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Review

Exploiting Venom Toxins in Paratransgenesis to Prevent Mosquito-Borne Disease

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Abstract: Mosquitoes are responsible for the transmission of numerous pathogens, including *Plasmodium* parasites, arboviruses and filarial worms. They pose a significant risk to public health with over 200 million cases of malaria per annum and approximately four billion people at risk of arthropod-borne viruses (arboviruses). Mosquito populations are geographically expanding into temperate regions and their distribution is predicted to continue increasing. Mosquito symbionts, including fungi, bacteria and viruses, have desirable traits for mosquito disease control including spreading horizontally and vertically through mosquito populations and potentially colonising multiple important vector species. Paratransgenesis, genetic modification of mosquito symbionts with effectors to target the pathogen rather than the vector, is a promising strategy to prevent the spread of mosquito-borne diseases. A variety of effectors can be expressed but venom toxins are excellent effector candidates because they are target specific, potent, and, stable. However, the only toxins to be explored in mosquito paratransgenesis to date are Scorpine and mutated phospholipase A2. To enhance the scope, effectiveness, and durability of paratransgenesis, an expanded arsenal of effectors is required. This review discusses other potential toxins effectors for future paratransgenesis studies based on prior *in vitro* and *in vivo* antiparasitic and antiviral studies and highlights the need for further research and investment in this area. In terms of mosquito-borne diseases, paratransgenesis strategies have been developed to target *Plasmodium*. We postulate the potential to apply this principle to target arboviruses using antiviral toxin effectors.

Keywords: *Plasmodium*; rift valley fever virus; yellow fever virus; Japanese encephalitis virus; West Nile virus; chikungunya virus; dengue virus; zika virus; snake; scorpion; spider; *Aedes*; *Anopheles*

Overall Introduction and Aims

Mosquito-borne pathogens such as parasites and arthropod-borne viruses (arboviruses) pose a significant risk to public health [1]. Malaria is one of the most common parasitic diseases globally with an estimated 249 million cases and 608,000 deaths reported in 2022, mostly in children under five in sub-Saharan Africa [2]. Over half the world's population are at risk of infection by arboviruses, including Rift Valley fever virus (RVFV), yellow fever virus (YFV), Zika virus (ZIKV), Japanese encephalitis virus (JEV), West Nile virus (WNV), Dengue virus (DENV), and Chikungunya virus (CHIKV) [3].

Due to climate change and other anthropogenic factors, the burden of mosquito-borne diseases is intensifying [4–6]. There is no single solution for the control of mosquito-borne disease and multiple strategies are required. This multi-pronged approach will require location-specific strategies influenced by environmental and economic factors, governing bodies and disease prevalence [7]. In this regard, novel strategies and tools are urgently required to develop an integrated control strategy.

Paratransgenesis, the genetic engineering of symbionts with anti-pathogenic effectors to control disease transmission, represents a potentially promising strategy. The technique was originally developed by Beard et al. to control *Rhodnius prolixus* (triatomine/kissing bug) from spreading the

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causal parasite of Chagas disease (*Trypanosoma cruzi*). A gram-positive bacteria, *Rhodococcus rhodnii*, that occurs at high concentrations within the hindgut of *R. prolixus* was genetically engineered to express a trypanocidal immune peptide, Cecropin A. This resulted in a decreased *T. cruzi* infection rate in *R. prolixus* and was approved as an integrated pest management program in South and Central America [8].

Paratransgenesis offers several advantages. It is scalable because transgenic microorganisms can be grown to large quantities at low cost. The technique is not limited to single mosquito species because symbiotics can potentially colonise multiple important vector species. Moreover, the symbiont can be maintained within the ecosystem by vertical, horizontal, and trans-stadial transmission, mitigating the need for re-introduction [9,10]. Finally, and perhaps most importantly, it is a manipulable system that can be altered to target different pathogens or keep pace with resistance by exploiting different effectors. As such, the discovery and development of novel anti-pathogen molecules is critical for paratransgenesis implementation.

