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Dengue virus, West Nile virus, and Zika virus: potential novel antiviral drugs targeting flaviviruses currently at discovery and preclinical development stages

Rafaela da Costa Ricardo

Orientado por:

Ivo Cristiano Rocha Martins

Co-Orientado por:

Nuno Correia Santos

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ABSTRACT

Dengue, West Nile and Zika viruses are vector-borne flaviviruses responsible for numerous disease outbreaks in human populations, in both the Northern and Southern Hemispheres. Despite overall low mortality, infection may lead to potentially severe situations such as (depending on the virus): hypovolemic shock, encephalitis, acute flaccid paralysis, Guillain-Barré syndrome, congenital malformations (*e.g.*, microcephaly) and, in some instances, fatal outcomes. In parallel, outbreaks also have major socio-economic repercussions, especially in already vulnerable societies. Thus far, no specific treatments are available, and management is symptomatic. Dengvaxia was the world's first dengue vaccine and has been approved by health entities in some countries. However, specific criteria regarding its administration imposes limitations on its widespread use and it is not fully effective. Other prophylactic approaches against these viruses are even more limited. Therefore, developing potential therapeutic strategies is an urgent necessity and will be discussed hereafter. To do so, we will, in the following sections, first describe the epidemiology and the virus life cycle and structure. Then, we will report on the clinical presentation, diagnosis approaches and, finally, available vaccines, for these three flaviviruses. Later, we will provide examples of potentially promising compounds at discovery and preclinical development stages, identified, and deposited at the GlobalData database. These are divided into three main types, according to the main therapeutic molecule involved: antibody-based, peptide-based molecules and, other potential approaches. Finally, we discuss and compare the most promising developments, with the aim of ascertaining feasible future potential therapies against these flaviviruses of major concern to human health.

KEYWORDS
Flaviviruses; Dengue virus; West Nile virus; Zika virus; therapeutics.

RESUMO

Os vírus da dengue, Nilo Ocidental e Zika são flavivírus transmitidos por vetores artrópodes, sendo responsáveis por múltiplos surtos de doença em populações humanas, em ambos os hemisférios. Apesar da baixa mortalidade associada, a infeção por estes vírus pode culminar em quadros potencialmente severos, os quais variam de acordo com o vírus em questão, incluindo: choque hipovolémico, encefalite, paralisia flácida aguda, síndrome de Guillain-Barré, malformações congénitas (*e.g.,* microcefalia), podendo ser letal. Em paralelo com os efeitos na saúde, os surtos tendem a ter grandes repercussões socioeconómicas, especialmente em sociedades empobrecidas, já de si muito vulneráveis. Até à data, não existem tratamentos específicos e a gestão da doença é sobretudo sintomática. A vacina Dengvaxia contra o vírus dengue já foi aprovada em alguns países. Contudo, os critérios específicos quanto à sua administração impõem limitações ao seu uso generalizado. Além disso, a sua efetividade é relativamente baixa, nomeadamente em algumas faixas etárias mais frágeis. Por conseguinte, as abordagens profiláticas são bastante limitadas e o desenvolvimento de estratégias terapêuticas configura uma necessidade urgente. Nas próximas secções, iremos descrever primeiramente a epidemiologia, o ciclo de vida e a estrutura viral. Seguidamente, detalharemos a apresentação clínica destes três flavivírus, assim como as formas de diagnóstico existentes e vacinas disponíveis. Posteriormente, apresentaremos uma descrição dos compostos em fase de descoberta e/ou pré-clínica, identificados na base de dados GlobalData. Estes dividem-se em três subcategorias, de acordo com a molécula terapêutica envolvida: moléculas baseadas em anticorpos, em péptidos, e ainda, outras abordagens diversas. Por fim, apresentaremos uma breve discussão e comparação dos desenvolvimentos mais promissores para futuras estratégias terapêuticas contra cada um destes três flavivírus, de grande impacto na saúde humana.

PALAVRAS-CHAVE
Flavivírus; vírus Dengue; vírus do Nilo Ocidental; vírus Zika; terapêutica.

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1. INTRODUCTION

Flaviviruses are single-stranded RNA viruses (Pierson et al., 2020), being one of the most clinically relevant virus group amongst arboviruses (Harapan et al., 2020). Viruses of the *Flaviviridae* family are responsible for a spectrum of human diseases ranging from mild self-limited illness to severe life-threatening syndromes (Pierson et al., 2020). Many of these viruses can easily adapt to different host and environmental conditions, making them an epidemiological challenge that is somewhat difficult to manage and contain (Beckham et al., 2015). The global widespread and epidemic transmission over the last seven decades of several members of the *Flavivirus* genus, namely dengue (DENV), West Nile (WNV) and Zika (ZIKV) viruses, has been noteworthy (Pierson et al., 2020). Increasing unplanned urbanization, which tends to create ideal arthropod breeding habitats, extensive global travel and international trade (facilitating virus and vector geographical spread), environmental changes (namely climate change), and biological challenges (inherent to viral vectors management) are some of the factors that contributed to these viruses' expansion (Pierson et al., 2020; Silva et al., 2020).

This is clear concerning dengue as, since the turn of the millennium, the scientific community witnessed an increase in its incidence. Human transmission of any of the four DENV serotypes (DENV-1 to DENV4) occurs through *Aedes* spp. mosquitoes, specifically *Aedes aegypti* and *Aedes albopictus* (Pierson et al., 2020; Harapan et al., 2020; Silva et al., 2020). According to the World Health Organization (WHO), about half of the world's population may be at risk of developing DENV infection (World Health Organization, 2022). Besides the worrisome impacts on the populations' health, dengue infections also have repercussions on the affected regions' economy. The estimated total annual aggregate economic burden of dengue reached 8.9 billion USD in 2013, showing the dimension of the problem (Harapan et al., 2020).

Regarding WNV, infections are also associated with economic losses, not only related to treatment costs and morbidity losses, but also with intensive preventive control programs, plus the loss of animals/animal products (Habarugira et al., 2020). Mosquitoes of the genus *Culex* are considered the primary vectors of WNV (Pierson et al.; Habarugira et al., 2020). Historically, WNV outbreaks causing febrile illness occurred

sporadically in regions of Africa, the Middle East, Asia and Australia. Notwithstanding, in the 1990s, cases in Eastern Europe were associated with neurological disease and deaths (Pierson et al., 2020) and, more recently, outbreaks have been reported in non-endemic regions (Habarugira et al., 2020).

Concerning ZIKV, this flavivirus shares the same main vectors as DENV, namely *Aedes albopictus* and *Aedes aegypti* mosquitoes (Silva et al., 2020). During the 2015-2016 ZIKV epidemic outbreak in Brazil, an association between ZIKV infection and microcephaly in newborns was reported (Silva et al., 2020). One year later, the WHO declared ZIKV infection as a Public Health Emergency of International Concern. Other outbreaks soon appeared and, thus far, a total of 86 countries and territories have disclosed to the WHO evidence of mosquito transmitted ZIKV infection (World Health Organization, 2018).

As previously mentioned for WNV infections, several control and prevention strategies aimed at vector control have also been implemented for DENV and ZIKV. These strategies encompass mechanical, chemical, and biological methods and include methods such as surveillance through geographical mapping of virus foci, oviposition-based techniques, use of insecticides and plant derivatives, bacterial infection of vectors (e.g., *Wolbachia*, a parasite that interferes with essential mechanisms of the vector species) and genetic manipulation of mosquitoes (Rather et al., 2017; Rather et al., 2017; Wilson et al., 2020). In any case, direct measures against the mosquito vectors are the most effective. These include simple approaches such as disposal of containers serving as stagnant water deposits, which are easy to implement and constitute one of the most reliable strategies to avoid vector proliferation (Rather et al., 2017). Other measures specifically aimed at ZIKV infection are also recommended, such as safe sexual practices (given the possibility of sexual transmission) and avoiding travelling to endemic regions during pregnancy (Rather et al., 2017; Singh et al., 2018). Community-based control programs that promote the education of populations are also important (Rather et al., 2017).

Overall, as the vectors responsible for infection spread to other than tropical and subtropical regions, the diseases they convey are becoming more acknowledged by

health services (Silva et al., 2020). Therefore, the continued threat posed by flaviviruses highlights the imperative need for prophylactic approaches, as well as effective treatments, to alleviate their major health impact and financial burden in affected regions (Pierson et al., 2020; Silva et al., 2020).

2. EPIDEMIOLOGY

In the last decades, we have witnessed the emergence and re-emergence of dengue, Zika and West Nile viruses in both the Northern and Southern Hemispheres. DENV and ZKV are now two of the most epidemiologically concerning viruses globally (Harapan et al., 2020). Hereafter the epidemiologic aspects surrounding these viruses (and which help to explain their global prominence) will be discussed, to understand the key issues to be considered. One key factor explaining these viruses global spread is the concomitant expansion of their vectors, as exemplified here for *Aedes aegypti* and *Culex quinquefasciatus* mosquitoes, which global distribution has been spreading (Figures 1 and 2, respectively).

