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Disruption of dengue virus transmission by mosquitoes

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Abstract

Current control efforts for mosquito-borne arboviruses focus on mosquito control involving insecticide applications, which are becoming increasingly ineffective and unsustainable in urban areas. Mosquito population replacement is an alternative arbovirus control concept aiming at replacing virus-competent vector populations with laboratory-engineered incompetent vectors. A prerequisite for this strategy is the design of robust anti-pathogen effectors that can ultimately be genetically driven through a wild-type population. Several anti-pathogen effector concepts have been developed that target the RNA genomes of arboviruses such as dengue virus in a highly sequence-specific manner. Design principles are based on long inverted-repeat RNA triggered RNA interference, catalytic hammerhead ribozymes, and trans-splicing Group I Introns that are able to induce apoptosis in virus-infected cells following splicing with target viral RNA.

Keywords

Aedes aegypti; dengue virus; antiviral effector gene; RNA interference; catalytic hammerhead ribozyme; trans-splicing Group I Intron

Introduction

Increasing global trade and human traffic cause arthropod-borne viruses (arboviruses) and their vectors to become ever more prevalent around the world [1–5]. Recent examples are mosquito-borne arboviruses such as dengue virus (*Flaviviridae*; *Flavivirus*; serotypes 1–4, DENV1–4) causing morbidity and mortality throughout the world's tropics, and the current emergence of chikungunya virus (*Togaviridae*; *Alphavirus*; CHIKV) in the Caribbean and the Americas [6,7*,8*]. No therapeutics are available for most of these arboviral diseases, and vaccines have been licensed against only a handful of arboviruses, with DENV or CHIKV being notable exceptions [9]. Current mosquito-borne arbovirus control efforts rely

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on intensive vector control measures such as removing potential mosquito breeding sites, use of insecticide treated bed nets/window curtains, and residual insecticide applications [6,10]. Using insecticides is becoming increasingly ineffective and unsustainable because many mosquito populations have developed elevated levels of insecticide resistance over time [11–13]. A promising, alternative strategy to suppress mosquito transmission of arboviruses such as DENV or CHIKV involves *Wolbachia*, an intracellular bacterial symbiont that lives inside insects and is transmitted vertically from mother to offspring [14]. *Wolbachia* has been successfully trans-infected into naïve *Ae. aegypti* populations [15,16]. Reproduction is inhibited when *Wolbachia*-infected males are mated with non-infected females, thus favoring spread of the symbiont within a population. Importantly, mosquitoes which are infected with certain *Wolbachia* strains have been shown to be resistant to arboviruses such as DENV and CHIKV [15,16]. *Wolbachia* can also negatively impact the longevity of infected mosquitoes. Currently, the effectiveness of *Wolbachia* as an arbovirus/mosquito control agent is tested in field studies in Australia [16,17].

Two novel genetic pest management concepts of arbovirus/mosquito control rely on the use of genetically-modified mosquitoes. One of them is population reduction (elimination) based on Release of Insects with Dominant Lethality (RIDL) [18–20,21**–22], the other concept includes population replacement strategies, which will be the focus of this article. Population replacement implies that an arbovirus-competent mosquito population is replaced with laboratory generated, incompetent mosquitoes harboring specifically engineered antiviral effector genes [23–25,26**,27**]. Until now, the majority of research on disrupting mosquito-borne viral disease transmission has been concentrated on DENV and *Ae. aegypti* because of their role in transmitting the most clinically important arbovirus affecting humans [28]. Anti-DENV effector gene strategies include inverted-repeat (IR) RNA, catalytic hammerhead ribozymes (hRz), or trans-splicing Group I Intron (GrpI), which target and destroy exposed viral RNA genomes during their replication in the vector cell environment.