Venom toxins are excellent candidates for effectors in paratransgenesis. Venoms are complex mixtures of toxic proteins, peptides, and small molecules that are delivered through the infliction of a wound [11,12]. Venoms of hymenopteran insects such as bees and wasps are diverse, consisting of peptides, enzymes, and neurotransmitters [13], whilst scorpion and spider venoms largely consist of neurotoxins, which modulate a variety of channels including voltage-gated potassium, sodium, and calcium ion channels, acid sensing ion channels, calcium-activated potassium channels, glutamate receptors, and glutamate transporters [14,15]. Snake venom consists of haemotoxins, cytotoxins, and neurotoxins that can be grouped into superfamilies by structure, with phospholipase A2s ($PLA_{2}s$), snake venom metalloproteinases, snake venom serine proteinases, and three-finger toxins being the most abundant [16]. Venom toxins have high specificity, potency, and stability [11], and are less susceptible to bioaccumulation than chemical insecticides [17]. The venoms of many hymenopteran insects, scorpions, spiders, and snakes have been studied for their potential antiparasitic [18–20] and antiviral [21–24] properties. This review highlights the untapped potential of venom toxins as effectors in paratransgenesis. We discuss successful paratransgenesis studies that have been undertaken with antimalarial venom toxins as proof of principle and the need for specific screening of venom toxins to identify effectors is highlighted. Regarding mosquito-borne diseases, paratransgenesis strategies have focused on targeting *Plasmodium*, the causal agent of malaria. However, we suggest that paratransgenesis could be applicable to target arboviruses through the use of antiviral venom toxins.

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Antiparasitic Venom Toxins as Effectors to Target *Plasmodium*

Previous mosquito paratransgenesis strategies have focussed on targeting *Plasmodium*, the casual parasite of malaria. The species of *Plasmodium* responsible for causing malaria in humans are *P. falciparum*, *P. vivax*, *P. ovale*, *P. knowlesi*, and *P. malariae*, with the former being responsible for more than 90% of the malaria deaths. Mosquitoes from the Anopheles genus are responsible for the transmission of malaria. Paratransgenesis targeting *Plasmodium* must use effectors that inhibit the parasite stages within the mosquito: gametes, ookinetes, oocysts or sporozoites (Figure 1) [25]. Two venom toxins have effectively been utilised as effectors (Table 1): Scorpine, an excitatory neurotoxin from *Pandinus imperator* with antibacterial and antiparasitic properties, and $mPLA_2$, a PLA_2 from bee venom with a point mutation (H67N) to prevent enzyme activity and toxicity to bacteria. $mPLA_2$ expressed in *Escherichia coli* induced a moderate reduction of oocyst numbers from *P. berghei*, a rodent malaria model, when fed to *Anopheles stephensi*, however, the bacterium survived poorly in the mosquito [26]. $mPLA_2$ and Scorpine expressed in *P. agglomerans* [27] and *Serratia* [28] were able to

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effectively colonise the midgut of *An. gambiae* and decreased the number of *P. falciparum* oocytes in infected mosquitoes. Scorpine has also been expressed in *Asaia* [29], a bacteria found in *Anopheles sp.*, *Aedes aegypti*, and *Aedes albopictus* [30–36] that is transmitted vertically, horizontally and transstadially [31]. Transgenic *Asaia* expressing Scorpine significantly reduced the number of *P. berghei* oocytes in the mosquito midgut, however, constitutive expression of the toxin compromised bacterial fitness. To improve bacterial fitness, blood meal inducible promoters within the mosquito microbiome were identified and used to conditionally express Scorpine. This enabled *Asaia* to maintain fitness and compete with wild type *Asaia*, whilst oocyst midgut number in *A. stephensi* decreased by approximately 90% and prevalence decreased by up to 20%, indicating a decrease in infection potential [37]. Future work should assess transgenic bacteria in semi-field trials to assess suitability for practical use within the field.

Table 1. Summary of the toxin effectors used in paratransgenesis studies that target *Plasmodium*. Abbreviation: $mPLA_2$, inactive mutant (H67N) PLA_2.

Venom Effector	Mosquito Species	Vector Symbiont	Effect	Ref
Scorpine	*An. stephensi*	*Asaia bogorensis*	63% reduction in oocyst number of *Plasmodium berghei*	[29]
Scorpine	*An. stephensi*	*Asaia bogorensis*	90% reduction in oocyst number of *Plasmodium berghei*	[37]
$mPLA_2$	*An. stephensi*	*Escherichia coli*	23% reduction in oocyst number of *Plasmodium berghei*	[26]
Scorpine & $mPLA_2$	*An.gambiea* *An. stephensi*	*Pantoea agglomerans*	97.8% reduction (scorpine) and 85.3% reduction ($mPLA_2$) in oocyst number of *Plasmodium falciparum*	[27]
Scorpine & $mPLA_2$	*An. gambiae*	*Serratia marcescens*	93% reduction (scorpine) and 86% reduction ($mPLA_2$) in oocyst number of *Plasmodium falciparum*	[28]