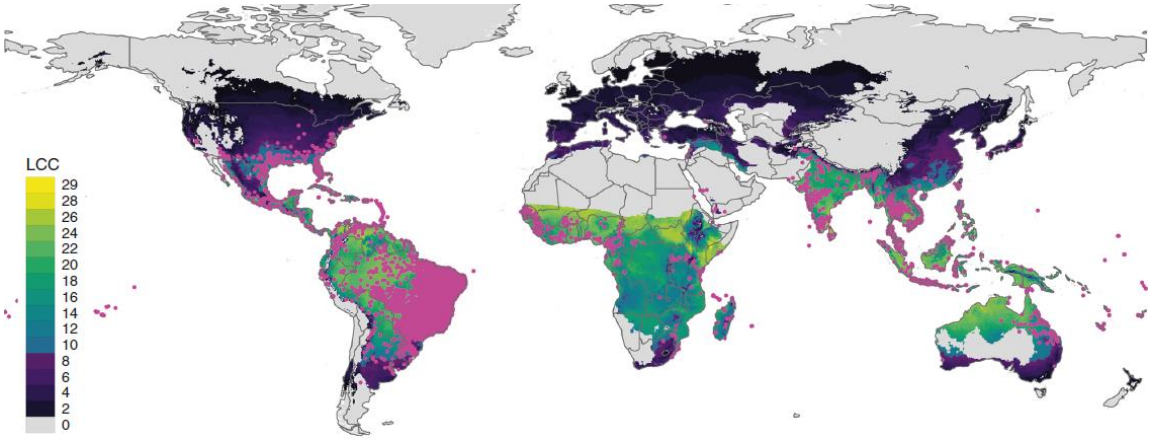

Figure 1: Distribution of *Aedes aegypti* worldwide. The map indicates the total number of annual life-cycle completions (LCC) per year of *A. aegypti* at the global scale, with occurrence data overlaid. *A. aegypti* is a recognized competent vector for both DENV and ZIKV. Adapted from Iwamura et al., 2020.

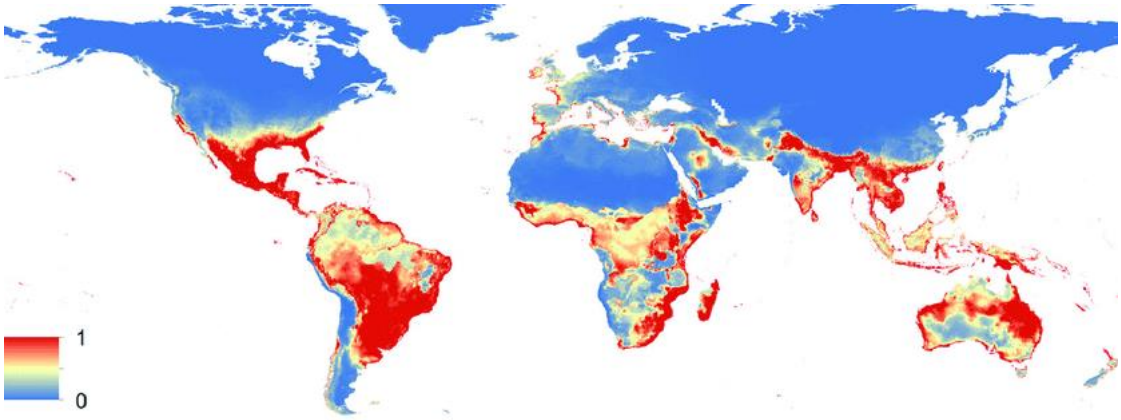

Figure 2: Estimated potential global distribution of *Culex quinquefasciatus.* The colours represent the suitability level from 0 (blue) to 1 (red). *C. quinquefasciatus* is a known competent biological vector of WNV. In addition, recent evidence suggested its potential as a vector for ZIKV (Alaniz et al., 2018; Pielnaa et al., 2020). Adapted from Alaniz et al., 2018.

2.1. DENV

Precise determination of DENV incidence is challenging, as most cases are asymptomatic or mild, adding to that the underreporting of cases due to misdiagnosis as other febrile illnesses. However, estimates of the number of annual infections worldwide range from 284 to 528 million (Harapan et al., 2020). A report including 76 countries indicates that, between 1990 and 2013, apparent cases of dengue more than doubled every decade (Harapan et al., 2020), with the number of cases reported to WHO in the last two decades increasing over 8-fold, reaching 5.2 million in 2019 (World Health Organization, 2022). DENV origins are thought to remount to non-human primates (sylvatic DENV) in Africa and Asia, estimated to have emerged 1000 years ago. Cross-species transfer to humans then occurred independently with all four serotypes (DENV1 to DENV4), and transmission in human populations has been established in the last few hundred years (Harapan et al., 2020). Nowadays, DENV is endemic in many regions of Africa, the Americas, Eastern Mediterranean, Southeast Asia and Western Pacific. The highest incidence rates occur in Southeast Asia, with an age-standardized yearly average of 34.3 cases per 1000 inhabitants (Harapan et al., 2020). In fact, the WHO stated that

Asia represents about 70% of the current burden of disease globally (World Health Organization, 2022). Studies suggest that silent infections play a substantial role during dengue epidemics and may contribute up to 84% of total DENV transmission (Halstead, 2019). In the last few years, outbreaks have also been reported in Europe, namely autochthonous dengue cases in Croatia and France in 2010 (Silva et al., 2020). More recently, in 2012-2013, 1080 dengue cases were confirmed in Madeira Island, Portugal, the largest European outbreak since 1928, when more than one million people were affected in Greece and Turkey (Silva et al., 2020).

2.2. WNV

WNV was first isolated in 1937, from a febrile patient in Uganda, West Nile Province (Chancey et al., 2015; Habarugira et al., 2020; Pierson et al., 2020; Hernández-Triana et al., 2014). Early epidemics studies associated WNV with relatively mild disease in humans. Serosurveys also suggest that WNV outbreaks may have by then occurred throughout much of Africa, the Middle East and South Asia, albeit no clear evidence of clinical cases is available (Chancey et al., 2015). The most prominent WNV outbreaks with clinical relevance have taken place in Israel, Romania, Russia, Greece, and the USA (World Health Organization, 2017). WNV was first isolated from a febrile child in 1951, during an outbreak near Haifa, Israel (Chancey et al., 2015). Later, in 1957, the first deaths due to WNV neuroinvasive disease (WNND) were reported in elderly Israeli patients. In 2000, 417 confirmed cases and 35 deaths were attributable to WNV and, ever since Israel suffers summertime outbreaks of varying severity (Chancey et al., 2015). In Europe, the first WNV cases occurred at Albania, in 1958 (Chancey et al., 2015). In 1962-1963, the first European WNV outbreak occurred, in Southern France, causing both human and equine disease (Chancey et al., 2015). Since then, Europe endured two large WNV epidemics (Chancey et al., 2015; World Health Organization, 2017). The first took place in Romania in 1996, with 17 deaths registered, and the second occurred in Russia in 1999, with 40 deaths from acute aseptic meningoencephalitis consequent to WNV infection (Chancey et al., 2015). Several other outbreaks and occasional cases have impacted European countries, and, as such, WNV surveillance programs are now

implemented in some countries (Chancey et al., 2015). Recently, in 2010, in Northern Greece (between the rivers Axios and Aliakmonas) an outbreak resulted in a total of 262 patients, 65 of which classified as West Nile fever while 197 suffered neurological disease (Hernández-Triana et al., 2014). The virus has also been isolated from mosquitoes in Portugal and the Czech Republic, migrating birds in Slovakia and Western Ukraine, and ticks in Hungary and Moldavia (Chancey et al., 2015). In the USA, a well-known WNV outbreak occurred in the 1999 summer in New York (Chancey et al., 2015; Pierson et al., 2020; Peterson et al., 2013), in a cluster of encephalitis patients (Chancey et al., 2015). In the following years, the virus spread to all 48 contiguous USA states, into Canada, Mexico (Chancey et al., 2015), the Caribbean and even part of South America (Pierson et al., 2020). WNV is now endemic in the USA, causing 3 of the largest arboviral neuroinvasive disease outbreaks in the country history (Peterson et al., 2013). WHO now considers WNV endemic in Africa, the Middle East, the USA, Australia, Europe and Asia (World Health Organization, 2017), demonstrating its capacity to successfully propagate around the globe.

2.3. ZIKV

Zika virus was first identified by chance in 1947 in a rhesus monkey of the Zika Forest of Uganda, amidst studies to discover the vector responsible for the transmission of the yellow fever virus (Silva et al., 2020; Plourde et al., 2016; Pielnaa et al., 2020; World Health Organization, 2016). Sometime later, the first cases of human infection were reported in Uganda, Tanzania and Eastern Nigeria (Silva et al., 2020; Plourde et al., 2016; World Health Organization, 2016). In the following years, scarce, geographically limited cases were reported, mostly describing patients with clinical presentations consistent with mild febrile illnesses (Silva et al., 2020; Pierson et al., 2020). Surveillance studies described possible human infections occurring throughout Africa, Asia and Oceania (Pierson et al., 2020; Plourde et al., 2016), although some authors consider that results may overestimate true prevalence of the virus, as serologic overlap often occurs between ZIKV and other flaviviruses (including DENV and WNV) (Plourde et al., 2016). The first major outbreak of human ZIKV infection was reported in 2007 in the Yap islands

(Federated States of Micronesia) (Silva et al., 2020; Plourde et al., 2016; Pielnaa et al., 2020). Estimates suggest that approximately 73% of the population was infected; however, only a relatively small number of infected individuals (≈18%) ended up developing symptomatic disease (Plourde et al., 2016). Since 2007, outbreaks have been reported in various regions of Asia and the Pacific, including French Polynesia, Cook Islands, Easter Island, New Caledonia, Singapore, Vietnam and Thailand (Silva et al., 2020; Plourde et al., 2016; Pielnaa et al., 2020). In 2015, ZIKV infections emerged in continental South America, in Brazil, this time being correlated with the possible occurrence of severe neurological complications, both in adults and infants (Pierson et al., 2020; Silva et al., 2020; Plourde et al., 2016). In the same year, Cape Verde also reported an outbreak (Silva et al., 2020; Pielnaa et al., 2020). In Europe, there are records of a small number of imported cases, either travel-associated or cases of sexual and vertical transmission (Silva et al., 2020; Plourde et al., 2016; Pielnaa et al., 2020). To date, we have knowledge of at least 86 countries and territories with reported evidence of ZIKV infection due to mosquito-mediated transmission (World Health Organization, 2018), and Zika virus has been declared a public health emergency (Silva et al., 2020; Plourde et al., 2016; World Health Organization, 2016).

