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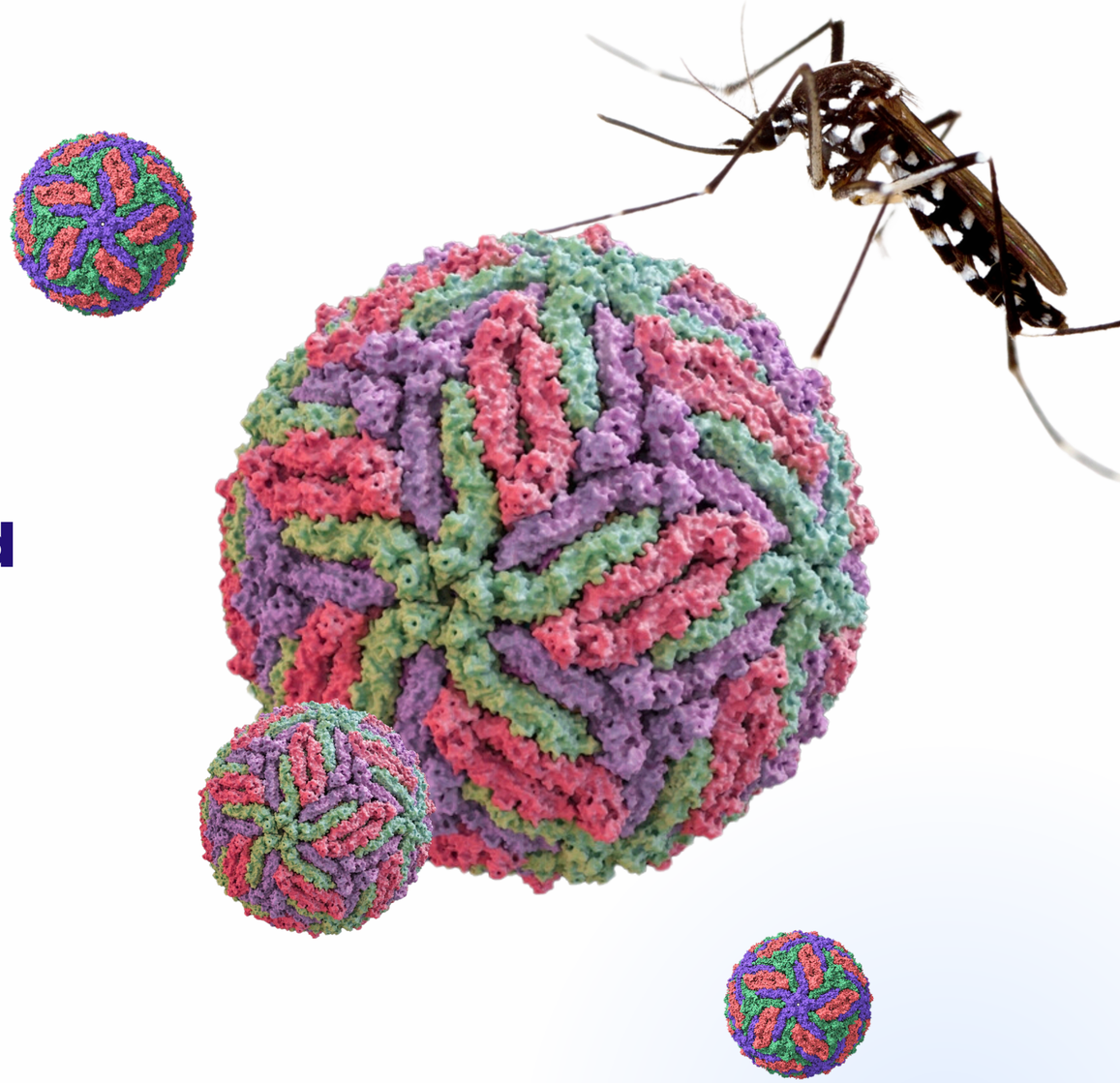

Effective suppression of Dengue fever virus in mosquito cell cultures using retroviral transduction of hammerhead ribozymes targeting the viral genome

Pruksa N et al. Virology Journal. 2009

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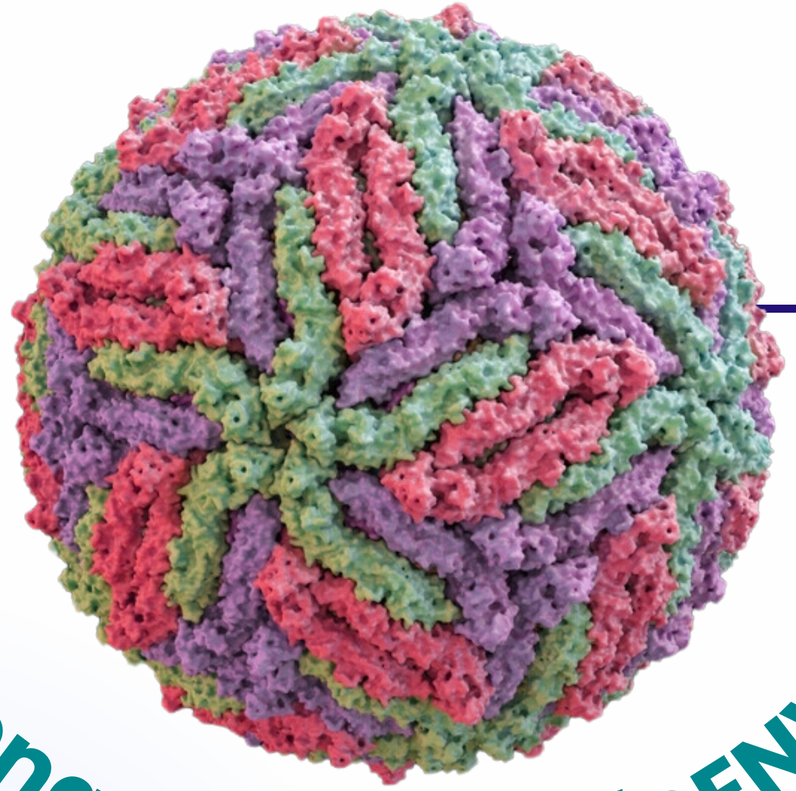
Medical Scientist, practitionor level

Insect biology research and test section



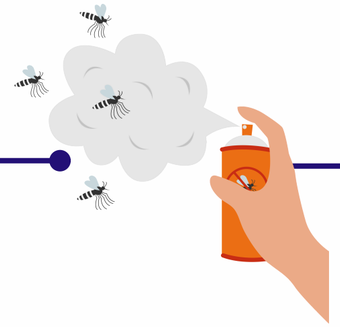
Dengue viruses (DENV)

- Family Flaviviridae
- DENV are transmitted to human populations by the mosquitoes *Ae.aegypti* and *Ae.albopictus.*
- An estimated 50–100 million cases of DF are reported each year.
- With 500,000 cases of DHF/DSS and more than 20,000 deaths.

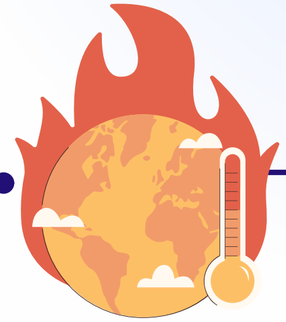
The factors that contribute to the emergence of this disease complex