Despite promising preliminary mosquito paratransgenesis data, only a limited number of effector molecules have been assessed with $mPLA_2$ and Scorpine being the only venom toxins effectors that have been experimentally tested in mosquito paratransgenesis. An expanded arsenal of molecules is required to allow a multi-faceted and adaptable approach to paratransgenesis. Importantly, expression of multiple effectors has been shown to enhance efficacy [27,28] and can enable several stages of the pathogen life cycle to be targeted, increasing robustness. The risk of resistance development can be reduced through identification and use of multiple effectors with different mechanisms of actions and/or broad-spectrum actions. There is also a potential to target multiple pathogens through co-expression of effectors or use of effectors with multiple mechanism of actions. Finally, it is important to have a diverse effector library available to mitigate resistance and enable new paratransgenesis replacement strategies.

Venom toxins have the potential to act as effectors due to their antiparasitic activity (Supplementary Table S1, Figure 1). However, the majority of these studies have focussed on the intraerythrocytic asexual stages of *Plasmodium* within the mammalian host [19], in line with research more applicable to the identification of antimalaria therapeutics. Few studies have screened toxins to identify effectors for paratransgenesis, but for effectors to be useful they must target the *Plasmodium* stages occurring in the mosquito [25].

Several α-helical linear peptides such as Anoplin and Mastoparan X from wasp venom [38], Melittin from European honeybee venom [38], and MeuTXKβ [39] from *Mesobuthus* scorpion venom inhibit ookinete development. Whilst, another linear helical peptide, specifically scorpion toxin VmCT1 from *Vaejovis mexicanus* is effective *in vitro* against *P. gallinaceum* sporozoites, a poultry model of the last stage of *Plasmodium* development within the mosquito [40]. Antimicrobial peptides from scorpions including Scorpine and synthetic peptides based on Vejovine and Hadrurin also inhibit

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ookinete development *in vitro* [41–43]. *In vivo* studies have found PLA_2 derived from the venom of the rattlesnake *Crotalus adamanteus* reduced the number of oocysts by 99% when mixed with cultured *P. falciparum* gametocytes and fed to *An. gambiae* or *An. stephensi* mosquitos [44]. A similar reduction in *P. gallinaceum* oocyst number in *Ae. aegypti* was achieved. Interestingly, the PLA_2 toxin did not affect ookinete viability, but acted on the midgut surface, preventing ookinete maturation to oocytes. A similar effect was observed for a PLA_2 from bee venom in *Ae. fluviatilis* [45] and Melittin [38].