2.4. A NOTE ON VECTORS

Part of the difficulty in dealing with DENV, ZIKV and WNV revolves around the characteristics of their insect vectors. Their ability to rapidly expand and establish novel mosquito populations in previously non-endemic areas (as exemplified in Fig. 1) increases the probability of new and more frequent outbreaks (Silva et al., 2020). Factors promoting viral amplification and human outbreaks are complex and depend on specific vector species. *A. albopictus* and *A. aegypti* mosquitoes are the most effective DENV and ZIKV vectors (Pierson et al, 2020; Harapan et al., 2020; Silva et al., 2020; Alaniz et al., 2018; Pielnaa et al., 2020). *Culex* spp. mosquitoes are the main WNV vectors (Peterson et al., 2013; Pierson et al., 2020; Habarugira et al., 2020; Martín-Acebes et al., 2012), although other mosquito species (*e.g.*, *A. albopictus*) may possess transmission competency (Habarugira et al., 2020; Chancey et al., 2015). Expansion of these vectors

is not only dependent on environmental changes, but also on enhanced globalization and socio-economic factors (Silva et al., 2020). Regarding environmental changes, factors such as higher temperature and higher humidity are known to benefit mosquitoes' populations (Martín-Acebes et al., 2012). Elevated temperatures shorten the incubation time in mosquitoes and increase viral transmission efficiency to hosts (Martín-Acebes et al., 2012; Peterson et al., 2013). However, *A. albopictus* mosquitoes have shown to be able to survive in more temperate regions, a particularity that potentially promotes their expansion to other than tropical and sub-tropical regions (Silva et al., 2020). Rapid travel and trade, associated with globalization, allow diseases and their associated vectors to overcome geographic barriers and promote their spread from endemic to non-endemic regions (Silva et al., 2020). As previously mentioned, socio-economic factors have also been associated with higher incidence of flaviviruses' infections in some locations (Pierson et al., 2020; Peterson et al., 2013; Harapan et al., 2020). In addition to the already mentioned factors, WNV cycles in nature between *Culex* mosquitoes and vertebrate animal hosts, namely birds, horses and other mammals (Pierson et al., 2020; Habarugira et al., 2020; Peterson et al., 2013). These hosts represent important reservoirs and are essential for the sustainability of the infection cycle, acting as virus amplifiers and source of infection for dead-end-hosts, like humans (Pierson et al., 2020; Habarugira et al., 2020). In fact, the role of migratory birds in WNV introduction and spread across Europe and the Americas has already been recognized (Martín-Acebes et al., 2012).

3. CLINICAL PRESENTATION, DIAGNOSIS AND VACCINES

Before proceeding, it is important to shortly elaborate on these viruses' life cycle and structure (Fig. 3), which highlights their common origin and partially explains their similar mode of transmission and infection. Briefly, flaviviruses are small spherical viruses of approximately 50 nm in diameter with a single positive-strand RNA genome, encoding three structural – capsid (C), pre-membrane (prM), which is a precursor to membrane (M), and envelope (E) – and seven non-structural viral proteins (NS1, NS2A,

NS2B, NS3, NS4A, NS4B, and NS5) (Pierson et al., 2020; Mukhopadhyay et al., 2005). The three structural proteins constitute the virus particle, wherein C protein encapsidates the ~10.8-kb genome and is surrounded by a host-derived lipid bilayer incorporating copies of the E and M proteins (Mukhopadhyay et al., 2005).

The flaviviruses life cycle includes as main steps the viral binding and entry, translation, replication, assembly, and release (Van Leur et al., 2021). The entry process begins with the attachment of viral particles to the cell surface and binding of the viral E protein to a cellular receptor (Pierson et al., 2020), Van Leur et al., 2021). Identifying the specific entry receptor involved in internalization of infectious virions in humans and other vertebrate animals remains a challenge (Pierson et al., 2020; Van Leur et al., 2021), but mannose and phosphatidylserine receptors have been reported as relevant for flavivirus pathogenesis (Harapan et al., 2020; Van Leur et al., 2021). Several cell surface markers have also been proposed as attachment factors [e.g., glycosaminoglycans, C-type lectins DC-SIGN (dendritic cell-specific intracellular adhesion molecule-3-grabbing non-integrin), heat-shock proteins, the chaperone BiP/GRP78, neolactotetraosylceramide and CD14] (Harapan et al., 2020; Van Leur et al., 2021).

After attachment, clathrin-dependent endocytic vesicles mediate virus internalization and membrane fusion is triggered by the endosomal acidic environment (Pierson et al., 2020; Harapan et al., 2020; Silva et al., 2020; Van Leur et al., 2021). Upon fusion of the viral envelope and cell membrane, the RNA genome is released into the cytoplasm and translation of the viral polyprotein it encodes occurs (Harapan et al., 2020; Silva et al., 2020). Then follows cleavage by host and viral proteases into the structural and non-structural proteins (Harapan et al., 2020; Silva et al., 2020; Van Leur et al., 2021).

Flaviviruses assembly and replicate on the endoplasmic reticulum (ER) membranes (Rajah et al., 2020). Immature virions are transported through the secretory pathway (Harapan et al., 2020) and maturation is then promoted by the acidic pH of the trans-Golgi network (Cordero-Rivera et al., 2021). It is at this stage that prM is cleaved into M by a host-encoded furin protease, causing the spiky cell surface (characteristic of immature virions) to transform into a smooth surface, the typical morphology of mature

virions (Harapan et al., 2020). Finally, mature virions are released from host cells through exocytosis (Harapan et al., 2020; Silva et al., 2020).

Concerning the viral proteins' function, the E protein is one of the most important for binding. This protein contains epitopes that bind cellular receptors, enabling virus entry (Wilder-Smith et al., 2019). The C protein has key roles in viral assembly, genome encapsidation and interaction with host lipid systems (Harapan et al., 2020; Martins et al., 2012; Carvalho et al., 2012). The prM protein interacts with the E protein preventing conformational changes that could allow fortuitous fusion of virions with host membranes during egress and its cleavage to M is required for formation of mature virions (Pierson et al., 2020). The seven non-structural proteins are necessary for effective viral replication (Wilder-Smith et al., 2019). Thus, these viruses display an overall common structural arrangement of the virion structure and a very similar proteome. However, and notwithstanding some symptoms that can be common among them, this shared structural resemblance does not imply similar clinical features, as discussed in the next section.

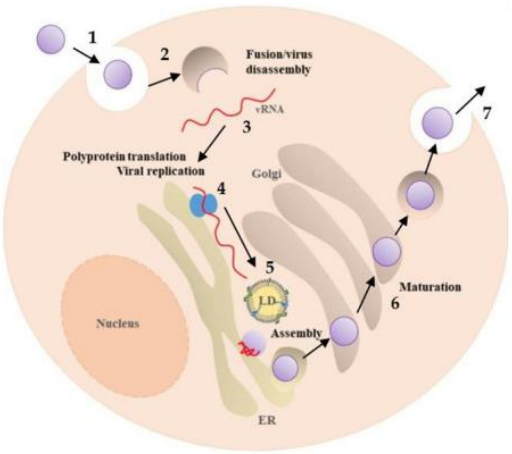

Figure 3: Viral life cycle. After entering host cells by clathrin-mediated endocytosis (1), membrane fusion of the viral envelope and the cell membrane occurs (2). Viral genome is released into the cytoplasm (3) and translated into a single polyprotein, later cleaved into the three structural and seven non-structural proteins (4). Next, replication occurs surrounding the endoplasmic reticulum (ER) and lipid droplets (LDs) (5), followed by viral packaging and assembly to form infectious viral particles (6), which are then released through exocytosis (7). The three

flaviviruses discussed here share similar virion structure and mode of infection, besides being also all mosquito-borne. Adapted from Silva el al., 2020.

3.1. CLINICAL PRESENTATION

Despite these viruses' similarity at the virion structure level, mode of infection and terms of transmission, symptoms can be quite different, both between viruses (WNV vs. ZIKV vs. DENV vs. each of the viruses serotypes/strains) and between infected people. Acute flavivirus infection in humans span conditions that range from asymptomatic to mild illness and up to severe diseases, which can be fatal (Pierson et al., 2020) (figure 4). Estimates vary widely, but, roughly, 40 to 80% of flavivirus infections are considered to be asymptomatic or to cause minimal illness (Silva et al., 2020; Peterson et al., 2013; Pierson et al., 2020; Martín-Acebes et al., 2012; Chancey et al., 2015; Pielnaa et al., 2020; Plourde et al., 2016).