The collapse of mosquito vector control

The demise of public health programs



Mosquito drug resistance



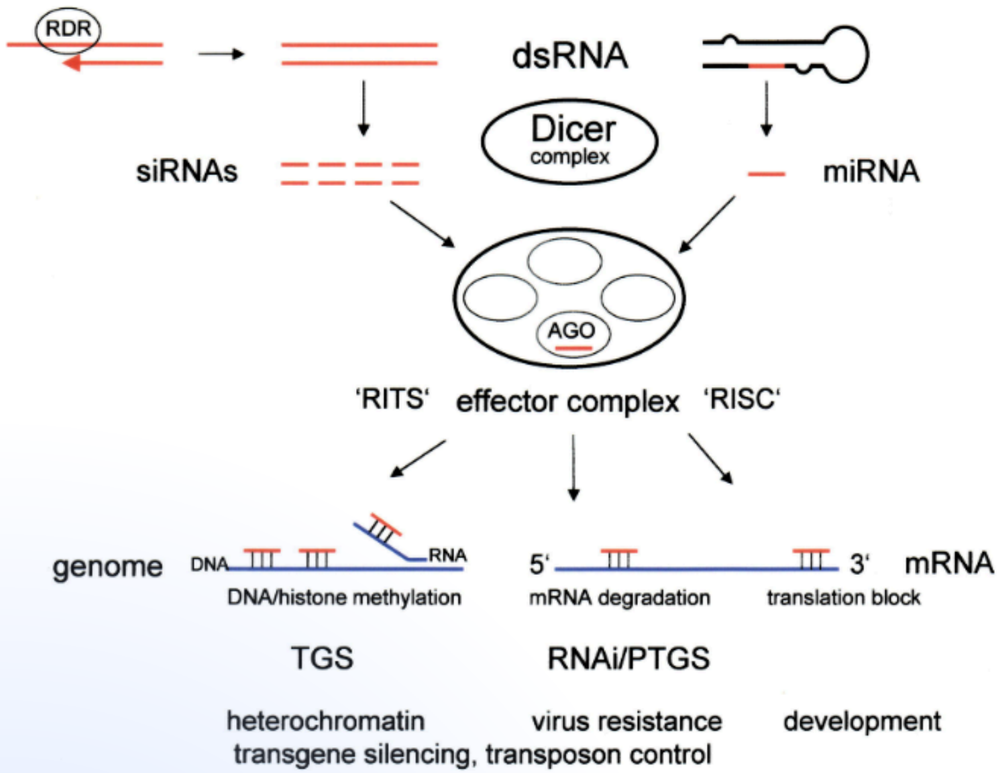
Climatic changes

Expanding urbanization

Increased global travel and commerce

Vaccines will not be available for general use

RNA interference or RNAi



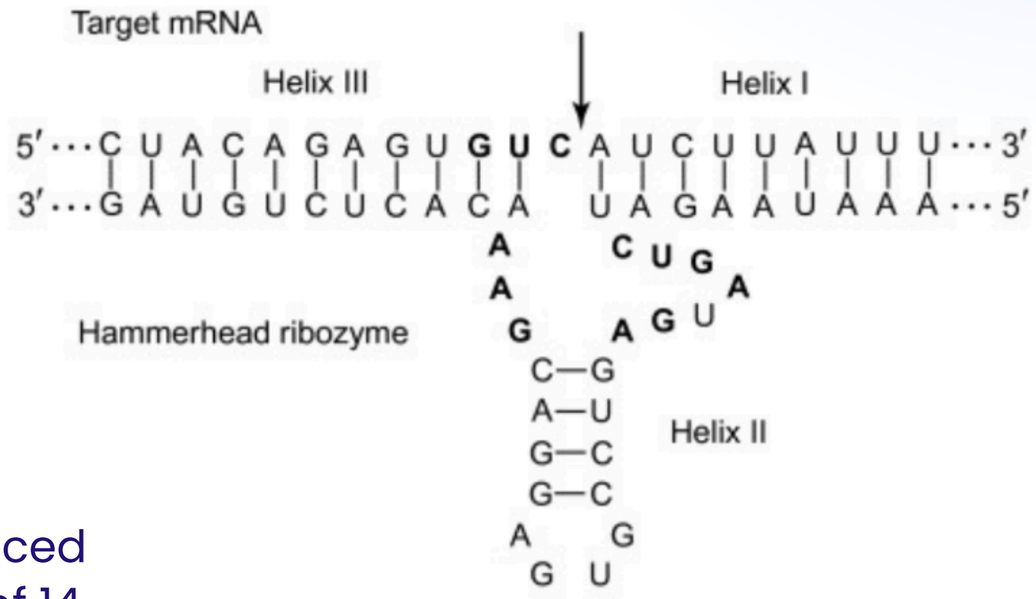
- Alternative strategies targeting DENV in mosquito cells and tissues have demonstrated some promise for suppression of the virus in mosquito vector populations.
- Modified antisense oligonucleotides, induction of RNA interference (RNAi) using both preM-derived sense and antisense encoding sequences expressed from dsSIN virus vectors and hairpin dsRNA to mediate RNAi in both mosquito cells and transgenic mosquitoes have each provided significant levels of DENV suppression.
- This study has focused efforts on RNA-enzyme (ribozyme) mediated viral suppression.

RNA-enzyme (ribozyme) mediated viral suppression

- We explore the utility of a genetic approach utilizing **hammerhead ribozymes (hRz)** for suppression of DENV in mosquito cells.

- HRz can inhibit the replication of a number of RNA viruses including human immunodeficiency virus. These molecules are capable of identifying targets as small as 15 nt in length, potentially allowing highly conserved sequences to be the focus of attack.

- In this report, Ae. albopictus (C6/36) cells were transduced with pantropic retroviral vectors, each expressing one of 14 anti-DENV hRz driven from the Ae. aegypti tRNAval promoter.

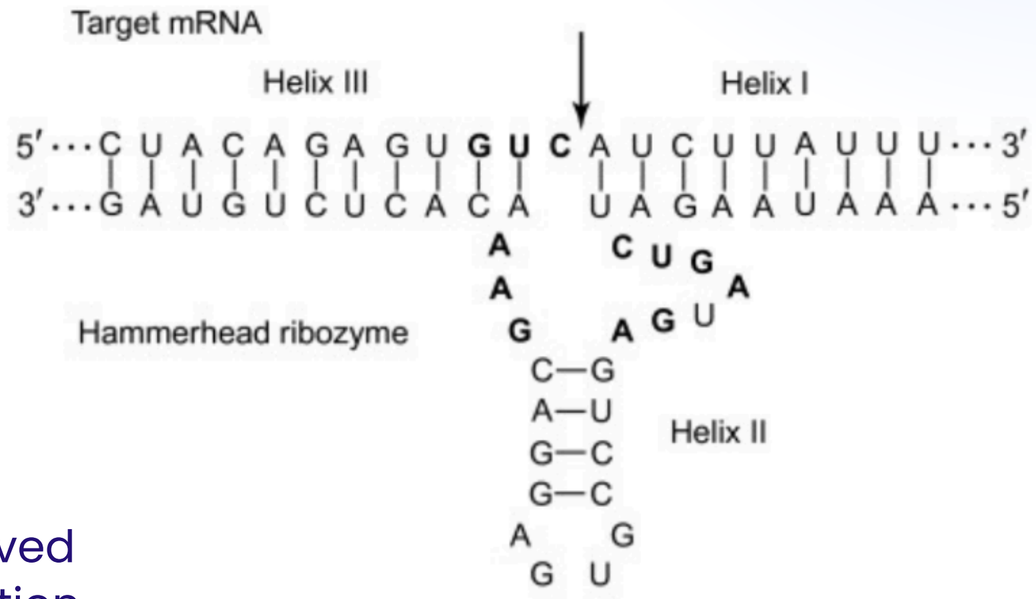


RNA-enzyme (ribozyme) mediated viral suppression

- These ribozyme-transduced cells were challenged with virus and assayed for productivity.

- Northern analyses, immunofluorescence assays, and quantitative real-time PCR were used to demonstrate that C6/36 cells expressing several hRzs were able to suppress DENV replication.

- Several of these targeted sequences are highly conserved among DENV serotypes, and may facilitate the application of this approach to transgenic mosquitoes.



Ae. aegypti tRNAval identification

- The *Ae. aegypti* tRNAval sequence from the GenBank database based upon homology to the Drosophila melanogaster tRNAval sequence was used as the promoter.
- The presumptive Ae. aegypti tRNAval (GenBank accession: CC142986), shared a 95% similarity (e = 5 × 10-27) to the D. melanogaster tRNAval, including both internal promoter sites **(Fig. 1)**.

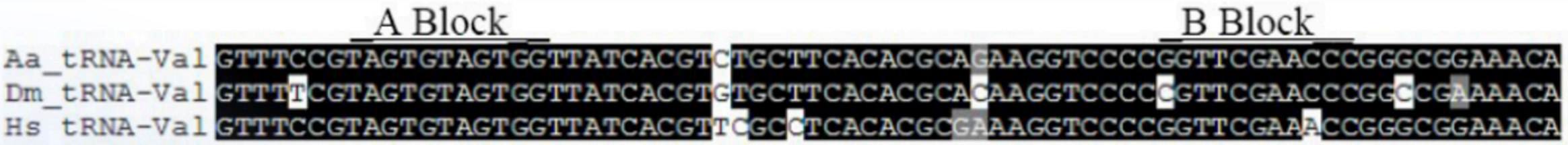


Figure 1 Nucleotide alignments of the Human (Hs), D. melanogaster (Dm), and Ae. aegypti (Aa) tRNAval. The position of the concensus internal A and B blocks of the RNA pol III promoter are indicated.

Selection of hRz target sites on DENV-2 genome

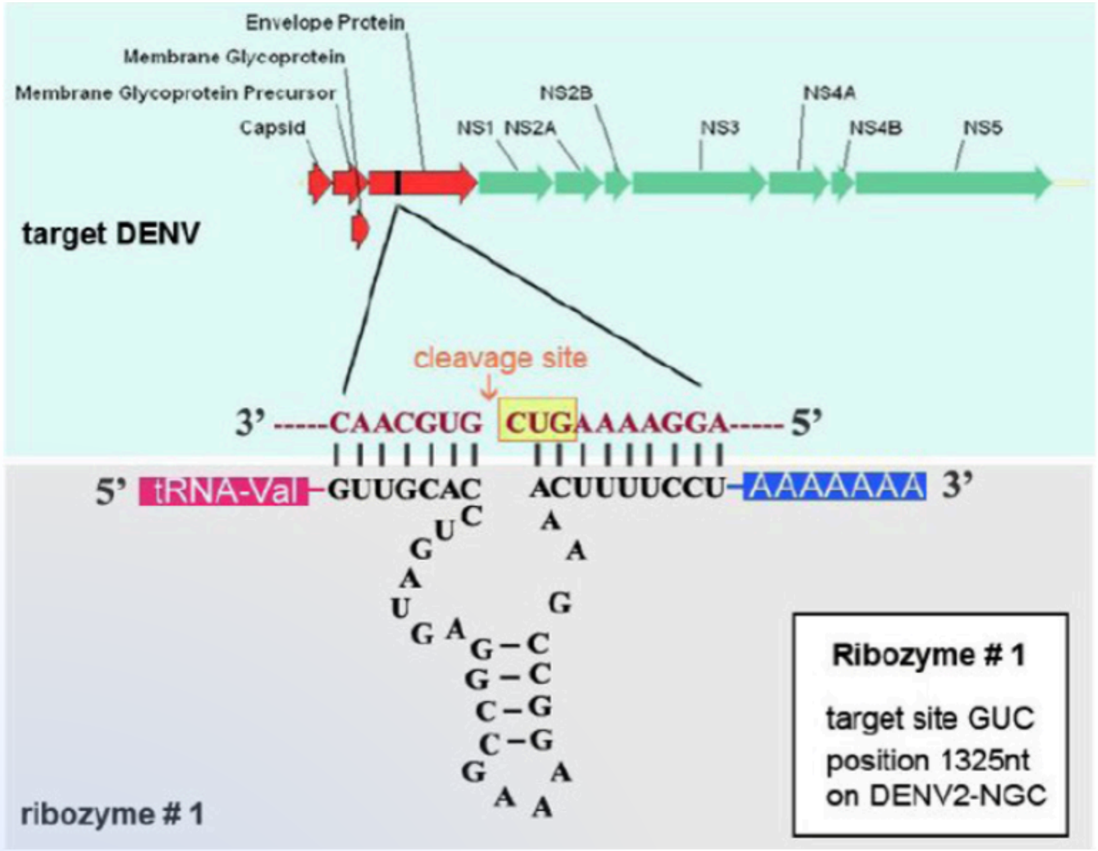
- Genomic sequences of 29 strains of DENV-2 were retrieved from GenBank, aligned in ClustalX
- hRz target sites were selected by scanning for conserved sequences that contain one of the following triplets; GTC, GTA, ATC or CTC.
- These triplets are believed to be the most effective target sites for hRz cleavage activity
- The primary criterion for selection was that the target site must be present in all 29 strains of DENV-2 or present only in DENV-2 NGC.
- 14 sequences of hRz templates, positions of the triplets on DENV genome, and the specific strains of DENV-2 NGC they match are listed in **Table 3.**