In the typical, urban disease cycle the four serotypes of DENV (DENV1–4) circulate between mosquitoes and humans [5,6,28]. Principal vectors are culicine mosquitoes, *Aedes aegypti* and *Ae. albopictus*. Following ingestion of a viremic human bloodmeal, DENV enters the epithelial cells of the mosquito midgut and replicates in this tissue. Within 4–5 days, the virus starts disseminating from the midgut to secondary tissues such as hemocytes, fat body, nerve tissues, and the salivary glands [29–31]. Once the salivary glands are infected and virus is released into the salivary ducts, the mosquito is able to transmit the virus to a new human host. Besides overcoming several tissue barriers in the mosquito, DENV has to cope with several innate immune defenses, Toll, JAK-STAT, and RNA interference (RNAi), the last of which is the most potent antiviral immune pathway capable of eliminating the virus [32*,33*,34*,35,36*]. Because the midgut is the initial tissue of DENV infection in the mosquito, it also constitutes the ideal site for over-expressing/activating an antiviral effector [25,26**]. This way DENV can be attacked at an early, vulnerable step of its replication cycle before the virus has established any infection foci.

RNAi-based antiviral effectors

Dicer2 of the endogenous, antiviral exo-siRNA pathway senses the presence of long dsRNAs, as they arise during replication of positive sense RNA viruses like DENV (Figure 1) [32*]. Dicer2 initiates the RNAi pathway by cleaving the dsRNAs into 21 bp duplexes. With the help of the RNA binding protein R2D2, the 21 bp duplexes are unwound and one strand (guide strand) is incorporated into the RNA induced silencing complex (RISC), of which the endonuclease argonaute2 is the catalytic component. The guide strand then guides RISC to homologous RNA molecules, which are sliced with the help of argonaute2 resulting in the destruction of viral genomes.

An anti-DENV effector gene that efficiently triggers the RNAi pathway against the virus consists of an inverted-repeat (IR) DNA encoding virus-derived sequences of 300–600 bp length in sense and antisense orientation [25,26**,27**]. A small, functional intron placed between the sense and antisense sequences is spliced out during transcription, thereby supporting stable dsRNA formation and establishing the trigger for RNAi. This approach has been used to generate transgenic resistance to DENV2 (Figure 1). In this case the virus-derived cDNA sequences, 578 bp in length, corresponding to the prM-M encoding region of the viral genome are separated by an intron originating from the *sialokinin I* gene [37]. Regulatory elements of this IR effector gene include the transcription termination signal of Simian virus 40 and an endogenous, tissue-specific promoter such as the bloodmeal-inducible *carboxypeptidase A* promoter of *Ae. aegypti* [38,39]. This promoter drives gene expression in midgut epithelial cells between 4 and 32 h following acquisition of a bloodmeal, an ideal time frame in which to tackle DENV2 before it is able to establish infection foci in this tissue. Germline transformation of the mosquito host is facilitated by insertion of the IR effector gene into a transgene insertion vector consisting of the non-autonomous class II DNA transposable element (TE), *mariner Mos1* [40–42]. This TE vector also contains an eye-specific selection marker such as enhanced green fluorescent protein (EGFP) to allow easy identification of transformants [43,44]. Unfortunately, TEs such as *mariner Mos1* follow quasi-random integration patterns due to their short recognition sequence motifs, which are abundantly present in the host genome. Consequently, TE-mediated transgene expression is often affected by position effect variegation, which could be overcome by using site-specific integration systems such as *PhiC31* or chromatin insulators such as the *scs scs'* elements of the *Drosophila gypsy* retrotransposon [45,46*,47,48*].

Using the Higgs White Eye (HWE) strain of *Ae. aegypti*, a transgenic line, Carb77, was previously generated according to the design principles described above [25]. Carb77 females, expressing the IR effector in midgut tissue of bloodfed females were highly resistant to orally acquired DENV2 but not to other serotypes of the virus or to other arboviruses, confirming the homology-dependence of this antiviral strategy. Carb77 females exhibited a midgut infection barrier to the virus, which could be circumvented by intrathoracic injection of the virus [25]. After 17 generations in laboratory culture, these Carb77 mosquitoes lost their resistance phenotype even though the transgene itself was not mutated [49], possibly due to hetero-chromatin rearrangement that silenced the IR effector gene. In a subsequent experiment a new transgenic line, Carb109, was generated, which

harbored the identical transgene as Carb77 [26**]. Carb109 females proved to be refractory to various DENV2 strains for at least 33 generations. Importantly, the resistance phenotype was maintained after introgression of the Carb109 transgene into a genetically diverse laboratory strain (GDLS) derived from 10 different *Ae. aegypti* wildtype populations from DENV-endemic regions of Southern Mexico. Furthermore, fitness studies showed that introgression of the Carb109 transgene into GDLS via consecutive backcrosses resulted in transgenic hybrids that exhibited only minimal fitness loads. After five consecutive backcrosses without selection for transgenic individuals, the transgenic allele frequency of introgressed cage populations was in equilibrium. Thus, the strong fitness loads observed for the original Carb109 transgenic strain seem to have been associated with the genetic background of the highly inbred HWE strain, which had been the recipient for germline transformation.