3.1.1. DENV

Concerning symptomatic DENV infection, typical clinical presentation consists of a self-limited flu-like syndrome, with patients experiencing fever, headache, myalgia, arthralgia and sometimes developing rash (Pierson et al., 2020) (figure 4). Such symptomatic dengue infection normally comprises three stages: the febrile, critical and recovery phases (Wilder-Smith et al., 2019; Harapan et al., 2020). The febrile phase is characterized by a sudden fever onset, often accompanied by malaise, vomiting, constitutional symptoms, and the previously mentioned symptoms (Wilder-Smith et al., 2019). The critical period begins at the time of defervescence (Wilder-Smith et al., 2019; Harapan et al., 2020). Individuals require close monitoring to promptly identify possible signs of vasculopathy, namely increased vascular permeability, plasma leakage, and intravascular volume depletion (Silva et al., 2020; Wilder-Smith et al., 2019). Identifiable signs include increased hemoconcentration, serosal effusions, most frequently pleural and peritoneal, and gall bladder wall oedema. Minor hemorrhagic complications may

also be seen during this critical phase (Pierson et al., 2020; Silva et al., 2020; Wilder-Smith et al., 2019). Dengue shock syndrome is evident when pulse pressure values reach 20 mmHg or lower and requires rapid fluid resuscitation (Wilder-Smith et al., 2019). Other complications, resulting from organ impairment, have also been documented, but most likely ensue in individuals with underlying conditions. According to some authors, recurrent episodes of shock can occur in the 48-78 h interval before resolution of the vasculopathy and are associated with increase in fatal outcomes. Following appropriate supportive care, full recovery typically happens within 1-2 weeks. However, sequalae such as fatigue, weakness, myalgia and depression may last up to several months after acute disease resolution in adult patients (Wilder-Smith et al., 2019). According to the 2009 WHO dengue case classification, severe dengue occurs when symptomatic individuals experience at least one complication related to plasma leakage, and that originates dengue shock syndrome or respiratory distress, severe hemorrhage, or organ impairment (Wilder-Smith et al., 2019). Overall, less than 5% of DENV infections progress to the life-threatening severe dengue clinical presentation (Silva et al., 2020).

3.1.2. WNV

As for other flavivirus infections, WNV infection cases are mostly asymptomatic, being estimated that less than 1% of infected individuals progress to severe disease (Martín-Acebes et al., 2012; Kramer et al., 2007; Chancey et al., 2015). Severe West Nile disease most commonly manifests as neuroinvasive conditions comprising (Fig. 4): West Nile meningitis, West Nile encephalitis, and acute flaccid paralysis (Martín-Acebes et al., 2012; Kramer et al., 2007). The clinical presentation of West Nile meningitis resembles those caused by other etiological agents. Individuals present with fever, headache, neck stiffness, nuchal rigidity, photophobia, and Kerning's and Brudzinski's signs reflecting meningeal irritation can be positive upon physical examination (Petersen et al., 2013; Kramer et al., 2007). Patients developing West Nile encephalitis may present an altered level of conscience (Habarugira et al., 2020; Kramer et al., 2007), and focal neurological signs and symptoms, such as dysarthria (Kramer et al., 2007), tremor (Kramer et al., 2007; Petersen et al., 2013), ataxia (Petersen et al., 2013; Kramer et al., 2007), and

parkinsonism (Kramer et al., 2007; Petersen et al., 2013). Albeit most West Nile fever patients have complete recovery, those with neuroinvasive disease have poorer outcomes (Martín-Acebes et al., 2012; Chancey et al., 2015; Kramer et al., 2007). Recent studies suggest that, within this patients' cluster, individuals with West Nile encephalitis had worse outcomes and required more assistance after hospitalization than patients who develop West Nile meningitis (Kramer et al., 2007). As previously mentioned, acute flaccid paralysis may also develop, most frequently as an acute asymmetric paralysis with normal sensory examination (Kramer et al., 2007). One study documented that most patients did not have viral prodrome or signs of meningitis or encephalitis before flaccid paralysis onset (Kramer et al., 2007). The same authors followed a group of paralysis patients and concluded that initial disease severity was not predictive of outcome (Kramer et al., 2007). Other studies indicate that neuroinvasive disease recovery time is highly variable, with physical and cognitive deficits persisting from 6 months to 2 years after initial diagnosis (Martín-Acebes et al., 2012; Chancey et al., 2015; Kramer et al., 2007). Among other risk factors of severe West Nile disease, immunosuppression and old age seem to be the most important (Martín-Acebes et al., 2012; Chancey et al., 2015). This is crucial information, relevant in epidemiological terms and monitoring, both at the population as well as at the individual level. The aging of the population (alongside the known and well-documented vector and virus worldwide global expansion) should thus be computed, namely when considering resources allocation to R&D, monitorization and public health WNV policies.

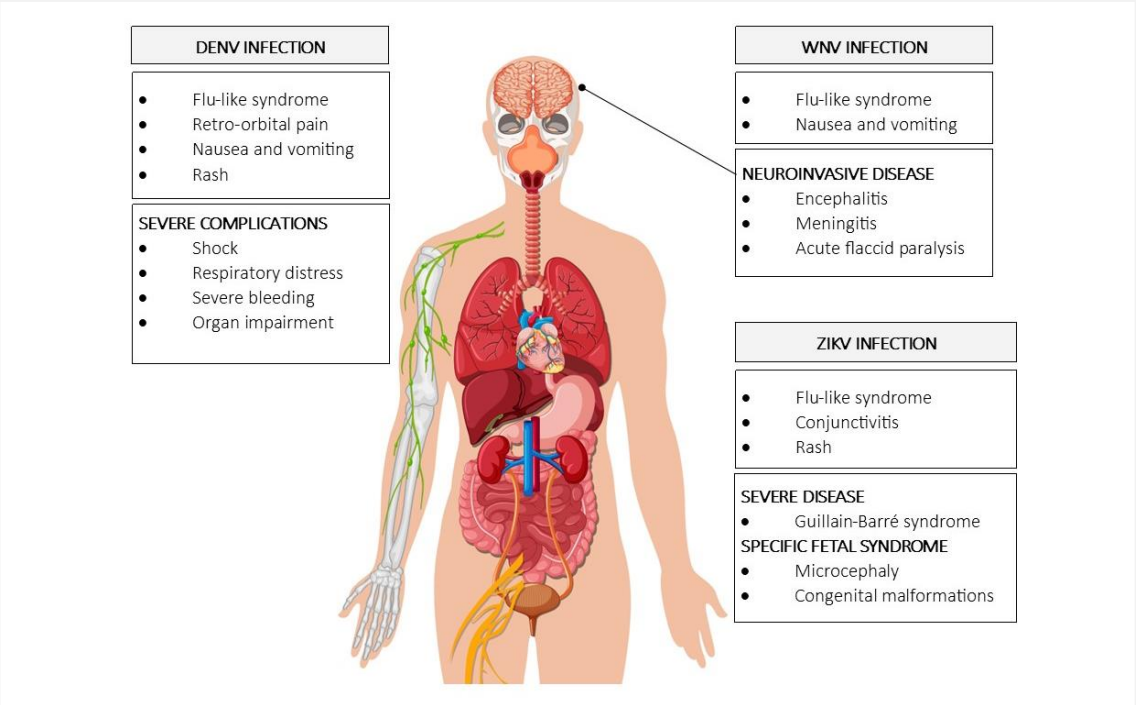

Figure 4: DENV, WNV, and ZIKV clinical manifestations. Flu-like syndrome includes signs and symptoms such as fever, headache, malaise, loss of appetite, fatigue, myalgia, and arthralgia. This vector has been designed using resources from Freepik (Vector created by brgfx from https://www.freepik.com).

3.1.3. ZIKV

Regarding ZIKV infections, only a small percentage seems to result in complicated clinical outcomes (Silva et al., 2020). Different flaviviruses are known to have different cellular and tissue tropism. ZIKV can cause both visceral and neurotropic disease, preferentially infecting progenitor cells, epithelium and myeloid cells, and produces injury on the reproductive tracts and eyes (Pierson et al., 2020). ZIKV has also tropism for placental tissue, which may explain its teratogenicity (Pierson et al., 2020). As shown in Fig. 4, ZIKV infection has been associated with cases of microcephaly and other congenital malformations (Silva et al., 2020). In adults, severe neurologic complications of infection described include Guillain- Barré syndrome (Silva et al., 2020; Plourde et al., 2016; Pierson et al., 2020), but also meningitis and meningoencephalitis (Plourde et al., 2016). Three ZIKV lineages have been identified but, despite being now clear that African lineage strains are more virulent than Asian ones, it is still not known whether increased

virulence of certain strains may result in more severe clinical outcomes (Silva et al., 2020). Overall, ZIKV monitoring is necessary to collect more data but, given the consequences to newborns and their families, this virus must also be considered in public health monitoring policies. Moreover, as Aedes spp. are also found throughout the globe, ZIKV incidence is only expected to increase, as already seen for DENV, which is transmitted by the same vectors.

3.2. DIAGNOSIS

Diagnosis of these flaviviruses infections is complicated due to the wide range of possible clinical presentations (described above). Moreover, the reason why some infected people develop more severe disease phenotypes than others is also still not fully understood, making it more difficult to reach a proper diagnosis. Host factors, including polymorphisms in key host genes, prior flavivirus immunity (primary vs. secondary infection), host immune status, age and the presence of certain comorbidities, such as hypertension and diabetes, have been suggested as predisposing to severe disease (Pierson et al., 2020; Harapan et al., 2020; Silva et al., 2020). Moreover, the specific tropism of each virus, the ability of evading host immunity and direct pathogenic effects are also mentioned as viral factors that likely contribute to the variability in pathogenicity amongst viral strains (Pierson et al., 2020). Ideally, all these factors must be considered to reach a proper diagnosis.