Table 3: Sequence, positions, and tropism of each of the 14 ribozymes used in this study

hRz #	Sequence of target RNA (5'→3')	Target triplet and position in genome	DENV-2 strains targeted
1	AGGAAAA**GUC**GUGCAAC	GUC 1325 (ENV)	NGC
2	GUUUUAG**GUC**GCCUGAU	GUC 1988 (ENV)	NGC
3	UAGCCCA**GUC**AACAUAG	GUC 2035 (ENV)	NGC
4	CAUAGGA**GUC**AUUAUCA	GUC 2323 (ENV)	NGC
5	UCACU**GUC**UGUGUC	GUC 2367 (ENV)	All strains
6	AUAGU**GUC**AUCAGUGAA	GUC 8272 (NS5)	All strains
7	AAAGAA**GUC**AGGCCA	GUC 10420 (3'NCR)	All strains
8	GCAUG**GUA**CCUGUGG	GUA 4503 (NS2B)	All strains
9	GACUCAAAA**CUC**AUGUCAGC	CUC 2974 (NS1)	All strains
10	AUGGAA**AUC**AGACCATT	AUC 3430 (NS1)	All strains
11	GGAAGCU**GUA**CGCAUGG	GUA 10528 (3'NCR)	All strains
12	UGAAGCU**GUA**GUCUC	GUA 10624 (3'NCR)	All strains
13	AUGCC**AUC**CAUGAA	AUC 10244 (NS5)	All strains
14	CUGUUGA**AUC**AACAGGU	AUC 10768 (3'NCR)	All strains

Underlined: core NUH triplet

Construction of retroviral transducing vectors expressing anti-DENV hRzs and establishment of transduced C6/36 cells



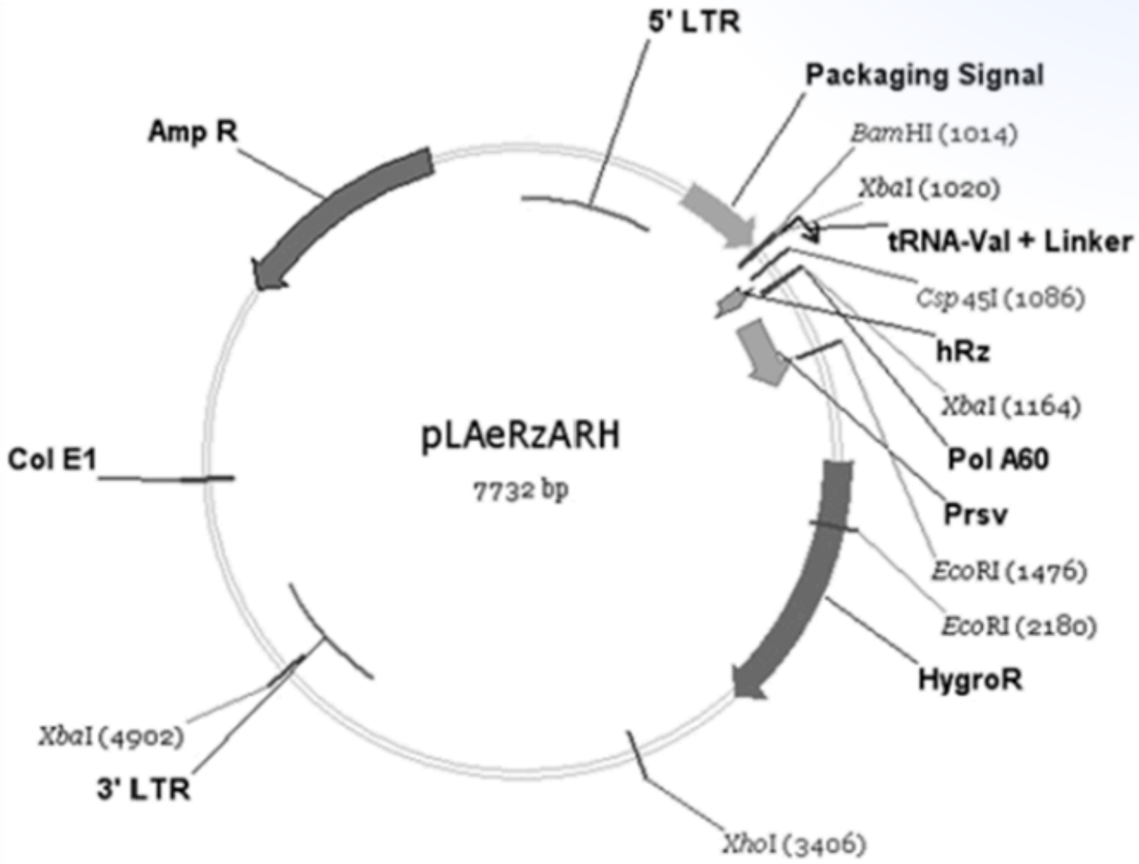
mechanism

- hRz are small ribonucleic-based enzymes that are capable of catalyzing target RNA cleavage in a sequence-specific manner.
- Their mechanism of action involves the pairing of the 5' helix I and 3' helix III arms of the hRz to complementary 3' and 5' base pairs, respectively, on the target RNA. **(Fig. 2)**
- The catalytic core of the hRz, or helix II, is responsible for cleavage at a 5'-NUH-3' triplet site on the target RNA, where N can be any of the four nucleotides and H can be A, C or U

Figure 2 Representative hRz structure and its DENV target sequence. hRz # 1 nucleotide sequence and structure is depicted. Nucleotides flanking the cleavage site (yellow box) in the envelope protein region of the DENV-2 target RNA are enlarged. The ribozyme cleaves the target RNA at the GUC triplet site following antisense recognition and base pairing of the two ribozyme arms.

Construction of retroviral transducing vectors expressing anti-DENV hRzs and establishment of transduced C6/36 cells

- The hRz expression plasmid, pLAeRzARH.
- 14 hRzs digested with Csp45 I and Mfe I were cloned to the plasmid and No-hRz control.
- tRNAval internal RNA pol III promoter was digested with Xba I to the plasmid to drive the expression of each hRz to optimize expression and translocation of the hRzs to the cytoplasm.
- A stretch of 60As is attached to the 3' end of the hRz sequence for recruitment of RNA helicase.
- RSV promoter was digested with Xba I and EcoR V and cloned to the plasmid to drive independent expression of the hygromycin resistance gene.



Construction of retroviral transducing vectors expressing anti-DENV hRzs and establishment of transduced C6/36 cells

hRz #	Template sequence for amplification (5' to 3')
1	CCGGTTCGAACCCGGGCACTACAAAAACCAACAAgt tgcac**CTGATGAGGCCGAAAGGCCGAAA**ctttttcctA CAATTGTTTTTTTGAATTCATC
2	CCGGTTCGAACCCGGGCACTACAAAAACCAACAAat caggc**CTGATGAGGCCGAAAGGCCGAAA**cctaaaac ACAATTGTTTTTTTGAATTCATC
3	CCGGTTCGAACCCGGGCACTACAAAAACCAACAAct atgtt**CTGATGAGGCCGAAAGGCCGAAA**ctgggctaA CAATTGTTTTTTTGAATTCATC
4	CCGGTTCGAACCCGGGCACTACAAAAACCAACAAtg ataat**CTGATGAGGCCGAAAGGCCGAAA**ctcctatgA CAATTGTTTTTTTGAATTCATC
5	CCGGTTCGAACCCGGGCACTACAAAAACCAACAAg acaca**CTGATGAGGCCGAAAGGCCGAAA**cagtgaAC AATTGTTTTTTTGAATTCATC
6	CCGGTTCGAACCCGGGCACTACAAAAACCAACAAtt cactgat**CTGATGAGGCCGAAAGGCCGAAA**cactatA CAATTGTTTTTTTGAATTCATC
7	CCGGTTCGAACCCGGGCACTACAAAAACCAACAAtg gcct**CTGATGAGGCCGAAAGGCCGAAA**cttctttAC AATTGTTTTTTTGAATTCATC
8	CCGGTTCGAACCCGGGCACTACAAAAACCAACAAc cacagg**CTGATGAGGCCGAAAGGCCGAAA**ccatgcA CAATTGTTTTTTTGAATTCATC
9	CCGGTTCGAACCCGGGCACTACAAAAACCAACAAg ctgacat**CTGATGAGGCCGAAAGGCCGAAA**gttttgag tcACAATTGTTTTTTTGAATTCATC
10	CCGGTTCGAACCCGGGCACTACAAAAACCAACAAa atggtct**CTGATGAGGCCGAAAGGCCGAAA**tttccatA CAATTGTTTTTTTGAATTCATC
11	CCGGTTCGAACCCGGGCACTACAAAAACCAACAAc catgcg**CTGATGAGGCCGAAAGGCCGAAA**cagcttcc ACAATTGTTTTTTTGAATTCATC
12	CCGGTTCGAACCCGGGCACTACAAAAACCAACAAg agac**CTGATGAGGCCGAAAGGCCGAAA**cagcttcaA CAATTGTTTTTTTGAATTCATC
13	CCGGTTCGAACCCGGGCACTACAAAAACCAACAAtt catg**CTGATGAGGCCGAAAGGCCGAAA**tggcatACA ATTGTTTTTTTGAATTCATC
14	CCGGTTCGAACCCGGGCACTACAAAAACCAACAAa cctgtt**CTGATGAGGCCGAAAGGCCGAAA**ttcaacag ACAATTGTTTTTTTGAATTCATC