The identical DENV2 targeting IR effector was also transgenically overexpressed in fat body tissue under control of the bloodmeal inducible *vitellogenin 1* promoter and in salivary glands from the *30K* promoter [27**,49]. Silencing DENV2 in fat body did not affect the mosquito's overall vector competence for the virus, indicating that this tissue can be circumvented by DENV2 during its route to the salivary glands. Constitutive silencing of DENV2 in the distal-lateral lobes of the salivary glands significantly reduced virus titer and infection rate in this tissue. Importantly, in two of five generated transgenic lines, the virus was completely absent in saliva [27**].

All these findings are highly encouraging, demonstrating that it is feasible to overexpress a synthetic antiviral effector gene to generate transgenic refractoriness in vector mosquitoes. It is possible to engineer complete resistance to an arbovirus in *Ae. aegypti*, which is stably maintained for many generations without imposing a strong fitness load onto the transgene harboring mosquitoes. Thus, the use of gene drive systems such as meiotic drive (based on *Medea*) or Homing Endonucleases (HEG), which are still not available for *Ae. aegypti*, may not be an absolute necessity as mathematical models generated with the recently developed *Ae. aegypti* population dynamics software, Skeeter Buster, suggest [50–51,52**,53,54,55**].

Hammerhead ribozyme strategy

Hammerhead ribozymes (hRz) are small catalytic RNA molecules that cleave their target RNAs in a sequence-specific manner (Figure 2). This class of catalytic RNA was initially identified as *cis*-cleaving molecules among plant viroids and plant virus satellite RNAs [56–59]. Most of these natural hRz consist of a central conserved core sequence flanked by three double-stranded regions with relaxed sequence requirements (helices I, II and III), two of which are capped by short loops. Hasellhoff and Gerlach [60] separated the catalytic portion of the hRz from its substrate by removing the connecting short loop resulting in a trans-cleaving ribozyme (Figure 2). Trans-cleaving ribozymes have endoribonuclease activity in the presence of divalent cations like Mg^{++} and can also perform multiple turn-over kinetics enhancing their catalytic activity [60–62].

Several features make hRz attractive as synthetic anti-arboviral effectors: i) hRz are derived from plant viroids, therefore arbovirus vectors do not exhibit any natural resistance against hRz; ii) the antiviral effect of hRz is mediated through the catalytic activity of the molecule itself and does not require additional host factors [63]; iii) typical hRz target sites are 18–19 nucleotides in length, allowing a relatively high target specificity, iv) hRz activity is not restricted to a particular cellular compartment; RNA in both the nucleus and the cytoplasm is efficiently attacked, v) hRz exhibit a high degree of target specificity, as single nucleotide polymorphisms can diminish hRz target recognition and cleavage [64]. In mammalian cells, hRz have demonstrated significant replication inhibition of many medically important viruses including human immunodeficiency virus (HIV), DENV2, influenza A virus, and hepatitis C virus (HCV) [65,66**,67,68]. The efficiency of a hRz as an antiviral effector in mosquitoes depends on several factors such as expression levels, co-localization with the target RNA, and target site accessibility. Several modifications have allowed optimization of hRz effectiveness. For example, the use of tRNA valine pol III as the promoter allows hRz expression to reach levels high enough to help overcome the rate-limiting step for catalysis in cells, the binding of the ribozyme to its target [69]. The clover leaf structure of the tRNA enables the protein exportin-X to transport the hRz to the cytoplasm, where it co-localizes with the infecting viral RNA and causes its degradation before the viral RNA has the opportunity to replicate (and to produce progeny RNA genomes) in the cell [70]. Target site accessibility is enhanced by attaching an Adenosine 60 tail to the hRz, allowing recruitment of RNA helices that can unwind potential interfering secondary structures in the target viral RNA (Figure 2) [71–74].