When DENV infection is suspected, the choice of diagnostic test depends on the time elapsed since disease onset (Wilder-Smith et al., 2019; Harapan et al., 2020). In the first 5 days, dengue may be diagnosed by virus isolation in cell culture, detection of viral RNA by nucleic acid amplification tests (NAAT) such as reverse transcription polymerase chain reaction (RT-PCR), or detection of viral antigens such as NS1 by enzyme-linked immunosorbent assay (ELISA) or rapid tests. After this period, specific IgM or IgG antibody detection through serological assays should be preferred, as viruses subside, and dengue-specific antibodies begin to appear (Wilder-Smith et al., 2019; Harapan et al., 2020). Dengue IgM antibodies may persist until 3 months after secondary infection

or longer in primary dengue infections. At point of care, combination of NS1 antigen detection and IgM testing offers a longer diagnostic period, although cross-reactivity with ZIKV has been reported for both (Wilder-Smith et al., 2019; Plourde et al., 2016).

Pertaining to ZIKV infection diagnosis, the same rationale applies. Studies estimated that ZIKV viremic period may be as brief as 5 days, and during this interval of time, molecular amplification using RT-PCR on serum samples seems to be the most specific diagnostic method (Plourde et al., 2016). Serologic approaches have limitations, as cross-reactivity with DENV is likely to occur, as previously mentioned. Currently, serum or cerebrospinal fluid are the samples of choice for testing; however, the utility of other specimens, such as urine, are being evaluated, and according to one study, ZIKV RNA may be detectable up to 20 days after viremia becomes imperceptible (Plourde et al., 2016).

For WNV neuroinvasive diseases, a definite diagnosis requires a positive IgM antibody test in the serum or cerebrospinal fluid, when clinical presentation is suggestive of either one of the three known syndromes (meningitis, encephalitis and acute flaccid paralysis) (Kramer et al., 2007). The diagnosis should be considered when epidemiological data suggests a likely context, as in endemic regions during the seasons when mosquito-borne diseases tend to occur (Kramer et al., 2007; Habarugira et al., 2020). For differential diagnosis with flaviviruses of the Japanese and thick-borne encephalitis complex, serological assays and PCR testing may be helpful, as clinical presentations do not differ (Kramer et al., 2007; Martín-Acebes et al., 2012). Additional acute flaccid paralysis differential diagnosis include conditions such as Guillain-Barré syndrome, myopathy, neuromuscular junction disorders and other motor neuron diseases caused by alternative viral agents. In this case electrophysiological and neuro imaging studies may provide helpful clues in distinction between possible etiologies (Kramer et al., 2007).

3.4. VACCINES

Before proceeding towards therapeutic possibilities, it is relevant to include a short description on prophylactics, namely available vaccines. The world's first dengue vaccine, CYD-TDV or Dengvaxia, is a live attenuated, tetravalent vaccine, based on the YFV-17D vaccine backbone, developed by Sanofi Pasteur (Pierson et al., 2020; Wilder-Smith et al., 2019). CYD-TDV performance efficacy depends on serotype, baseline serostatus and age (Silva et al., 2020; Wilder-Smith et al., 2019). A large phase 3 clinical trial in Asia and Latin America revealed an increment in risk of severe dengue in seronegative vaccine recipients in relation to seropositive recipients not previously vaccinated (Wilder-Smith et al., 2019). In 2018, the WHO stated that pre-vaccination screening should be performed in countries considering CYD-TDV vaccination (Wilder-Smith et al., 2019). CYD-TDV is currently approved in several countries, with indication for individuals aged 9 to 45 years, who had at least one previous DENV infection (Pierson et al., 2020; Silva et al., 2020; Wilder-Smith et al., 2019). Although the verdict is still to be issued a mounting body of evidence indicates that dengue vaccine (Dengvaxia) can promote the formation of cross-reactive antibodies that may have a role in triggering antibody-dependent enhancement (ADE) of flavivirus infections subsequent to the vaccination in otherwise seronegative patients (Shuckla et al., 2020; Idris et al., 2021; Alves et al., 2021; Dans et al., 2018). Nevertheless, other researchers suggest that ADE due to vaccination is a rare phenomenon, if it occurs at all (Huang et al., 2021). Further research is necessary.

As for WNV and ZIKV human infection, there are currently no approved human vaccines or other specific treatments available (Pierson et al., 2020; Silva et al., 2020; Kramer et al., 2007). Even though progress towards development of potential WNV vaccines has been made, their cost-effectiveness for human treatment remains uncertain (Martín-Acebes et al., 2012). Notwithstanding, a WNV vaccine for equine use has been approved. It is based on immunization with formalin-inactivated WNV, a recombinant canarypox virus vector, and a DNA plasmid expressing WNV prM and E proteins (Kramer et al., 2007). Considering all of this, developing effective therapeutic approaches remain a major need, even if more prophylactic vaccination strategies become available, as, so far, even in the case of DENV, no vaccine is either fully efficient against all viral serotypes, or recommended and effective in all age groups, namely the

most vulnerable. Thus, the most promising advances in terms of possible therapeutic approaches are described hereafter.

4. POTENTIAL NOVEL DRUGS FOR THE TREATMENT OF FLAVIVIRUS INFECTIONS

Given the above, it is important to determine the most promising biomedical advances in terms of future treatments against these flaviviruses. To do so, we accessed the GlobalData database on April 22, 2021 and searched for treatments disclosed as being currently developed against flavivirus infections. Selected data included molecules at discovery and preclinical development stage consisting of peptides, oligonucleotides, and proteins targeting flaviviruses. As keywords for targets we included Flavivirus as well as the particular virus mentioned (search terms: flavivirus, dengue, West Nile, Zika, DENV, WNV, ZIKV). This yielded 10 relevant hits, which were further studied and classified. These include, when classified at the molecular level, antibody-based, peptide-based and other approaches, as described ahead.

4.1. ANTIBODY-BASED THERAPEUTIC APPROACHES

Hereafter, five antibody based-therapeutic approaches are presented. All have been evaluated in detail, being in different stages of the clinical development process. In the concluding remarks section, a comment on those showing the most promising advances is available.

4.1.1. AC-10

In their studies, Bailey et al. characterized several neutralizing monoclonal antibodies (mAbs) isolated from a patient with acute Zika virus infection (Bailey et al., 2019). Their purpose was to map the epitopes targeted by neutralizing antibodies and try to understand whether certain germline rearrangements provided better neutralizing responses (Bailey et al., 2019). AC-10 was one of four antibodies that demonstrated high neutralizing potency against ZIKV (Bailey et al., 2019). The

rearrangement of VH1-2/VL2-8 (with VH and VL referring to heavy and light variable regions, respectively) was a common ground in potently neutralizing antibodies, including AC-10, as well as the presence of a motif composed of at least three tyrosine residues in the complementary-determining region 3 (Bailey et al., 2019). AC-10 was shown to be potently neutralizing, with 50% inhibitory concentration (IC_{50}) values below 25 ng/ml, most likely inhibiting viral binding and/or fusion, as its neutralization efficiency was superior when added before or at the time of infection (Bailey et al., 2019). AC-10 and other potently neutralizing antibodies induce escape mutations in the lateral ridge region of domains III and I of ZIKV envelope (E) protein (Bailey et al., 2019). On the other hand, less neutralizing antibodies tended to induce escape mutations in E protein domain II (Bailey et al., 2019), suggesting that this domain may be less determinant for antibody-mediated protection. Interestingly, point mutations in site 162 of domain I and in site 368 of domain III were detected in escape variants to AC-10, suggesting that these positions may play a more important role in neutralization (Bailey et al., 2019). Authors concluded that residue S368 in the E protein lateral region was required for complete inhibition by AC-10 and other mAbs containing the same germ line rearrangements (Bailey et al., 2019). This is an important discovery, as this region is conserved in 97.6% of the ZIKV sequences analyzed (Bailey et al., 2019). Therefore, AC-10 may be widely effective against different ZIKV strains. In addition, the S368R mutation correlated with the appearance of another mutation in the viral prM gene (D57N), suggesting that prM residue 57 may be key to viral replication (Bailey et al., 2019). Besides, other mutations were detected in regions encoding nonstructural proteins, namely NS2A, NS3 and NS5 (Bailey et al., 2019). However, the significance of these mutations is still not fully understood (Bailey et al., 2019). Regarding Fc-mediated functions, low concentrations of neutralizing antibodies increased ZIKV virions internalization, but did not lead to antibody-dependent cellular cytotoxicity on infected cells (Bailey et al., 2019). E protein-specific neutralizing antibodies also did not elicit protective Fc-mediated effector functions (Bailey et al., 2019).