Construction of retroviral transducing vectors expressing anti-DENV hRzs and establishment of transduced C6/36 cells

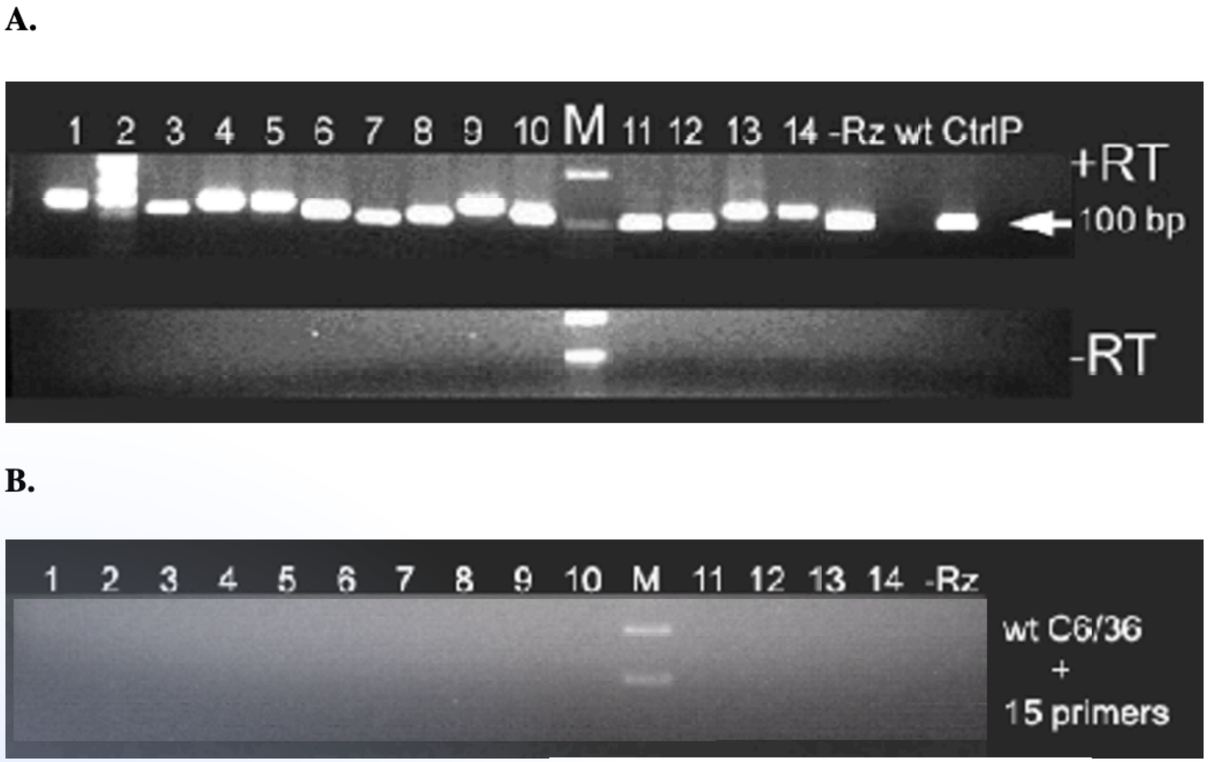


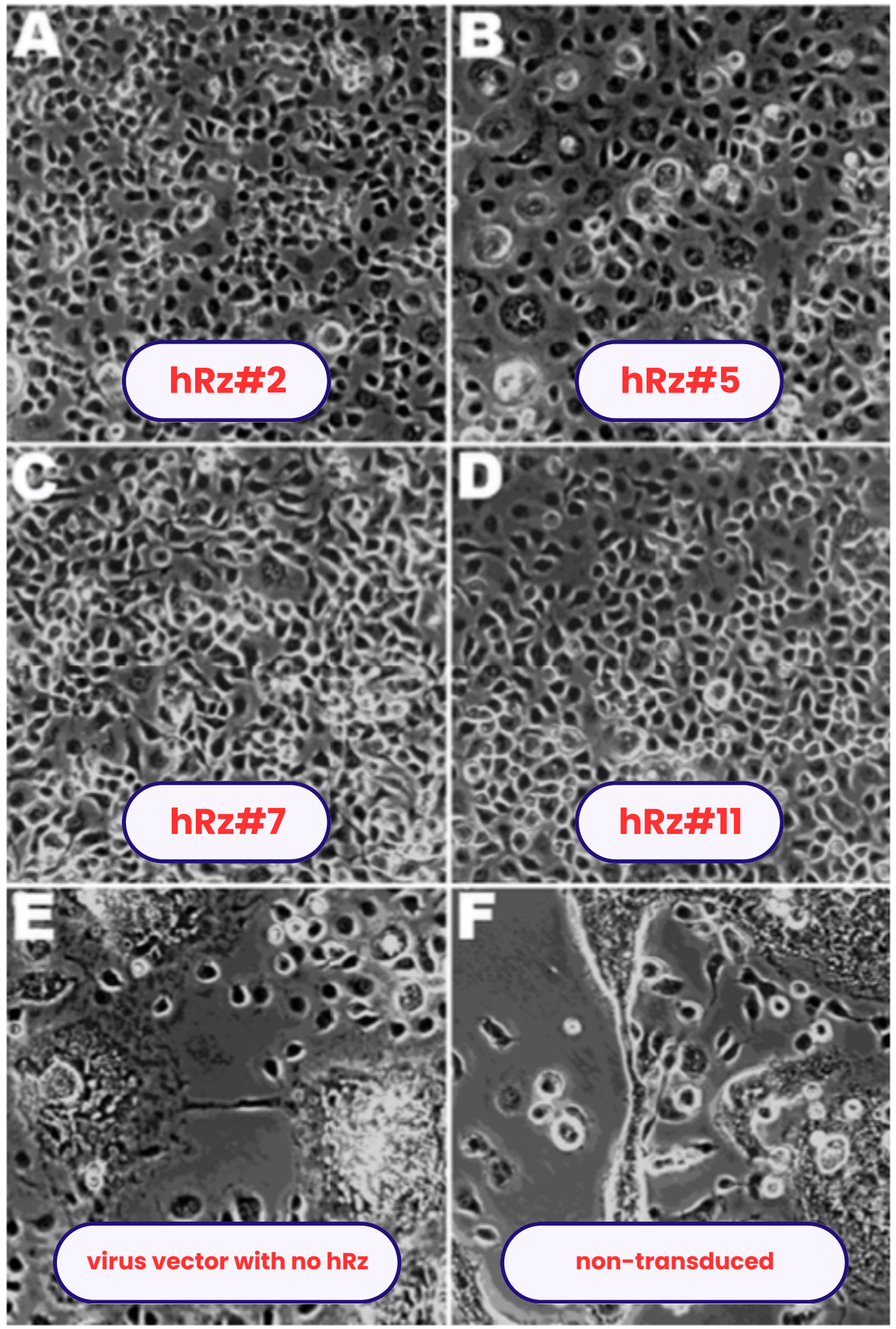
Figure 2
RT-PCR of total RNA extracted from hRz-C6/36 cells. (A) hRz expression in the cells was detected by the presence of an hRz-specific band at about 100 bp. Primers for each hRz-C6/36 cells were specific to the hRz insert except for the control lacking a hRz sequence (-Rz) for which the primers were specific to $tRNA^{val}$ and poly(A) tail. (B) Wild-type C6/36 cells failed to give a PCR product when tested with 15 sets of primers (each primer was specific to each hRz). *1–14*: 14 different hRz-C6/36 cells; *-Rz*: C6/36 cells without the hRz insert; *wt*: wild-type C6/36 cells; *CtrlP*: plasmid DNA control for PCR amplification; *M*: 1 Kb Plus DNA ladder; *+RT*: reactions with reverse transcriptase; *-RT*: reactions without reverse transcriptase.

- Fourteen ribozyme-encoding retroviruses and one control lacking a ribozyme sequence were used to transduce wildtype C6/36 cells by infecting at an MOI of 30 as described in Materials and Methods.
- Retrovirus-infected C6/36 cells were placed under hygromycin selection for 4–8 weeks and then analyzed for hRz expression by RT-PCR of total cellular RNA
- hRz expression in the cells was detected by the presence of an hRz-specific band at about 100 bp.

DENV infection in the hRz-transduced C6/36 cells

- **Wild-typeand hRz-transduced C6/36 cells** were seeded at **a density of 0.5 × (10)^6 cells/mL** in 6-well plates and incubated for 2 hr at 28°C to allow attachment.

- Once attached, cells were washed twice with medium and challenged with **DENV-2 NGC stock at an MOI of 0.01. Infection was allowed to continue for 7 days** at 28°C before **viral RNA was extracted both from cells and cultured supernatants** using TRI reagent (Invitrogen), and purified QIAamp Viral RNA Mini Kit (Qiagen), respectively.