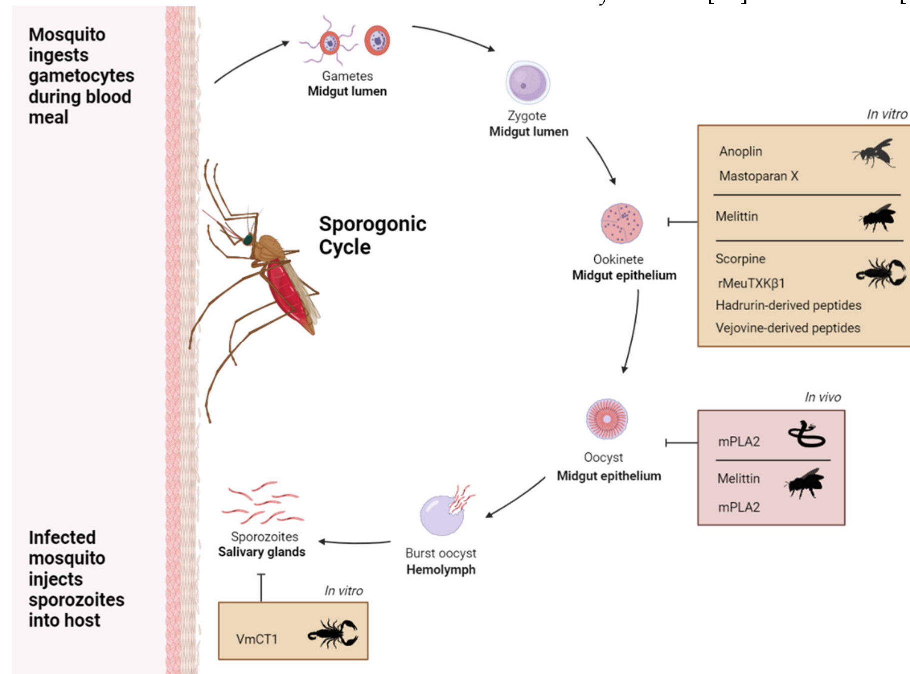

Figure 1. Overview of *in vitro* and *in vivo* studies showing activity of toxins against the mosquito stages of *Plasmodium sp*. Venom toxins that have shown anti-plasmodial activity within mosquitos (*in vivo*, pink panel), and anti-plasmodial activity when added directly to isolated *Plasmodium* stages (*in vitro*, orange panels). The cycle shows the developmental stages of *Plasmodium* sp. within the mosquito vector and inhibitory arrows indicate the stage targeted by the venom toxin. *Plasmodium* male and female gametocytes, the sexual stages that differentiate into gametes, are taken up by the mosquito in a bloodmeal and merge to form a zygote which develops into a motile ookinete. The ookinetes invade the epithelial lining of the mosquito midgut and differentiate into oocysts that generate sporozoites which are released into the hemolymph, invade the salivary gland and are injected in the skin of the next host during a blood meal.

Ookinete to oocyst development in the midgut, the bottleneck of malaria transmission [46], is the best target for effector screening. The emphasis should be on *P. falciparum*, the species primarily infecting humans and causing most malaria deaths. Although experiments with *P. falciparum* are challenging and limited to *in vitro* culture of the intraerythrocytic stages of the parasite, ookinetes can be generated *in vitro* by gametocyte differentiation in specialised medium enabling toxins to be rapidly screened [47]. However, studies have mainly been performed with *P. berghei* and *P. gallineceum*, species that infect rodents and poultry, respectively, as described above. This is because they can be maintained as intraerythrocytic stages in mice or chickens to generate a high density of gametocytes, the sexual stage that is required for oocyst development within the mosquito. An ideal high-throughput pipeline would be the screening of toxins on ookinete development *in vitro* with successful candidates taken forward to *in vivo* development of *Plasmodium* in the mosquito, as described by Carter et al., 2013 [38].

In addition, downstream assays must be undertaken to ensure the toxin is specific for the pathogen. This should involve assessing any potential effect of mosquito fitness, as performed in a previous study to identify effectors [38]. Assays included basic *in vitro* cell viability assays on mosquito cell lines and feeding toxins to mosquitoes. However, the compatibility of the toxins with

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the mosquito symbiont must also be assessed by performing minimum inhibitory concentration assays. From an ecological perspective it is important to assess effects on other insects, especially pollinator species, using similar assays. From a safety perspective, mammalian toxicity should also be reviewed using *in vivo* and *in vitro* assays. These types of studies have largely been neglected to date.

Could Paratransgenesis Be Used to Target Arboviruses?

Paratransgenesis to target arboviruses has not been attempted thus far but the antiviral properties of venom toxins are encouraging for this strategy. Antiviral compounds can target various stages of virus infection including pre-entry and/or post-entry stages [48]. Compounds can inactivate the virus pre-entry by inactivating the virus before it attaches to the cell, a process known as neutralisation; inhibiting surface proteins required for attachment; inhibiting virus endocytosis or inhibiting fusion of the viral envelope and host cell membrane. Alternatively, compounds can act at the post-entry stages, by inhibiting viral uncoating, replication, transcription, translation, virus assembly, and virus release. Antivirals can also induce the host immune response, through stimulating the production of interferons, other cytokines, and chemokines, affecting both pre- and post-entry stages. Targeting any of these stages within the mosquito midgut, as the location of arbovirus infection after the mosquito takes a blood meal from an infected host, has the potential to prevent viral dissemination into salivary glands. Blocking this step, as with *Plasmodium*, would prevent the mosquito becoming infectious and transmitting the arbovirus and has been suggested previously as a strategy to control arbovirus transmission [49].