In cell culture experiments, 14 hRz designed to target various conserved nucleotide sequences within the DENV2 genome have been analyzed. The *Ae. aegypti* tRNA valine pol III promoter and 60 adenosines were incorporated into the 5' and 3' regions of hRz, respectively, followed by its insertion into a retrovirus transgene vector, pLaeARH. Transformed *Ae. albopictus* C6/36 cell lines expressing the hRz were then challenged with DENV2. Analysis of cell morphology, virus titers and viral RNA copy numbers revealed that four of the transformed, hRz-expressing cell lines (hRz2, hRz5, hRz7 and hRz11) suppressed DENV2 replication by at least 100-fold. Catalytic hRz activity was demonstrated by performing in vitro cleavage assays in which hRz2 and hRz7 yielded specific cleavage products after mixing them with synthesized DENV2 target RNAs [75].

Trans-splicing Group I Introns

As an alternative to hRz, trans-splicing Group I introns (GrpI) have been shown to be highly effective catalytic RNAs requiring a minimal target sequence of only 9 nucleotides (nt). Thus, a GrpI can be more easily designed to target all four DENV serotypes simultaneously, as there are plenty of 9 nt long stretches with perfect sequence homology present among the genomes of the four different serotypes. Initially, a GrpI was identified in the ciliate protozoa *Tetrahymena thermophila* as a self-splicing intron [76]. Since then GrpI have been used for various applications such as repair of defective mRNA transcripts or as antiviral effectors targeting HIV-1, cucumber mosaic virus, HCV, or DENV1–4 [77–79,80**,81**]. A GrpI consists of an external (EGS) and an internal guide sequence (IGS), a trans-splicing domain, and a 3' exon-of-choice (which can be derived from a pro-apoptotic gene) (Figure

2). The catalytic reaction of the GrpI involves two trans-esterification steps: i) complementary base pairing of IGS and EGS with the viral target RNA causing formation of p1 and antisense helices. The subsequent, Guanosine-mediated trans-esterification 1 reaction (TES-1) results in cleavage of the target viral RNA downstream of the Uracil position; ii) the distal region of the cleaved target RNA is then displaced by nucleotides upstream of the 3' exon, which undergoes complementary base-pairing with IGS including the spacing sequence between IGS and EGS to form the P10 helix. This initiates TES-2, culminating in the ligation of the 5' region of the target sequence to the 3' (heterologous) exon.

Carter *et al.* [80**] designed six different GrpI targeting the highly conserved cyclization (CS) sequences (5'CS, CS1, and CS2) in the genomes of DENV1–4. In vitro assays revealed that two of the six GrpI, α DENV-U143-FL and α DENV-U134-FL, targeting U143 and U134 respectively, of the DENV2 5'CS were highly effective, with α DENV-U143-FL being able to cleave the viral RNA of all four DENV serotypes, whereas α DENV-U134-FL was specific to DENV2. The GrpI were expressed in C6/36 cells from the *Drosophila actin 5c* promoter. Another variant of the GrpI contained the coding sequence of the firefly luciferase gene as 3' exon leading to luciferase expression upon ligation with the 5' target viral RNA. DENV2 infection of GrpI transformed C6/36 cells resulted in significantly increased luciferase activity in comparison to the control. Importantly, DENV2 titers in cells expressing α DENV-U143-FL and α DENV-U134-FL were significantly reduced [80**]. Eventually, the luciferase encoding 3' exon was replaced with an exon encoding N bax, a truncated version of the pro-apoptotic gene tbax showing increased pro-apoptotic potency [82]. Infection of stably α DENV-U134- N bax -transformed clonal C6/36 cell lines with the four serotypes of DENV resulted in complete elimination of DENV. Furthermore, TUNEL staining and annexin V staining confirmed that N bax was able to induce premature cell death [81**].

In summary, GrpI and the earlier described hRz have a high potential to function as robust antiviral effectors when expressed in transgenic mosquitoes.