CPE due to DENV infection of C6/36 cells at 5 dpi

- The CPE of DENV-2 NGC infection in C6/36 cells, characterized by syncytium formation and decreased cell proliferation (E,F), was clearly visible 5 days post infection (dpi).
- Those cells expressing certain hRz exhibited a clear reduction in CPE at 5 dpi, allowing them to grow to confluency, while cells that lack hRz, (i.e. No-hRz and wild-type), exhibited the expected CPE.
- The most effective hRz constructs were those that appeared to completely suppress CPE. These were hRz-C6/36 cell lines # 2, 5, 7 and 11.

Figure 3 CPE due to DENV infection of C6/36 cells at 5 dpi. Images were taken at the 40× magnification. Cells were those transduced with hRz-encoding retroviruses and selected in hygromycin for stable integration of the transgene. Representative infected cell cultures are shown. These are cells transduced with (A) hRz # 2, (B) hRz # 5, (C) hRz # 7, (D) hRz # 11, (E) No Rz (transduced with lentivirus vector lacking a hRz) or (F) non-transduced C6/36 cells.

Northern analyses for DENV genome

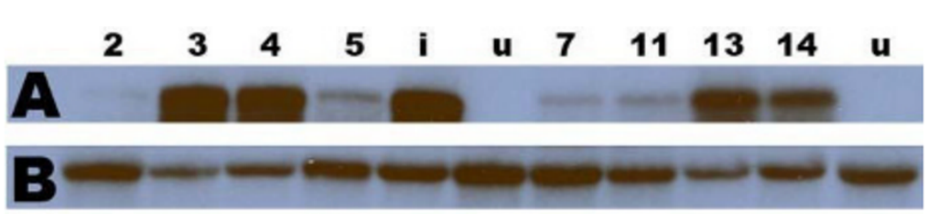


Figure 4 Northern hybridization analysis of DENV-2 replication in cells expressing hRz constructs.
(A) Total RNA samples hybridized with DENV-specific probes.
(B) Actin RNA from the same samples hybridized with a β-actin-specific probe. Each construct is indicated by numbers; i: wildtype C6/36 infected with DENV
u: uninfected wild-type C6/ 36.
The autoradiograph was exposed for 6 hr prior to development.

- Those transduced cultures that gave at least moderate CPE suppression were analyzed by Northern blot with DENVspecific probes **(probe sequence)** to determine the impact of hRz expression on DENV RNA replication.
- Infected and uninfected wildtype C6/36 cells were included as positive and negative controls, respectively, with β-actin serving as an internal hybridization and loading control.
- Autoradiographs **(Fig. 4A and 4B)** were scanned and analyzed by densitometry to estimate the relative amounts of DENV RNA in each sample

Northern analyses for DENV genome

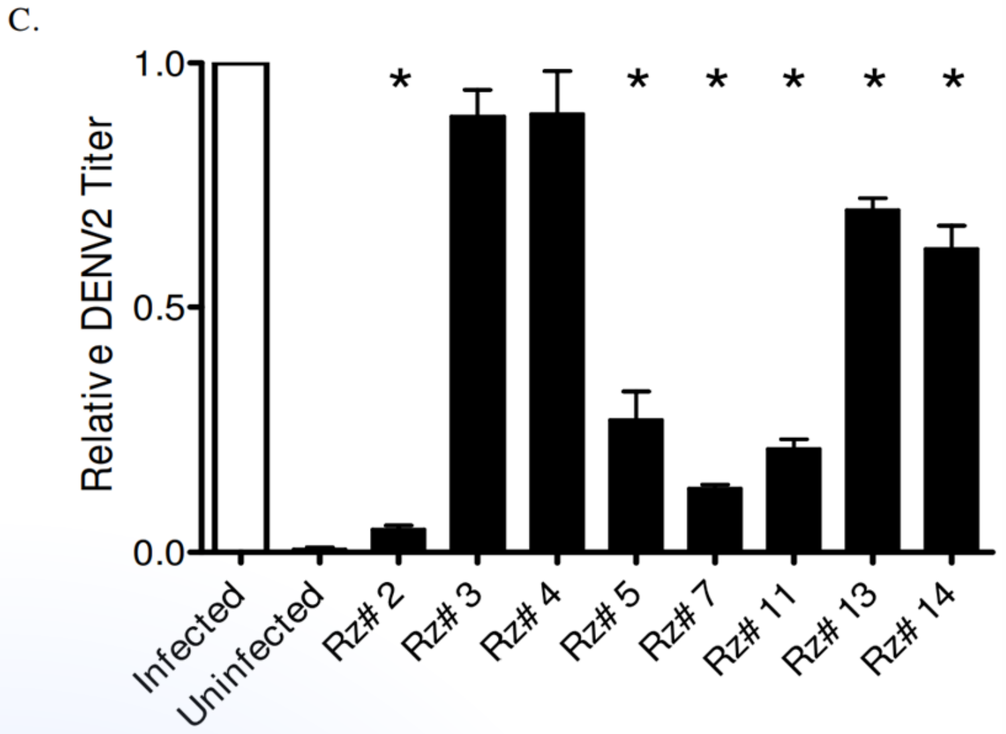


Figure 4 Northern hybridization analysis of DENV-2 replication in cells expressing hRz constructs. (C) Quantification of relative DENV-2 RNA levels from the Northern blot analysis. The scanned autoradiograph was processed in ImageJ software and the relative amount of DENV-2-specific RNA in each sample was compared against that of infected wild-type cells using an ANOVA test (GraphPad Prism 3.0). Statistically significant differences relative to the Infection control (Dunnett's posttest, $p < 0.01$) are indicated with asterisks. Infected: infected, non-transduced C6/36 cells; Uninfected: uninfected, nontransduced C6/36 cells; Rz #: Different infected hRz cells.

- The rapid degradation of ribozyme cleavage products coupled with the very effective suppression of DENV replication in the transduced cells, made the detection hRz cleavage product of RNAs difficult by Northern blots.
- The efficacy of the hRzs was estimated by comparing the relative amount of the target DENV RNA to the infected and uninfected C6/36 control cultures **(Fig. 4C).**
- These analyses confirm that hRz-C6/36 cell lines # 2, 5, 7 and 11 suppressed the replication of DENV by at least 25% relative to the infected wild-type cells

In vitro analyses of hRz cleavage activity

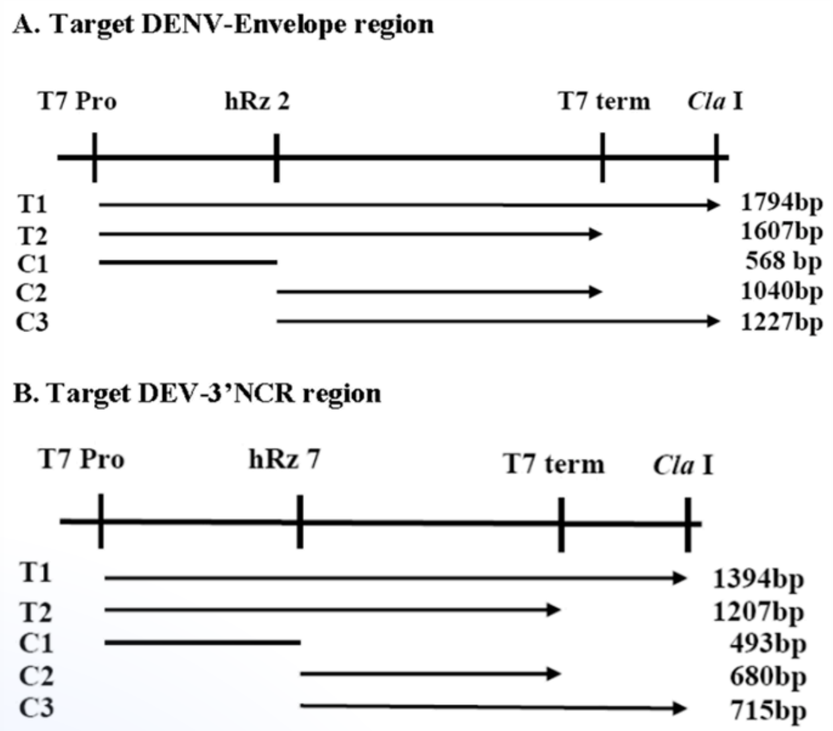


Figure 5 Confirmation of cleavage activities for representative hRzs. (A and B) Maps showing the in vitro transcripts generated from the linearized pET11a vectors for the hRz # 2 (A) and hRz # 7 (B) target substrates. Positions of the T7 promoter (T7- Pro), hRz-T7 cleavage site (hRz7), T7 terminator (T7 Term), and Cla I site used for linearization (Cla I) are indicated. T1 and T2 show the extent of two transcripts that are generated in the in vitro transcription reaction for each substrate. C1 shows the extent of the single 5' cleavage product from both transcripts from each substrate, while C2 and C3 show the extent of the two 3' cleavage products generated from the two different transcripts produced from each substrate.