Venom toxins have shown antiviral activity against ZIKV, DENV, YFV, JEV, and CHIKV. However, there is limited research on the antiviral properties of venom toxins against RVFV. Many antiviral venom toxins have been shown to target the pre-entry stages, the most studied of which being group I and II snake venom PLA_2 toxins (Figure 2, Supplementary Table S2). Group I PLA_2, consisting of PLA_2 produced by *Elapidae* (cobras, mambas, coral snakes) and *Hydrophidae* (sea snakes) whilst group II PLA_2 are produced by *Viperidae* (rattlesnakes) [12]. Group II PLA_2s derived from *Bothrops alteratus* [50], *B. leucurus* [51], and *B. asper* venom [52] can neutralise several strains of DENV, whilst group II PLA_2s from *B. jararacussu* [53,54] and *Crotalus durissus terrificus* venom have shown inhibition activity against YFV, CHIKV, DENV, and ZIKV [55–59]. LaPLA_2-1, a group III PLA_2 from the scorpion *Liocheles australasiae*, can neutralise DENV and JEV [60]. Interestingly, DENV propagated in mosquito cell lines was more sensitive to Mt-I, a catalytically inactive PLA_2 from *B. asper* venom, than viruses propagated in mammalian cells [52]. Neutralisation by group I, II, and III PLA_2 likely occurs by hydrolysis of the virus lipid bilayer [55,59,60]. Viral neutralisation has also been shown with to occur with ZY13, a peptide analogue of cathelicidin from *Bungarus fasciatus* venom [61] and the Scorpine-like peptide Smp76 from *Scorpio maurus palmatus* venom [62,63].

Venom toxins can also inhibit virus post-entry stages (Figure 2). Ev37, a Scorpine-like peptide from scorpion *Euscorpiops validus* venom that selectively inhibit $K_v1.3$ potassium channel, prevents viral genome release into the cytoplasm by acidifying viral genome-containing vesicles preventing membrane fusion [64]. The host defense peptide Av-LCTX-An1a from *Alopecosa nagpag* spider venom can inhibit viral protease activity preventing virus maturation [62]. Studies assessing the host immune response have found that Scorpine-like peptide rSmp76 from scorpion *Scorpio maurus palmatus* venom and ZY13 have antiviral effects by activating interferon signaling [61,62]. However, it is important to stress that most of these studies have been undertaken with mammalian cell lines, and their translatability into mosquito cells is unknown. Promisingly, recombinant Scorpine generated in *Anopheles gambie* cells can inhibit DENV serotype 2 replication in mosquito cells [42] showing the potential of venom toxins to have antiviral activity within mosquitoes. Similar studies with the aforementioned venom toxins are necessary to determine if the antiviral activity seen within mammalian cells is transferable to mosquitoes. *In vivo* studies assessing viral load, for example by RT-qPCR and plaque assays, in mosquitoes fed with toxins and virus must also be conducted to confirm *in vitro* findings.

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Screening to identify new potential antiviral effectors should involve assessing the ability of toxins to inhibit virus at all stages of infections. Viral neutralization should be assessed by incubating the test compound with the virus then assessing virus titre. Effect of the toxins at pre-entry stages should be evaluated by simultaneously adding the toxin and virus to mosquito cells at 4 °C (to prevent virus internalisation) and quantifying the levels of bound virus, as well as simultaneously adding the compounds and virus at 37 °C to determine effects on virus internalization and entry. Toxins should also be added after viral infection to evaluate post-entry antiviral activity. Finally, the ability of the toxin to induce a cellular antiviral response can be determined by addition of the toxin to the host cells pre-viral infection. Similarly, with antiparasitic effectors, any potential candidates should be further tested to ensure the toxin does not affect the fitness of the mosquito and symbiont. This is a vital step before moving forward with genetically engineering the symbiont.