Future directions

These data and those of other research groups strongly suggest that efficient blocking of arboviruses in mosquito vectors requires the design of synthetic effector molecules to which viruses are unable to mount any level of resistance. Less promising would be attempts to block arboviruses in the vector through manipulation of expression patterns of endogenous genes, having the potential to antagonize arbovirus replication/infection (i.e., innate immunity-related or virus receptor encoding genes). Such approaches would likely trigger the selection of variants with increased virulence from the viral quasi-species to overcome the manipulated (enhanced) antiviral response. *Wolbachia*'s utility as an arbovirus control agent in *Ae. aegypti* is already being investigated in limited field trials. However, so far it is not well understood how the endosymbiont causes resistance to arboviruses and whether arboviruses will be able to adapt to *Wolbachia*'s presence in *Ae. aegypti* over time.

Engineering tetravalent resistance to all four serotypes of DENV in mosquitoes using the RNAi-based synthetic effector design remains a challenge. Current research efforts focus on

highly conserved target regions of the DENV genome and on a new design concept for the IR molecules. Major efforts are also underway to generate and test transgenic mosquitoes harboring a GrpI such as α DENV-U134- N bax, which has the potential to target all four serotypes of DENV in *Ae. aegypti*. Furthermore, the RNAi-based strategy and the hRz strategy are currently being adopted to block transmission of other *Ae. aegypti*-borne arboviruses. Thus, it remains to be seen, which of these antiviral effector strategies will be most efficient in inhibiting replication of an arboviruses-of-interest in *Ae. aegypti* and whether the inclusion of multiple effectors might provide a more potent strategy for developing refractory transgenic vectors. The existing data clearly show that any arbovirus needs to be eliminated with ~100% efficiency in the mosquito to profoundly block virus transmission in the field when solely relying on a population replacement strategy. Test results with Carb109 mosquitoes indicate that this may be feasible. An additional advantage of the α DENV-U134- N bax GrpI strategy lies in its combined DENV1–4 resistance/death-upon-infection effect as revealed in cell culture assays. Based on this concept, DENV infected mosquitoes would be unable to transmit the virus and at the same time be eliminated from the population.

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Highlights

- Complete resistance to DENV2 was achieved in transgenic mosquitoes harboring an inverted-repeat construct triggering RNA interference against the virus. The transgene imposed only minimal fitness costs onto the mosquitoes.
- A hammerhead ribozyme was engineered that efficiently blocked DENV2 in cell culture.
- A trans-splicing Group I Intron was designed, which efficiently targeted all four DENV serotypes in cell culture. This approach can be combined with a death-upon infection strategy by overexpressing a pro-apoptotic gene in virus-infected cells.

Figure 1. Schematic representation of the *AeCPA/prM-M sense/intron/prM-M antisense/SV40A* inverted-repeat (IR) construct and its processing by the endogenous RNAi pathway of *Ae. aegypti*

The IR construct was inserted into the *mariner Mos1* TE, which was used as insertion vector for germline transformation of *Ae. aegypti*. Following acquisition of a bloodmeal, the IR effector is transcribed in the nucleus of midgut epithelial cells and processed into a long dsRNA molecule. The long dsRNA is exported from the nucleus and sensed by dicer2, which cleaves the long dsRNA into 21 bp duplexes. With the help of the RNA-binding protein R2D2 the 21 bp duplexes are unwound. One of the strands (guide strand) is

incorporated into RISC (RNA-induced silencing complex), the other strand (passenger strand) is discarded. Argonaute2 of RISC guides the complex to RNA molecules showing sequence homology to the guide strand (i.e. the viral RNA genome of DENV2 acquired along with the bloodmeal) and destroys the viral RNA before the virus is able to replicate and establish infection foci.

Abbreviations: *AeCPA* = *Aedes aegypti* carboxypeptidase A promoter; prM-M s, prM-M as = 578 bp cDNA of the DENV2 prM protein encoding region in sense and antisense orientations, respectively; i = minor (62 nt) intron of *Ae. aegypti* sialokinin I; svA = polyadenylation signal of Simian virus 40 VP1 gene.

Abbreviations: UTR = untranslated region, EGS = external guide sequence, IGS = internal guide sequence, BL = bulge loop, TSD = trans-splicing domain, TES = trans-esterification reaction, Rz = ribozyme (hammerhead ribozyme or Group I intron), Pol II/III = polymerase II or III promoter, CAP = 5'-methyl guanosine, SL I, II, III = stem loop I, II III, CC = catalytic core, LS = linker sequence, hRz = hammerhead ribozymes, H = RNA helicase.