- Four most effective ribozymes for their cleavage activity in vitro.
- DNA molecules encoding each hRz construct, including the tRNAval and polyA tail, were synthesized downstream of a T7 promoter sequence, cloned, and expressed in vitro as described in Materials and Methods.
- These in vitro transcribed ribozymes were combined with in vitro transcribed target RNA molecules containing extensive regions of the DENV-2 NGC genome that encompass hRz # 2 and 5, or hRz # 7 and 11 cleavage sites.
- The results for two of these ribozymes, hRz # 2 and # 7, are presented in **Fig. 5.**

In vitro analyses of hRz cleavage activity

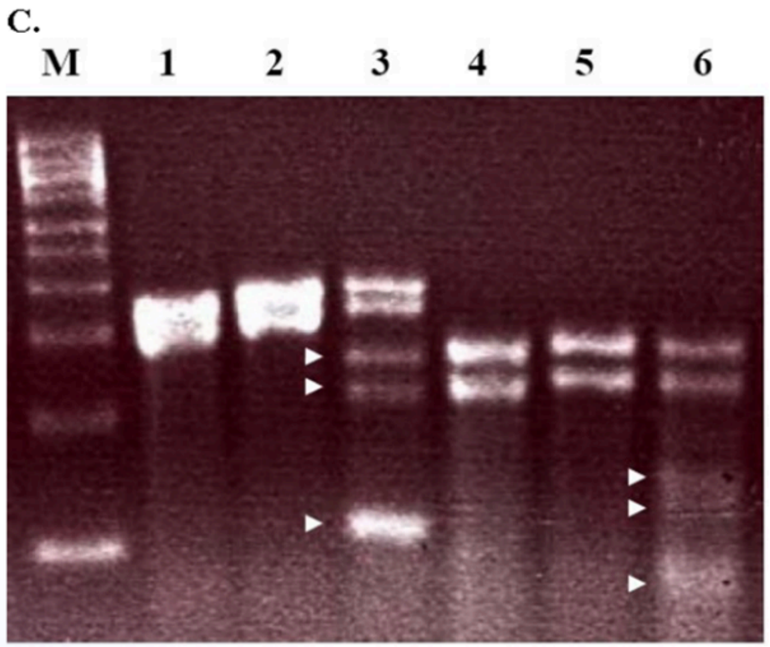


Figure 5 Confirmation of cleavage activities for representative hRzs Confirmation of cleavage activities for representative hRzs. (C) Agarose gel of in vitro cleavage reaction products. In vitro transcribed targets and their respective hRz # 2 and hRz # 7 were incubated for 30 min at 37°C. Cleavage products were separated in 2% agarose gels stained with ethidium bromide. Lane M: Millenium™ RNA Marker. Lanes 1–3: In vitro transcribed DENV-ENV region target without MgCl2 (lane 1), hRz # 2 and without MgCl2 (lane 2), and hRz # 2 with MgCl2 (lane 3). Lanes 4–6: In vitro transcribed 3'NCR region target without MgCl2 (lane 4), hRz # 7 without MgCl2 (lane 5), and hRz # 7 with MgCl2 (lane 6). Arrows in lanes 3 and 6 show the expected hRz # 2 and hRz#7 cleavage products, respectively

- The cleavage products and hRzs are apparent as distinct bands in the lanes corresponding to each reaction.
- A third band of unknown identity was detected in each experimental lane as well.
- We believe this extra fragment is the result of alternative cleavage of the target RNA since the size of the hRz transcripts (50 nt) are too small to appear on these gels, and because these fragment do not appear in the control lanes lacking hRz

TCID50 immunofluorescence assays

Table 1: Tabulation of data for $TCID_{50}$ and qRT-PCR analyses of hRz effectiveness

	TCID50		qRT-PCR Cells		qRT-PCR Supernatant		
	Avg	SE	Avg	SE	Avg	SE	Avg % Red
Infected	4.39×10^6	7.12×10^5	1.77×10^6	4.17×10^5	2.92×10^6	1.38×10^6	0
Uninfected	2	1	3	2	3	1	N/A
No-hRz	1.88×10^6	5.94×10^5	1.37×10^6	2.53×10^5	1.60×10^6	6.46×10^5	41.71
Rz # 1	8.77×10^5	3.4210^6	1.62×10^6	3.13×10^5	9.46×10^5	2.70×10^5	52.03
Rz # 2	2.78×10^4	1.09×10^4	5.88×10^4	3.95×10^4	5.37×10^4	1.65×10^4	98.07
Rz # 3	1.16×10^6	6.8910^5	1.68×10^6	$3.38, \times 10^5$	1.39×10^6	7.62×10^5	43.62
Rz # 4	2.37×10^6	1.14×10^6	1.99×10^6	2.61×10^5	1.51×10^6	7.58×10^5	27.39
Rz # 5	3.08×10^4	9.1×10^3	9.65×10^4	4.90×10^4	9.44×10^4	2.18×10^4	96.87
Rz # 6	2.33×10^5	1.16×10^5	1.65×10^6	1.97×10^5	6.45×10^5	1.76×10^5	59.74
Rz # 7	2.72×10^4	1.13×10^4	9.56×10^4	2.04×10^4	4.98×10^4	2.49×10^4	97.42
Rz # 8	1.87×10^5	1.26×10^5	6.46×10^5	2.51×10^5	4.95×10^5	2.43×10^5	80.76
Rz # 9	8.50×10^4	3.46×10^4	2.51×10^5	1.24×10^5	3.24×10^5	1.45×10^5	90.92
Rz # 10	4.07×10^5	2.34×10^5	5.11×10^5	1.39×10^5	1.36×10^5	4.71×10^4	85.74
Rz # 11	2.47×10^4	4.01×10^3	3.55×10^4	1.59×10^4	2.11×10^4	7.41×10^3	98.90
Rz # 12	5.49×10^5	1.61×10^5	5.51×10^5	3.17×10^5	3.43×10^5	2.06×10^5	81.54
Rz # 13	3.06×10^5	1.08×10^5	3.60×10^5	2.11×10^5	1.90×10^5	1.65×10^5	88.73
Rz # 14	1.98×10^5	1.22×10^5	2.63×10^5	1.07×10^5	2.63×10^5	2.20×10^5	90.53

Averages of results from four separate infections (Avg) are presented for each type of analysis along with Standard Error of the Mean (SE). The average percent reduction for all tests (Avg % Red) relative to the Infected cell control is calculated in the final column.

- Each ribozyme was determined the effectiveness in suppressing overall infectious virus production using an immunofluorescence-based TCID50 assay.
- Cell culture medium collected at 4 dpi from infected cells was assayed by fixing with acetone:DPBS (3:1).
- Positive DENV-infected cells was detected by using a DENV envelope protein-specific monoclonal antibody.
- Cells that showed fluorescence within the cytoplasm were scored as positive for DENV infection. The TCID50 was calculated based on Karber's method. The titer was expressed as log10TCID50/0.1 ml

TCID50 immunofluorescence assays

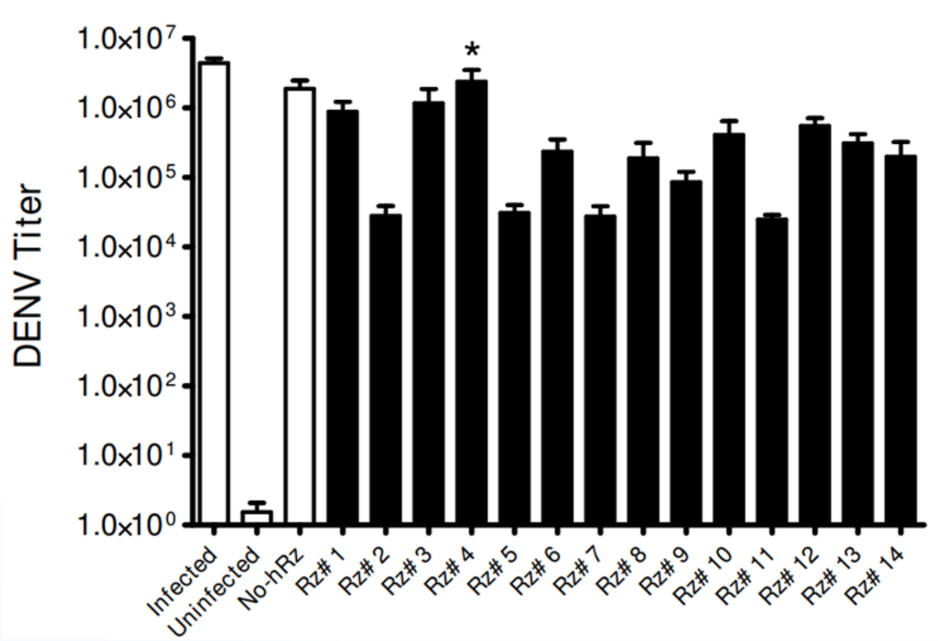


Figure 6 immunofluorescence assay TCID50 immunofluorescence assay. At 4 day dpi, cells were fixed and stained. Primary antibody against ENV protein and biotinylated-secondary antibody and streptavidin were employed as a fluorescence detection system. A oneway ANOVA test was performed using GraphPad Prism 3.0 software. Asterisk indicate no significant differences relative to the Infected control (Dunnett's, $p < 0.01$). Rz # 1–14: 14 different infected hRz cells; No Rz: infected C6/36 cells transduced with the lentivirus vector lacking a hRz insert.

- While all ribozyme transformed, and even the No-hRz transformed control cells, exhibited statistically significant reductions in overall infectious virus production, hRz-C6/36 cell lines # 2, 5, 7 and 11 had remarkably reduced DENV-2 titers, as much as 2 orders of magnitude, compared to infected wild-type cells **(Table 1 and Fig. 6)**.

- The fact that the No hRz control did have reduced yields of virus can be attributed to the hygromycin selection protocol, which did impact the virus infectivity in transformed cells to some extent.

Real-time PCR quantitation of DENV titer in whole cell RNA

- The ability of each hRz to suppress DENV genome replication was quantitatively evaluated for the hRz expressing C6/36 cells using qRT-PCR to detect virus genomes in cell lysates. Total cellular RNA was extracted 7 dpi from infected hRz-transduced cells following the protocol described in Materials and Methods.

- First-strand cDNA was prepared using the Capsid2 primer (Table 2), followed by 40 rounds of PCR amplification using primers Capsid2 F and R.

- The absolute quantity of viral RNA was determined based upon comparison of infected cell Ct values against those of viral RNA standards.

