomic RNA [76]. In trials in humans and non-human primates AVI-7288, a PMO complementary to Marburg mRNA nucleoprotein coding sequences, was effective at reducing morbidity/mortality when administered up to 4 days post-infection [77]. Thus, the PMO/PPMO and siRNA/miRNA technologies, perhaps combined with new delivery systems employing lipid nanoparticles [78], may have great promise as therapeutics.

6. Arthropod Vector-Targeted Strategies to Block Viral Transmission

For the majority of Flaviviruses mosquitoes act as both viral vector and amplifying host, which opens the door to several novel strategies aimed at breaking the cycle of virus transmission. As reviewed in [79] the number of human infections could be significantly reduced by decreasing the vector mosquito population or individual insect lifespan, by blocking/inhibiting human-to-mosquito or mosquito-to-human virus transmission, or by reducing viral replication in the mosquito vector. One novel method worthy of mention is the release of insects carrying a dominant lethal mutation (RIDL), a technology whereby genetically modified male mosquitoes carrying a late-acting, lethal, developmental gene are released into the environment to allow the lethal gene to be transmitted to mosquito progeny (reviewed in [80]), aimed at long term reduction in vector population and lifespan. Another promising strategy, which may or may not affect vector fitness, is the release of mosquitoes infected with the intracellular bacterium *Wolbachia pipientis* or other *Wolbachia* species (reviewed in [80]). In *Aedes aegypti*, Wolbachia colonization alters the reproductive system to enhance its vertical transmission between generations, but can sometimes reduce insect lifespan and, importantly, interfere with DENV replication at least partly through siRNA pathways, thereby reducing viral transmission. As noted in the earlier cited review field releases of Wolbachia-infected *Aedes aegypti* have occurred in Northern Australia, Vietnam, and Indonesia with the aim to suppress DENV transmission. More recently Wolbachia-infected *Aedes aegypti* were released in the US (Florida) in an attempt to suppress Zika transmission ('*The Scientist*': April 19, 2017). Although a promising approach, possible evolutionary effects on vector, virus or bacteria, which may lead, for example, to increased viral virulence remain theoretical concerns [81]. One additional strategy that deserves mention is based on the observation that antibodies directed against mosquito C-type lectins (mosGCTLs) are able to dramatically reduce mosquito infection rates in vitro [82](Liu et al, 2014), which suggests that passive or active immunization of people against mosGCTLs could be a novel way for blocking viral transmission. However, except for the use of Wolbachia, which has shown promising results in the field, the other approaches discussed above are very early in pre-clinical development.

7. Strategies for the use of Non-Vaccine Interventions with Vaccination

It may be feasible to employ multiple antiviral drug approaches together with viral vaccines in a complementary or synergistic way. In this way one could potentially mitigate any gaps in protection that might exist following primary vaccination until completion of the vaccine series with the final booster. Antiviral drugs could also be used for short-term protection, e.g., in vacationers to endemic areas. However, in all such cases care needs be used to ensure that the antiviral drug intervention will not interfere with

vaccination and the subsequent development of active immunity, as might occur with live-attenuated vaccines, for example.

Summary and Future Directions

While antiviral drug research is still in its relative infancy, considerable progress has been made in our understanding of Flavivirus replication, the identification of promising new drug targets, and in the development of protocols for drug discovery and clinical safety and efficacy trials. This ever-increasing body of knowledge, which builds upon and extends the current data, will undoubtedly set the groundwork for exciting new discoveries in the future. Current approaches with the potential to move relatively quickly through to licensure and commercialization include prophylactic and therapeutic antibodies, particularly if modified biochemically or genetically so as to mitigate the risk for ADE, and the PMO/PPMO class of antisense oligonucleotides, which already have shown promising clinical efficacy data. Also exciting are the novel strategies being developed aimed at reducing virus transmission by targeting the arthropod vector-hosts. Assuming that there are no adverse ecological side effects, this line of research has the potential to bear fruit in the near future, with tangible effects on reducing infection rates and disease in endemic areas. Another potentially fruitful area for future research is the development of drugs and other strategies to bolster the human innate immune response to viral infections, including use of compounds found in nature, to increase resistance to infection or lessen disease severity. However, continued progress will depend upon renewed commitment on the part of governments, public health authorities, and private companies.

Conflict of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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