4.1.2. EDE

Another study, by Barba-Spaeth et al., explored neutralizing antibodies against DENV serotypes 1 to 4, which also targeted a quaternary site at ZIKV E protein exposed surface (Barba-Spaeth et al., 2016). Antibodies were first isolated from a dengue patient (Barba-Spaeth et al., 2016). Crystal structure analysis of these antibodies complexed with ZIKV E protein situated its epitope in the interface between the two subunits of the E protein dimer, at a location believed to be the interaction site of prM with E dimers during virus replication (Barba-Spaeth et al., 2016). Two subsets of E-dimer epitope (EDE) antibodies were identified, EDE1 and EDE2, which display a differential requirement for glycosylation on the variable 150 loop of E protein: EDE2 affinity required glycosylation, while EDE1 did not (Barba-Spaeth et al., 2016). Neutralization assays suggested that EDE1 antibodies neutralize ZIKV more potently than EDE2 antibodies. EDE1 antibodies neutralized ZIKV African strain HD78788, as well as the French Polynesia PF13 strain (in this case, not showing glycosylation), with IC_{50} values in the nanomolar range (Barba-Spaeth et al., 2016). EDE2 binding capacity increases with glycan present, however EDE2 antibodies can equally neutralize both strains (Barba-Spaeth et al., 2016). Most antibodies initially isolated from dengue patients targeted the fusion loop epitope (FLE), contrarily to EDE1 and EDE2 antibodies. Thus, anti-EDE may be appropriate for epitope-focused vaccine against ZIKV/DENV viruses' serogroup (Barba-Spaeth et al., 2016), since other antibodies, namely anti-FLE antibodies, display cross-reactivity that may promote antibody-dependent enhancement (Shukla et al., 2020). Briefly, antibody-dependent enhancement (ADE) of infection occurs when cross-reactive antibodies or sub-neutralizing concentrations of antibodies generated in a primary infection facilitate viral entry in a secondary infection by a heterologous serotype or cross-reactive strain (Bournazos et al., 2020; Huisman et al., 2009; Tirado et al., 2003; Wilder-Smith et al., 2019). Pathogenesis of viral infection is enhanced through binding of antibodies to Fc receptors expressed on cells of the mononuclear phagocyte system, enabling not only the entry of the virus, but also viral evasion from host antiviral and immune responses (Bournazos et al., 2020; Wilder-Smith et al., 2019; Harapan et al., 2020). Moreover, being EDE antibodies binding region on prM-E dimers interaction site (and essential for viral replication being conserved amongst strains), it will have low risk of inducing escape mutations (Barba-Spaeth et al., 2016). Given EDE2 antibodies

poorer affinity in contact points of the variable 150 loop, these are thus somewhat inferior to EDE1 antibodies (Barba-Spaeth et al., 2016). All considered, EDE1 might thus be the preferential option.

4.1.3. ZKA190

The mAb ZKA190 was isolated from a panel of anti-ZIKV neutralizing human antibodies (Wang et al., 2017). The epitope of ZKA190 was located in the lateral region of domain III of ZIKV E protein, specifically loops BC, DE and FG, and part of the DI-DIII linker (Wang et al., 2017). These residues are conserved in 217 ZIKV strains (Wang et al., 2017). Therefore, they may be relevant regions for the development of future antibody vaccines against ZIKV. Surprisingly, ZKA190 also neutralizes Uganda 1947 MR766 strains, which contain substitutions in these residues (Wang et al., 2017), suggesting the antibody may target other regions of the virus. ZKA190 was shown to neutralize ZIKV strains from Africa, Asia and the Americas, with IC_{50} values in the nanomolar range (0.004 to 0.05 nM). It seems to act at a post-attachment step, likely membrane fusion (Wang et al., 2017). Moreover, Wang et al. referred the possibility of an additional neutralization mechanism. The observation of increasing viral amounts on the cell surface associated with increasing antibody concentrations suggests virus inactivation through aggregation, by simultaneously engaging epitopes on different particles of ZIKV, as later confirmed by dynamic light scattering (Wang et al., 2017). Pertaining to ADE phenomena, *in vivo* results indicated that ZKA190 did not elicit ADE, even at doses expected to provide only partial neutralization (Wang et al., 2017). Despite *in vivo* evidence, ZKA190 triggered ADE *in vitro* (Wang et al., 2017). Prophylaxis with ZKA190 protected mice from mortality and morbidity, with survival rates of 80 to 100% (15 mg/kg) and reduction of viral titers, after challenge with ZIKV strain MP1751 (African lineage) (Wang et al., 2017). Furthermore, one resistant mutant containing a DIII E370K mutation was identified (Wang et al., 2017). The emergence of resistant mutants poses a challenge for antibody-based vaccines, as they can render potential therapeutics obsolete. To minimize escape mutations risk, Wang et al. combined the potently neutralizing ZKA190 with the mAb ZKA185, creating the bispecific antibody FIT-1 (Wang

et al., 2017). ZKA185 was chosen as it cross-neutralizes ZIKV strains and does not compete with ZKA190, because it targets a different epitope, in domain II of the E protein (Wang et al., 2017). FIT-1 preserved the parental antibodies neutralizing potency against ZIKV strains and similar IC_{50} values. Moreover, FIT-1 bound E protein with an affinity superior to that of its parental antibodies and no escape mutations were documented, both *in vitro* and *in vivo*, even at lower dosages (Wang et al., 2017). As FIT-1 did not elicit immune evasion after 8 rounds of serial passages, authors considered it would be an unlikely event, since escape mutations were reported after 3 to 4 passages in studies with ZKA190 and ZKA185 (Wang et al., 2017). Virus inhibition by FIT-1 seems to occur by the same mechanisms described for ZKA190 (Wang et al., 2017). In addition, FIT-1 demonstrated capacity to block *in vitro* ADE mediated by prM mAb DV62 and revealed its therapeutic potential in *in vivo* studies, increasing survival without apparent morbidity and reducing ZIKV viral titers (Wang et al., 2017).

4.1.4. WNV-86

WNV-86 is a human monoclonal antibody selected from a cluster of 10 mAbs isolated from WNV infected individuals (Goo et al., 2019). *In vitro*, WNV-86 demonstrated effective neutralization of WNV and was shown to neutralize 50% of virus infectivity at 2 ng/mL (Goo et al., 2019). According to Goo et al., WNV-86 likely aimed to an epitope located in domain I or II of WNV E protein (Goo et al., 2019). Other anti-WNV antibodies have been reported, with several of these antibodies displaying preferential neutralization of partially mature virions, which still contain prM proteins in their surfaces (Goo et al., 2019). Partially mature virions contain structural characteristics of both mature and immature virions, namely the smooth surfaces characteristic of mature virions, plus the prM-E heterotrimeric spikes identified in immature virions (Goo et al., 2019). This distinct structure allows exposure of hidden epitopes, which can be better targeted by neutralizing antibodies (Goo et al., 2019). However, WNV-86 preferentially targets epitopes displayed on mature virions (that do not have prM), as IC_{50} values required for neutralization of virus particles lacking prM was 4-fold lower than for virus particles containing prM (Goo et al., 2019). *In vitro* selection of escape variants identified

a single threonine to asparagine nucleotide change in residue 64 of E protein domain II, resulting in incorporation of an N-linked glycosylation site, and a second threonine amino acid substitution to lysine in residue 208, also in domain II (Goo et al., 2019). Further analysis revealed that WNV particles carrying this second mutation at amino acid residue 208 still displayed neutralization potency and that both mutations were required to inhibit neutralization by WNV-86 (Goo et al., 2019). Despite these findings, the precise binding footprint of mAb WNV-86 is still unknown (Goo et al., 2019). Besides *in vitro* evidence, WNV-86 also demonstrated *in vivo* efficacy and was shown to reduce WNV-infected mice mortality (Goo et al., 2019). Furthermore, mice protection was attributed to WNV-86 direct inhibition of virus infection and dissemination, since both wild-type and LALA (a Leu234Ala/Leu235Ala mutation, commonly used to disrupt antibody effector functions) versions of WNV-86 were able to reduce viral titers in the spinal cord and brain of challenged mice (Goo et al., 2019).

4.1.5. ZIKV-117

ZIKV-117 is a monoclonal antibody isolated from a cluster of mAbs demonstrating affinity for ZIKV E protein (Sapparapu et al., 2016). Sapparapu et al. localized the epitope of ZIKV-117 at domain II of E protein, in a region across two adjacent dimers at the dimer-dimer interface (Sapparapu et al., 2016). No escape mutants of ZIKV-117 were reported and the mAb demonstrated capacity to neutralize several ZIKV strains, with IC_{50} values ranging from 5 to 25 ng/ml (Sapparapu et al., 2016). Neutralized strains included MR 766 and Dakar 41519 (African lineage), Malaysia P6740 and H/PF/2013 (Asian lineage), and Brazil Paraiba 2015 (American lineage) (Sapparapu et al., 2016). ADE of disease is one of the main concerns associated with the development of flavivirus antibody-based vaccines (Sapparapu et al., 2016). Regardless, ZIKV-117 possesses a restricted type-specific binding pattern and demonstrated not to be cross-reactive with DENV serotypes 1 to 4, as well as WNV E protein (Sapparapu et al., 2016). *In vivo*, ZIKV-117 was shown to protect mice (previously treated with anti-IfnarI mAbs) challenged with the ZIKV African strain Dakar (Sapparapu et al., 2016). ZIKV-117 also demonstrated to improve fetal outcome in pregnant mice when administered

before ZIKV inoculation at a single dose of 250 μg (Sapparapu et al., 2016). Other conducted experiments suggested a possible effect in prevention of vertical transmission of ZIKV, as ZIKV-117 treated pregnant mice displayed lower virus levels in the placenta and reduced viral titers were also found in fetal brain in the progeny of treated mice (Sapparapu et al., 2016). ZIKV-117 titers in the placenta and fetal brain were shown to be superior to the IC_{50} neutralization value, an unexpected result since levels of Fc receptor in the mouse placenta tend to be inferior to those of other mammalians (Sapparapu et al., 2016). Viral RNA levels in dams' brain and serum were also reduced by ZIKV-117 treatment (Sapparapu et al., 2016). The apparent protective role of ZIKV-117 in the pregnancy model was thought to be due to direct neutralization by the mAb, as studies with the LALA version of ZIKV-117 lead to similar results (Sapparapu et al., 2016). Additionally, post-exposure efficacy was reported by Sapparapu et al., as administration of ZIKV-117 resulted in a marked reduction of viral burden in dams, in the placenta and in the fetus at embryo day 13.5 (Sapparapu et al., 2016). Lastly, pathophysiological analysis reinforced the previous results – decreased placental damage, trophoblast cell death, and increased body size of fetuses was observed in comparison to control-treated dams (Sapparapu et al., 2016). Notwithstanding, the possibility of extrapolation of these observations to humans remains unclear, due to significant differences in placental architecture (Sapparapu et al., 2016).