Table 2: Designations and base sequences of twelve primer sets evaluated to select optimal primer pairs for detection of the DENV-2 NGC RNA genome

Designation	Primer sequences (5'→3')	
Capsid1	2F: caatatgctgaaacgcgaga	2R: ccatcactgttggaatcagc
Capsid2	3F: caatatgctgaaacgcgaga	3R: cgccatcactgttggaatc
Capsid3	4F: gcgagaaatacgcctttcaa	4R: ccatcactgttggaatcagc
Capsid4	5F: tatgctgaaacgcgagagaa	5R: cgccatcactgttggaatc
Capsid5	6F: gcgagaaatacgcctttcaa	6R: cgccatcactgttggaatc
Capsid6	7F: atgctgaaacgcgagagaaac	7R: ccctgctgttggtgggatt
NS51	2F: tcaaaagcattcagcacctg	2R: cacatttgggcgtaggactt
NS52	3F: gcaatgtatgccgatgacac	3R: caggtgctgaatgcttttga
NS53	4F: gcaatgtatgccgatgacac	4R: tcaggtgctgaatgcttttg
NS54	5F: tggaggagccttagtgagga	5R: acgtcccaaggttttgtcag
NS55	6F: tgagcaagaaagagggagga	6R: caggtgctgaatgcttttga
NS56	7F: caaaagcattcagcacctgaca	7R: gttaaagcgcttgcgaacct

F and R: forward and reverse primer, respectively.

Table 2: Designations and base sequences of twelve primer sets evaluated to select optimal primer pairs for detection of the DENV-2 NGC RNA genome

Designation	Primer sequences (5'→3')	
Capsid1	2F: caatatgctgaaacgcgaga	2R: ccatcactgttggaatcagc
Capsid2	3F: caatatgctgaaacgcgaga	3R: cgccatcactgttggaatc
Capsid3	4F: gcgagaaatacgcctttcaa	4R: ccatcactgttggaatcagc
Capsid4	5F: tatgctgaaacgcgagagaa	5R: cgccatcactgttggaatc
Capsid5	6F: gcgagaaatacgcctttcaa	6R: cgccatcactgttggaatc
Capsid6	7F: atgctgaaacgcgagagaaac	7R: ccctgctgttggtgggatt
NS51	2F: tcaaaagcattcagcacctg	2R: cacatttgggcgtaggactt
NS52	3F: gcaatgtatgccgatgacac	3R: caggtgctgaatgcttttga
NS53	4F: gcaatgtatgccgatgacac	4R: tcaggtgctgaatgcttttg
NS54	5F: tggaggagccttagtgagga	5R: acgtcccaaggttttgtcag
NS55	6F: tgagcaagaaagagggagga	6R: caggtgctgaatgcttttga
NS56	7F: caaaagcattcagcacctgaca	7R: gttaaagcgcttgcgaacct

F and R: forward and reverse primer, respectively.

Real-time PCR quantitation of DENV titer in whole cell RNA

Table 1: Tabulation of data for $TCID_{50}$ and qRT-PCR analyses of hRz effectiveness

	TCID50		qRT-PCR Cells		qRT-PCR Supernatant		
	Avg	SE	Avg	SE	Avg	SE	Avg % Red
Infected	4.39×10^6	7.12×10^5	1.77×10^6	4.17×10^5	2.92×10^6	1.38×10^6	0
Uninfected	2	1	3	2	3	1	N/A
No-hRz	1.88×10^6	5.94×10^5	1.37×10^6	2.53×10^5	1.60×10^6	6.46×10^5	41.71
Rz # 1	8.77×10^5	3.4210^6	1.62×10^6	3.13×10^5	9.46×10^5	2.70×10^5	52.03
Rz # 2	2.78×10^4	1.09×10^4	5.88×10^4	3.95×10^4	5.37×10^4	1.65×10^4	98.07
Rz # 3	1.16×10^6	6.8910^5	1.68×10^6	$3.38, \times 10^5$	1.39×10^6	7.62×10^5	43.62
Rz # 4	2.37×10^6	1.14×10^6	1.99×10^6	2.61×10^5	1.51×10^6	7.58×10^5	27.39
Rz # 5	3.08×10^4	9.1×10^3	9.65×10^4	4.90×10^4	9.44×10^4	2.18×10^4	96.87
Rz # 6	2.33×10^5	1.16×10^5	1.65×10^6	1.97×10^5	6.45×10^5	1.76×10^5	59.74
Rz # 7	2.72×10^4	1.13×10^4	9.56×10^4	2.04×10^4	4.98×10^4	2.49×10^4	97.42
Rz # 8	1.87×10^5	1.26×10^5	6.46×10^5	2.51×10^5	4.95×10^5	2.43×10^5	80.76
Rz # 9	8.50×10^4	3.46×10^4	2.51×10^5	1.24×10^5	3.24×10^5	1.45×10^5	90.92
Rz # 10	4.07×10^5	2.34×10^5	5.11×10^5	1.39×10^5	1.36×10^5	4.71×10^4	85.74
Rz # 11	2.47×10^4	4.01×10^3	3.55×10^4	1.59×10^4	2.11×10^4	7.41×10^3	98.90
Rz # 12	5.49×10^5	1.61×10^5	5.51×10^5	3.17×10^5	3.43×10^5	2.06×10^5	81.54
Rz # 13	3.06×10^5	1.08×10^5	3.60×10^5	2.11×10^5	1.90×10^5	1.65×10^5	88.73
Rz # 14	1.98×10^5	1.22×10^5	2.63×10^5	1.07×10^5	2.63×10^5	2.20×10^5	90.53

Averages of results from four separate infections (Avg) are presented for each type of analysis along with Standard Error of the Mean (SE). The average percent reduction for all tests (Avg % Red) relative to the Infected cell control is calculated in the final column.

- Four independent experiments were compared for each hRz-C6/36 transduced cell line to insure consistency and reproducibility.
- The results **(Table 1 and Fig. 7A)** for most of the hRz were consistent with the IFA determination of virus titer, with hRz-C6/36 cell lines # 2, 5, 7 and 11 exhibiting suppression of DENV replication by up to nearly 100 fold compared to the infected wild-type samples.

Real-time PCR quantitation of DENV titer in whole cell RNA

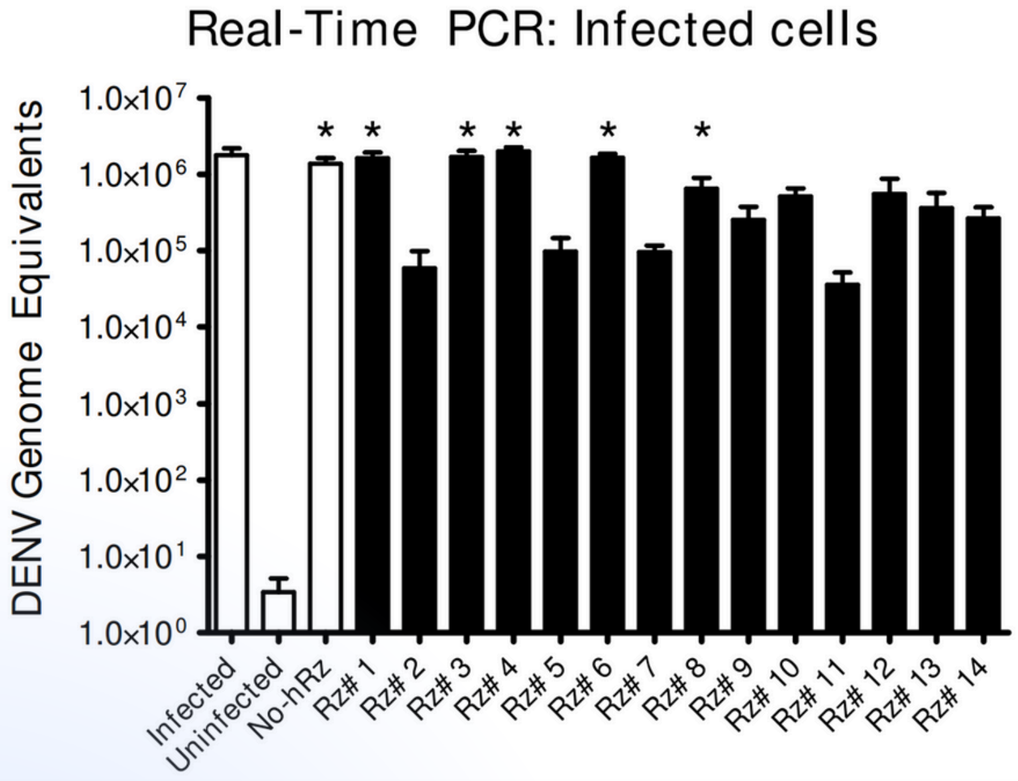


Figure 7 in cells infected with DENV and expressing hRzs A: Absolute quantitation of genome equivalents in cells infected with DENV and expressing hRzs.

- Viral RNA samples were obtained from total cell RNA extraction at 7 dpi. ANOVA test was performed using GraphPad Prism 3.0 software. Asterisk indicate no significant differences relative to the Infected control (Dunnett's, $p<0.01$) Plot is based on the average titer from 4 independent experiments. Rz # 1–14: 14 different infected hRz cells; No Rz: infected C6/36 cells transduced with the lentivirus vector lacking a hRz insert

Real-time PCR quantitation of DENV titer in cellular medium

Table 1: Tabulation of data for $TCID_{50}$ and qRT-PCR analyses of hRz effectiveness

	TCID50		qRT-PCR Cells		qRT-PCR Supernatant		
	Avg	SE	Avg	SE	Avg	SE	Avg % Red
Infected	4.39×10^6	7.12×10^5	1.77×10^6	4.17×10^5	2.92×10^6	1.38×10^6	0
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Averages of results from four separate infections (Avg) are presented for each type of analysis along with Standard Error of the Mean (SE). The average percent reduction for all tests (Avg % Red) relative to the Infected cell control is calculated in the final column.