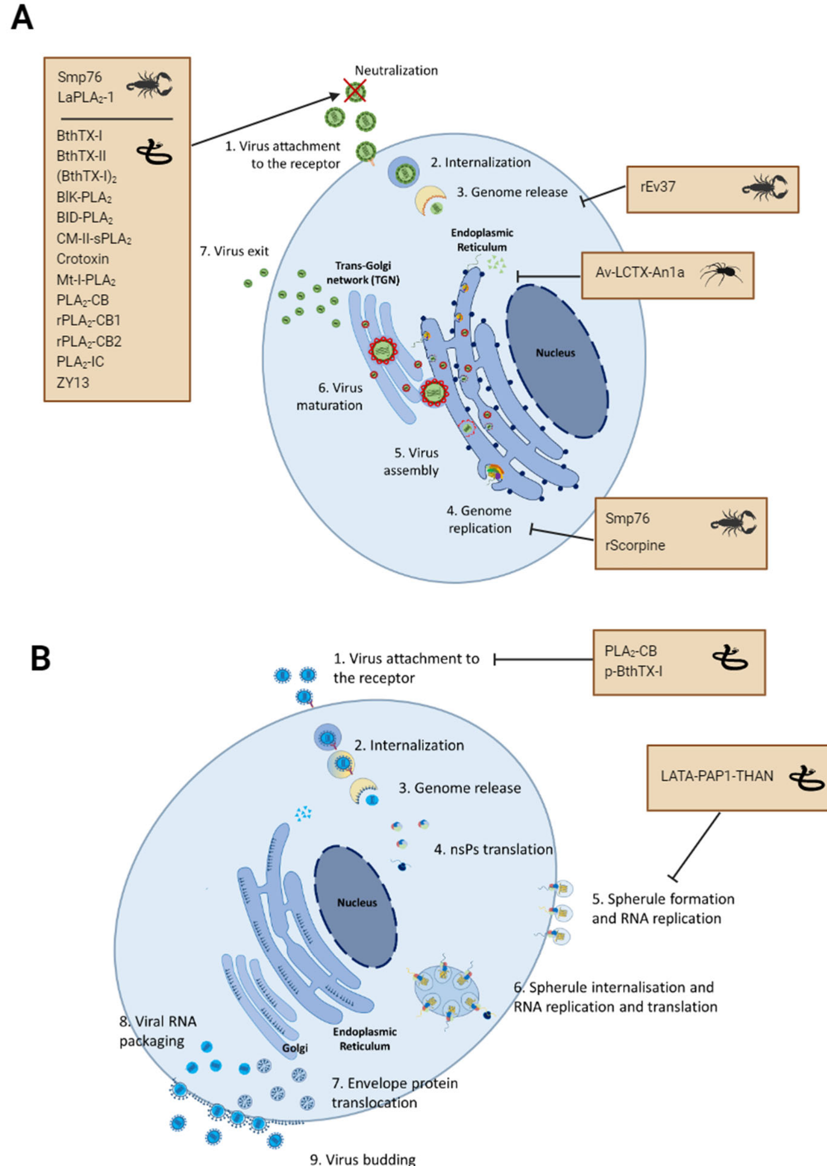

Figure 2. *Orthoflavivirus* (ZIKV, DENV, YFV, JEV) and *Alphavirus chikungunya* (CHIKV) stages of infection and venom toxin targets. Venom toxins that have shown antiviral activity against (**A**) *Orthoflavivirus* and (**B**) *Alphavirus chikungunya*. The illustration shows the infection stages of the two

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classes of virus and inhibitory arrows indicate the stage targeted by the venom toxin. Viral particles attach and are internalized through clathrin-mediated endocytosis. Acidification of the endosome facilitates membrane fusion. (**A**) Once the *Orthoflavivirus* capsid protein is released, the capsid is disassembled, and virus genomic RNA transported to the ER for translation and replication. Immature virions bud from the ER and undergo maturation in the trans-golgi network. Virus exits by exocytosis [65]. (**B**) After the CHIKV genome release, viral RNA is directly translated into non-structural proteins (nsPs) and forms replication spherules, where viral genome replication occurs. Viral RNA is then translated to produce structural proteins. Capsid proteins and genomic RNA are assembled in the cytoplasm to form icosahedral nucleocapsid, and other structural polyproteins are translocated into ER for post-translational modification and delivered to the cell surface through the secretory pathway. The virus budding occurs when the nucleocapsid assembles with the modified structural proteins [66].

Conclusions

Venoms contain a highly diverse library of bioactive and stable peptides with antiparasitic and antiviral properties. Studies have shown that using venom toxins as transgenes in paratransgenesis can be useful for the control of mosquito-borne pathogens, specifically *Plasmodium*. However, few studies have screened toxins with the goal of identifying effector molecules and therefore the choice of potential effectors is limited. Here, we have reviewed the toxin literature and have highlighted potential effector candidates for future paratransgenesis studies. However, we stress that additional screening with the aim of identifying effectors is vital. These studies should involve *in vitro* and *in vivo* studies to select antiviral and antiparasitic toxins that target appropriate stages of the pathogen life cycle, and that do not affect mosquito or symbiont fitness. We also argue the paratransgenesis strategy should be expanded to attempt to target arboviruses. The studies discussed here provide a strong foundation for further research in this area to identify toxin effector candidates.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org.

List of Abbreviations

Arbovirus, arthropod-borne viruses; CHIKV, chikungunya virus; DENV, dengue virus; JEV, Japanese encephalitis virus; $mPLA_2$, inactive mutant (H67N) phospholipase A2; PLA_2, phospholipase A2; RVFV, Rift Valley fever phlebovirus; WNV, West Nile virus; YFV, yellow fever virus; ZIKV, Zika virus.

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