4.1.6. OTHER ANTIBODY-BASED APPROACHES

There is still more work in progress that can inspire other antibody-based drugs and/or therapeutic approaches, albeit in a slightly more conceptual phase still. For example, Schenker and Sagiv provided methods for a potential ZIKV infection treatment and/or prophylactic intervention that focus on protecting both fetus and pregnant women against ZIKV infection. The treatment would consist of enriched anti-ZIKV human immunoglobulin preparation, potentially effective against different genotypic variants or strains of ZIKV. This was tested with anti-ZIKV IgGs purified from the plasma of seven convalescent donors, at 92 mg/mL, with complete neutralization of ZIKV in

K562 cells (Schenker et al., 2020). The proposed treatment should be able to prevent cross-reactions with a second species of Flaviviridae, due to its neutralizing capacity, reducing the possible occurrence of ADE upon a subsequent infection by another flavivirus species, strain and/or serotype (Schenker et al., 2020). To determine a therapeutic dose, young immunocompromised mice lacking the receptor for type I interferon (Ifnar I -/-) were infected with 1×103 PFU/mouse by subcutaneous route (Schenker et al., 2020). According to the natural course of disease, by day 5 post-infection (p.i.) mice began to lose weight, by day 6 p.i. hindlimb weakness was observed, and by day 7 p.i. the weight reduction was about 15-25% of the starting weight and partial to complete paralysis was expected (Schenker et al., 2020). Different anti-ZIKV antibodies doses were administered via intraperitoneal or IV route at days 1 and 7 p.i., and samples of blood, spleen, liver, brain, and ovary collected for virology and microscopic analysis (Schenker et al., 2020). To determine whether the treatment is effective in protecting the fetuses, pregnant Ifnar I -/- female mice are treated with the previously determined therapeutic dose of anti-ZIKV IgG at embryonic day 5.5 and infected with 1×103 PFU/mouse at embryonic day 6.5 (Schenker et al., 2020). After birth, newborns were evaluated for intrauterine growth restriction, ZIKV infection and injury to the fetal brain (Schenker et al., 2020). Another proposed method for preclinical studies in a pig model was also presented, consisting of trans-uterus injection of treatment into the amniotic sac, peritoneal cavity and intra-allantoic injection of selected fetuses (Schenker et al., 2020). At the 14th day after treatment sows and fetuses were sacrificed and tissue samples of fetuses were collected for examination and, if the treatment was successful, prevention of ZIKV transmission from infected fetuses to the adjacent treated fetuses would be expected (Schenker et al., 2020).

Overall, as mentioned above, several promising antibody-based findings have been achieved, both *in vivo* and *in vitro*. Moreover, ongoing studies to develop better animal models, may give rise to further improved methodologies. As such, all this paves the way for future antibody-based therapeutic approaches, which can be complemented with other strategies, described hereafter.

4.2. PEPTIDE-BASED THERAPEUTIC APPROACHES

Compared to antibody-based approaches, peptide-based developments are somewhat lagging. There are however several advances. As before, please refer to the concluding remarks section for a comment on the most advanced developments.

4.2.1. Ri57

Michael et al. proposed short-chain peptides directed against flaviviruses, capable of obstructing key regions of envelope glycoproteins (Michael et al., 2020). Ri57 is a peptide with 28 amino acid residues arranged in an enantiopure D-amino acid sequence. Ri57 was shown to display inhibitory activity against DENV serotypes 1 to 4, at 20 µM, with percentages of inhibition of 100% ± 0.0, 97.8% ± 1.4, 90.1% ± 8.5, and 94.4% ± 6.2, for DENV1, DENV2, DENV3 and DENV4, respectively (Michael et al., 2020). The peptide also inhibits ZIKV infection with high inhibitory percentages (71.0% ± 22.5 and 95.8% ± 2.8 for RI57 concentrations of 20 and 35 µM, respectively) (Michael et al., 2020). Next, Michael et al., studied Ri57 inhibition mechanism. The peptide's inhibitory activity was not attributable to cellular toxicity effects, as observed by mitochondrial reductase activity (Michael et al., 2020). Instead, Ri57 directly inhibited virus binding to cells (Michael et al., 2020). As to its mechanism of action, experimental evidence suggests that Ri57 acts as inhibitor of DENV and ZIKV virus fusion, blocking entry into the host cell and consequently infection (Michael et al., 2020). Researchers aimed at obtaining a peptide capable of resisting to peptidase activity (Michael et al., 2020). Normal human serum is composed of numerous proteolytic enzymes with capacity to degrade potential therapeutic peptides (Michael et al., 2020). Therefore, to be an antiviral candidate with potential *in vivo* capacity, peptides must be resistant to such enzymes (Michael et al., 2020). Ri57 remained completely intact in a solution of peptide in 1:2 dilution of normal human serum at 37°C for 24 h and retained its inhibitory activity against DENV2 when exposed to trypsin (Michael et al., 2020). This shows that this peptide has potential to be used in drug development strategies.

4.2.2. Tat-beclin-1

Tat-beclin-1 is an autophagy-inducing peptide, containing HIV-1 Tat protein transduction domain and amino acid residues 267 to 284 of beclin 1, a protein involved in autophagosome formation (Levine et al., 2014). In addition, three substitutions (H267E, S279D and Q281E) are included to increase Tat-beclin-1 hydrophilicity and solubility (Levine et al., 2014). The rationale behind relates to the known importance of pathways of autophagy in defense against infection (Levine et al., 2014). In fact, mice lacking autophagy genes or with hypomorphic alleles of these genes were more susceptible to lethal viral infections, and genetic knockout or knockdown of such genes led to increased replication of several viral infections (Levine et al., 2014). Thus, strategies capable of increasing infected cells autophagy could represent a possible mechanism for prevention and/or treatment of human viral diseases (Levine et al., 2014). Levine et al. demonstrated that cells treated with Tat-beclin-1 30 μM, 4 h post-WNV infection, had lower viral titers when compared to control (Levine et al., 2014). Tat-beclin-1 also demonstrated to be effective against WNV *in vitro*, with 10 μM of Tat-beclin-1 resulting in 10 to 50-fold reductions of WNV titers (Shoji-Kawata et al., 2013). Reduction of viral titers by Tat-beclin-1 was not due to peptide cytotoxicity, but rather the result of antiviral effects leading to increased autophagy (Shoji-Kawata et al., 2013). Additionally, Kawata et al. reported that prophylactic treatment with Tat-beclin-1 demonstrated antiviral activity of this peptide against a variety of positive strand RNA viruses (Shoji-Kawata et al., 2013). Besides, *in vivo* efficacy of Tat-beclin-1 has also been shown, with Tat-beclin-1 D-form improving the clinical outcome in a neonatal mouse model of WNV central nervous system infection, reducing the mortality of WNV-infected mice (Shoji-Kawata et al., 2013). Further analysis demonstrated that Tat-beclin-1 treatment let to lower WNV antigen levels in mice brains, lower registers of neuropathology, and less cell death (Shoji-Kawata et al., 2013). Taken together, findings seem to support the efficacy of the autophagy-inducing peptide Tat-beclin-1 both i*n vitro* and *in vivo*.

4.2.3. WLBU-2

WLBU-2 is a 24 residue cationic peptide with antimicrobial activity predicted to result of its interaction with negatively charged lipid membranes, leading to bilayer disruption (Hasek, 2014). A similar antiviral activity has also been suggested against a diversity of enveloped viruses (Hasek, 2014). Mammalian virus membranes do not tend to have negative surface charge but are richer in cholesterol (than host cells), and this higher cholesterol content seems to be needed for viral infectivity (Hasek, 2014). As such, adding a cholesterol recognition amino acid consensus (CRAC) motif to WLBU-2 could direct its activity to cholesterol-rich viral envelopes, prompting virus inactivation (Hasek, 2014). These CRAC modified peptides (LWYIK, LWYIK2 and VWYVK2) were shown to be up to 10-fold more potent antivirals than unmodified WLBU-2 (Hasek, 2014). VWYVK2 was the most active of these molecules, being active even at the lowest concentration tested (0.39 μM), with 77% reduction of plaques (Hasek, 2014). At the same concentration, WLBU-2 reduced viral plaques only by 16% (Hasek, 2014). In addition, IC_{50} values of CRAC-modified peptides were approximately 10-fold lower than for unmodified WLBU-2 (Hasek, 2014). To understand if unmodified WLBU-2 and CRAC modified peptides led to significant cytotoxicity in treated cells, hemolysis and MTS assays were performed (Hasek, 2014). Despite data reporting increased CRAC modified peptides hemolytic activity when compared to unmodified WLBU-2 (≈3-fold higher), the concentration at which modified peptides induced 50% hemolysis was still higher than that needed to induce 50% viral inactivation (Hasek, 2014). For all CRAC modified peptides, less than 10% hemolysis was observed at concentrations below 0.78 μM (Hasek, 2014). As to MTS assays, it was shown that CRAC modified peptides and WLBU-2 had similar cytotoxicity (Hasek, 2014). Therapeutic levels of CRAC modified peptides were lower than the cytotoxic levels (Hasek, 2014). Researchers also found that adding two CRAC motifs to WLBU2 did not alter activity as compared to peptides with a single CRAC motif, suggesting the absence of an additive effect with multiple CRAC motifs (Hasek, 2014). Furthermore, other mechanisms of inactivation besides lipid disruption were hypothesized. Contrary to expected, DENV (with high protein-to-lipid ratio in its envelope) was the most sensitive to CRAC modified peptides inactivation (Hasek, 2014). Therefore, other mechanisms could be at play, namely viral entry blockage due to these highly cationic peptides interaction with negatively charged cellular receptors of DENV,

leading to inhibition of dynamics between viruses and their receptors on the cell surface (Hasek, 2014). Overall, this supports further studies of potential peptide-based drug development approaches.