- The results **(Table 1 and Fig. 7B)** demonstrated that the titers of extracellular virus genomic RNA obtained for hRz-C6/36 cell lines # 2, 5, 7 and 11 were consistent with genome copies detected in whole cell extracts.
- Similar levels of reduction were seen for most of the other hRz as well.
- Together, these qRT-PCR findings confirmed that most of the highly effective hRzs affected viral RNA replication and not DENV assembly and release from the cells.

Real-time PCR quantitation of DENV titer in cellular medium

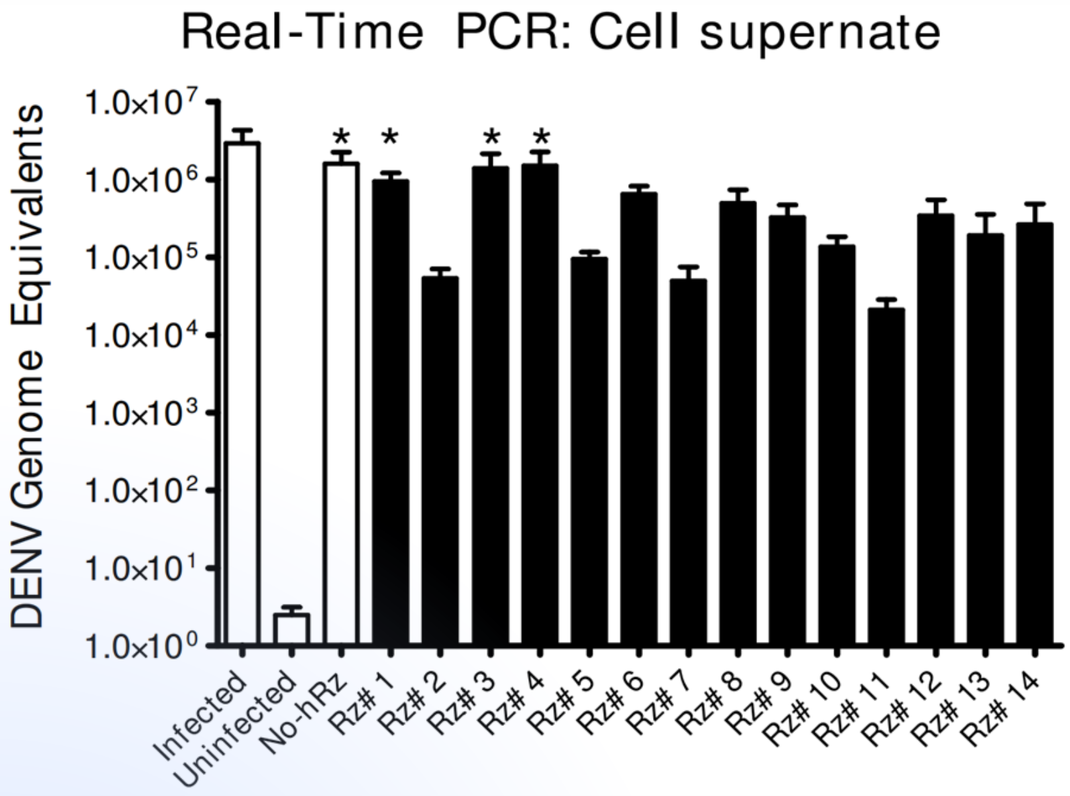

Figure 7 in cells infected with DENV and expressing hRzs
B: Absolute quantitation of viral titers from different hRz cells. Viral RNA samples were obtained from collected cell supernatant at 7 dpi. ANOVA test was performed using GraphPad Prism 3.0 software. Asterisk indicate no significant differences relative to the Infected control (Dunnett's, $p < 0.01$). Rz # 1–14: 14 different infected hRz cells; No Rz: infected C6/36 cells trasnduced with the lentivurs vector lacking a hRz insert. Plots are based on the average titer from 4 independent experiments.

- The levels of extracellular virion RNA for hRz # 6 were more closely related to the TCID50 results than to the qRTPCR results of total cellular RNA, reinforcing the possibility that this ribozyme's effect was related to interference with virion production rather than direct suppression of viral genomes.

Discussion

- In this study, it has confirmed the effectiveness of expressed hRzs as suppressive agents of DENV in transduced mosquito cells.

- These ribozymes can cleave their target RNA at an NUH triplet site, and may recycle themselves provided there are short sequence homologies between the ribozyme arms and the corresponding target sequence.

- Northern analyses, immunofluorescence assays, and quantitative real-time PCR demonstrated that C6/36 cells expressing certain hRzs were able to suppress DENV-2 NGC viral replication by at least 25%, with hRz-C6/36 cell lines # 2, 5, 7 and 11 having a more pronounced effect, of nearly 2 logs (100 fold) reduction in viral titer compared to the untransduced C6/36 cells.

- This research demonstrate that the hRz approach has significant potential as a strategy for suppressing DENV in transgenic mosquitoes, either alone or in combination with alternative genetic suppression strategies. While complete suppression of virus infectivity in infected mosquito tissues may not be possible, significant reduction of virus titers within mosquito tissues could result in a decreased efficiency of transmission for the virus, thereby reducing the prevalence of the disease among associated human populations.

กรมวิทยาศาสตร์การแพทย์
Department of Medical Sciences

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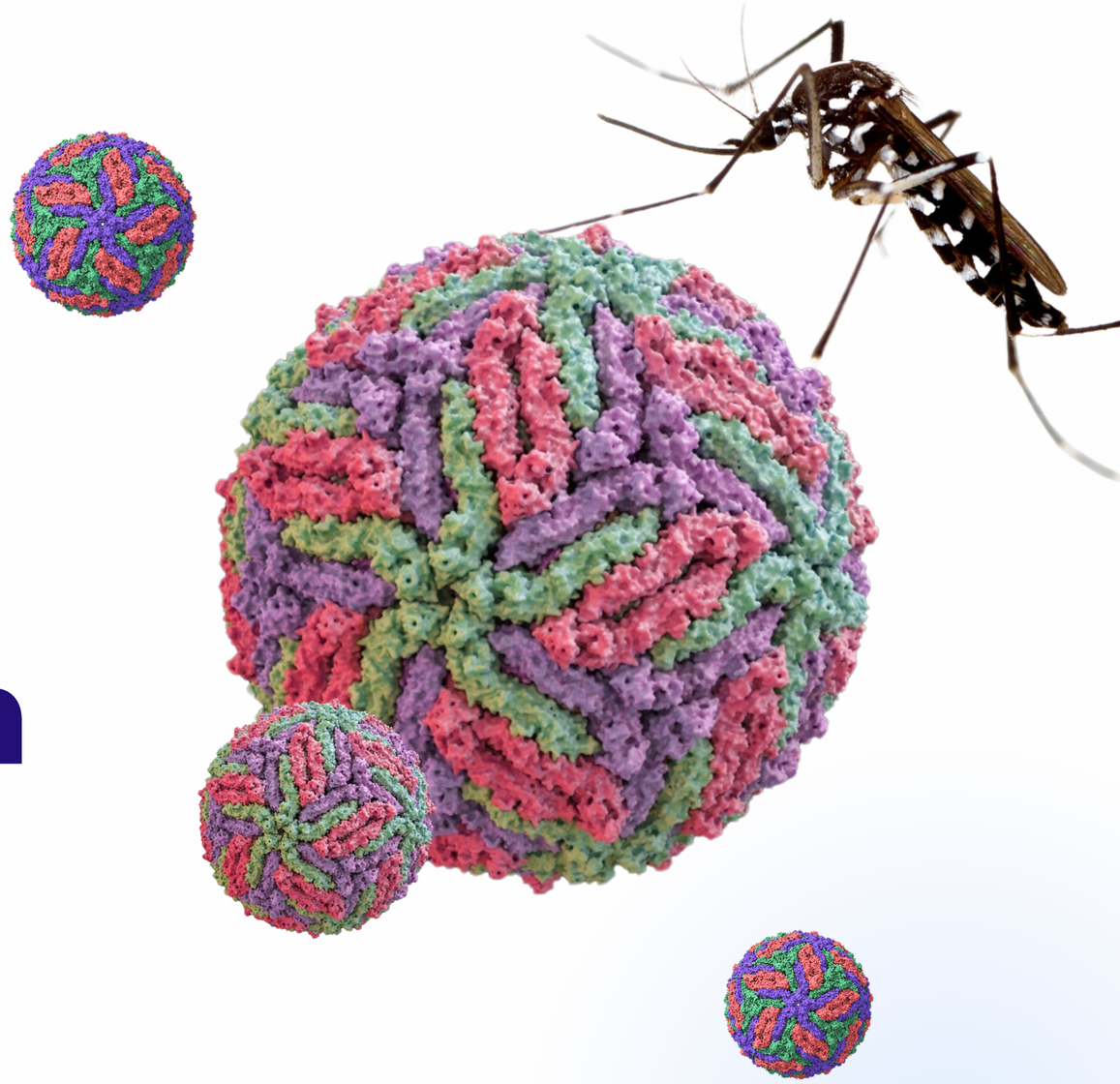
Thank you for your attention



TCID50

Infected 4390000

Rz#2 27800

4390000 – 27800 = 4362200

$$\frac{4362200 \times 100}{4390000} = 99.36$$

qRT-PCR Cells

Infected 1770000

Rz#2 58800

1770000 – 58800 = 4362200

$$\frac{4362200 \times 100}{1770000} = 96.68$$

qRT-PCR Supernatant

Infected 2920000

Rz#2 53700

2920000 – 53700 = 2866300

$$\frac{2866300 \times 100}{2920000} = 98.16$$

$$\frac{99.36 + 96.68 + 98.16}{3} = 98.07$$

Avg% Red = 98.07