4.3. OTHER THERAPEUTIC APPROACHES

Type I interferon (IFN) family is a multi-gene cytokine family encoding 13 partially homologous IFNα subtypes in humans (McNab et al., 2015). IFNα subtypes are recognized for inducing an antiviral state in both virus-infected and uninfected cells, doing so by inducing a programme of gene transcription that interferes with various stages of the viral replication cycle (McNab et al., 2015). Furthermore, studies with IFNAR1 (type I IFN receptor)-deficient mice provided evidence of the protective role of IFNα against viruses *in vivo* (McNab et al., 2015). This property of IFNα was also reinforced by studies in which exogenous IFN was used to treat viral infections (McNab et al., 2015). In fact, most viruses devote part of their limited genome to mechanisms that perturb IFNα/β production and/or IFNα/β-mediated signaling, inhibiting the induction of IFN-stimulated genes (McNab et al., 2015). This alone demonstrates these cytokines importance in protection against viral infection (McNab et al., 2015). Examples of prototypic viruses that benefit from inactivation of IFNα include flaviviruses such as WNV, alongside avian Influenza, SARS-CoV-1 and smallpox viruses, among other (Carter et al., 2011).

Ampligen is a synthetic double stranded ribonucleic acid (dsRNA) molecule, containing a rugged structure that increases its resistance to molecular unfolding, and acts as a selective Toll-like receptor 3 agonist (Carter et al., 2016). Binding of dsRNA to Toll-like receptor 3 induces expression of α and β interferons, and cytokine production, leading to an antiviral state within various cells (Olsen et al., 2007). Thus, Ampligen acts as an interferon-inducing molecule. Its efficacy has been demonstrated against both flavivirus and alphavirus-associated encephalitis in experimental animal models (Morrey et al., 2004). This molecule could serve as a WNV prophylactic treatment: it has been administered (intraperitoneal injection) to mice exposed to WNV (at a dose of 13 mg/kg), preventing mortality in mice (Morrey et al., 2004). Ampligen administration was shown to reduce viral titers to levels below the detection limits, supporting its drug

efficacy (Morrey et al., 2004). Notwithstanding, Ampligen prophylactic treatment (4 to 8 h pre-infection) did not result in statistically improved survival (Morrey et al., 2004). Moreover, in a separate experiment, Ampligen administered 4 to 6 h before viral challenge was shown to display no statistical difference compared to saline control (Morrey et al., 2004). Despite that, Ampligen treatment was associated with improved weight change (Morrey et al., 2004). Thus, it was efficacious *in vivo* only when treatment began at least one day before WNV exposure (Morrey et al., 2004). As other antiviral gene modulation strategies resorting interferon have been reported, this may become another tool in the interferon-based prophylactic/therapeutic arsenal, although likely not fully effective on its own.

Summing up, a list of potential therapeutic approaches is presented below (Table1).

Table 1: Summary of key characteristics of the compounds presented.

TYPE	COMPOUND NAME	MODE OF ACTION	EVIDENCE OF EFFICACY	DEVELOPMENT STAGE	REFERENCE
ANTIBODY-BASED	AC-10	Likely inhibits viral binding and/or membrane fusion by targeting epitopes in the lateral ridge of domain III and domain I of E protein	*In vitro* against ZIKV	Preclinical	Bailey et al., 2019
	EDE1	Targets epitopes in the E protein	*In vitro* against DENV1-4 and ZIKV	Preclinical	Barba-Spaeth et al., 2016
	FIT-1	Inhibition of a post-attachment step (likely fusion) by targeting an epitope in the lateral ridge of domain III of ZIKV E protein	*In vitro* and *in vivo* against ZIKV	Preclinical	Wang et al., 2017
	WNV-86	Most likely targets an epitope in domain I or domain II of E protein. Preferentially recognizes epitopes of mature virions.	*In vitro* and *in vivo* against WNV	Preclinical	Goo et al., 2014
	ZIKV-117	Targets an epitope on domain II of E protein	*In vitro* and *in vivo* against ZIKV	Preclinical	Sapparapu et al., 2016
PEPTIDE-BASED	Ri57	Inhibits viral fusion by targeting regions of E protein	*In vitro* against DENV1-4 and ZIKV	Preclinical	Michael et al., 2020
	Tat-beclin-1	Induction of autophagy	*In vitro* and *in vivo* against WNV	Preclinical	Levine et al., 2014; Shoji-Kawata et al., 2013
	WLBU-2 modified peptides	Inhibition through interaction with viral membranes	*In vitro* against DENV	Discovery	Hasek, 2014
OTHER	Ampligen	Induction of interferon expression	*In vitro* and *in vivo* against flaviviruses	Preclinical	Carter et al., 2016; Morrey et al., 2004

5. CONCLUDING REMARKS

The continued expansion of vectors beyond previously known endemic regions and the establishment of competent mosquito species in regions of Europe has raised the awareness of the potential risk of flavivirus infections. Increase in the frequency of outbreaks and the emergence of cases in regions with a more temperate climate have highlighted flaviviruses' changing epidemiology and ability to successfully adapt to new contexts. Such concerns regarding flavivirus infections are no longer circumscribed to the scientific community, as proven by increasing implementation of surveillance programs in several countries and territories. Regardless of the overall mortality rates associated with these infections, the economic burden is substantial and especially harmful to socio-economically disadvantaged regions. Although slow, progress towards the development of potential therapeutics is ongoing. As mentioned herein, several antiviral agents directed against DENV, WNV and ZIKV are currently under investigation. Antibody-based compounds have yielded the most promising results, with demonstration of both *in vitro* and *in vivo* efficacy. In our view, antibody-based therapies are the most advanced and promising therapeutics presently, specially those based on monoclonal antibodies against specific domains of the structural E protein. This suggests that this protein is indeed a good target of interest for the development of future antiviral drug therapies. Further studies are necessary but three monoclonal antibodies mentioned (FIT-1, WNV-86, and ZIKV-117) decreased mice mortality and one of them (ZIKV-117) improved the outcomes in progeny of pregnant mice facing ZIKV challenge. However, the use of antibody-based vaccines and/or treatments faces challenges, such as cross-reactivity amongst epitopes of different flaviviruses, leading to ADE. Although, given the divergent evidence regarding *in vivo* demonstration of ADE in mouse models, it is not yet certain and may depend on the flavivirus being assessed (King et al., 2007). Additionally, virion proteins can also be targeted by peptide-based compounds, disrupting crucial steps of the viral life cycle (e.g., Ri57). Other compounds with alternative mechanisms of action have also been explored, including peptides inducing autophagy of infected cells (e.g., Tat-beclin-1) and modulators of interferon expression (e.g., Ampligen), with *in vivo* activity against WNV. Reports of the efficacy of such

heterogeneous approaches reflect the multiple potential targets encoded by flaviviruses, as well as the potential of host-directed antivirals. As a whole, the compounds presented demonstrate that several treatments based on a variety of approaches may become feasible options in the near future. Nevertheless, more research regarding efficacy and safety is needed for the development of a potential antiviral therapy. Strategies employing alternative targets are currently under development. For example, approaches directed against the structural C protein of flaviviruses, more precisely through blockage of interactions between the latter and host and/or viral elements, as reviewed elsewhere (Silva et al., 2020). As an example, we developed pep14-23 (Martins et al., 2012; Faustino et al., 2015). pep14-23 is a promising drug lead, which seems to inhibit the interaction of DENV C protein with host intracellular lipid droplets (LDs) in *in vitro* studies (Martins et al., 2012). This is an essential interaction for viral replication (Carvalho et al., 2012), thus, approaches such as that of pep14-23 may be of particular interest in the development of anti-flavivirus drugs (Martins et al., 2012; Faustino et al., 2015; Faustino et al., 2019; Silva et al., 2020).

To conclude, along with continued investigation efforts, implementation of vector control strategies and other countermeasures that limit emergence and re-emergence of flavivirus outbreaks are also crucial to lessen the burden caused by their infection. Thus, a combined multifactorial approach is the best to follow, especially given flaviviruses adaptability and the vector role in epidemics. Such multi-pronged strategies are more likely to yield good results, both for prophylactic as well as therapeutic policy planning and are thus always recommended, alongside additional research on this topic.

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