Environment Responsive Peptide Dimers Bind and Stabilize dsRNA

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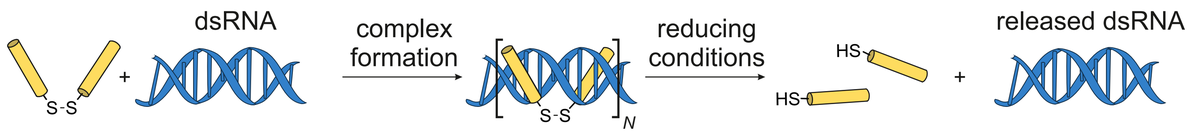
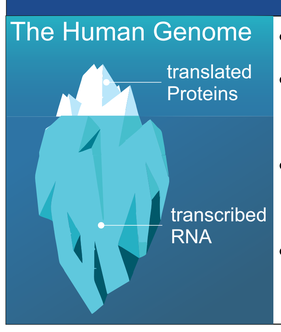
Abstract: Double-stranded RNAs (dsRNA) possess immense potential for biomedical applications.[1] However, their therapeutic utility is limited by **low stability** and **poor cellular uptake**.[2] Different strategies have been explored to enhance the stability of dsRNA, including the incorporation of modified nucleotides, and the use of diverse carrier systems. Nevertheless, these have not resulted in a broadly applicable approach thereby preventing the wide-spread application of dsRNA for therapeutic purposes. Herein, we report the design of **dimeric stapled peptides** based on the RNA-binding protein TAV2b. These dimers are obtained via **disulfide** formation and mimic the natural TAV2b assembly. They **bind and stabilize dsRNA** in the presence of serum, protecting it from degradation. In addition, peptide binding also **promotes cellular uptake of dsRNA**. Importantly, peptide dimers **monomerize under reducing conditions** which results in a loss of RNA binding. These findings highlight the potential of **peptide-based RNA binders** for the stabilization and protection of dsRNA, representing an appealing strategy towards the **environment-triggered release of RNA**. This can broaden the applicability of dsRNA, such as short interfering RNAs (siRNA), for therapeutic applications.



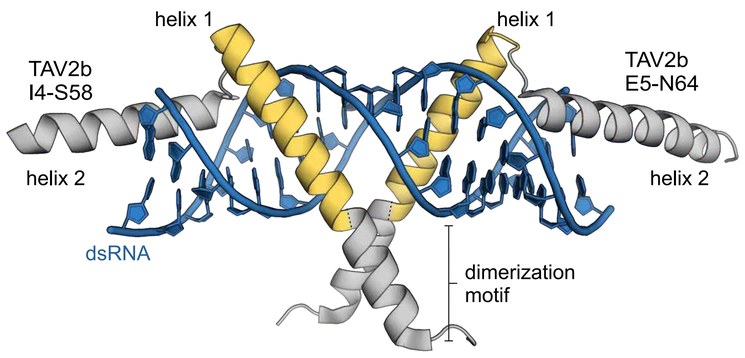
1. The Potential of RNA Therapeutics



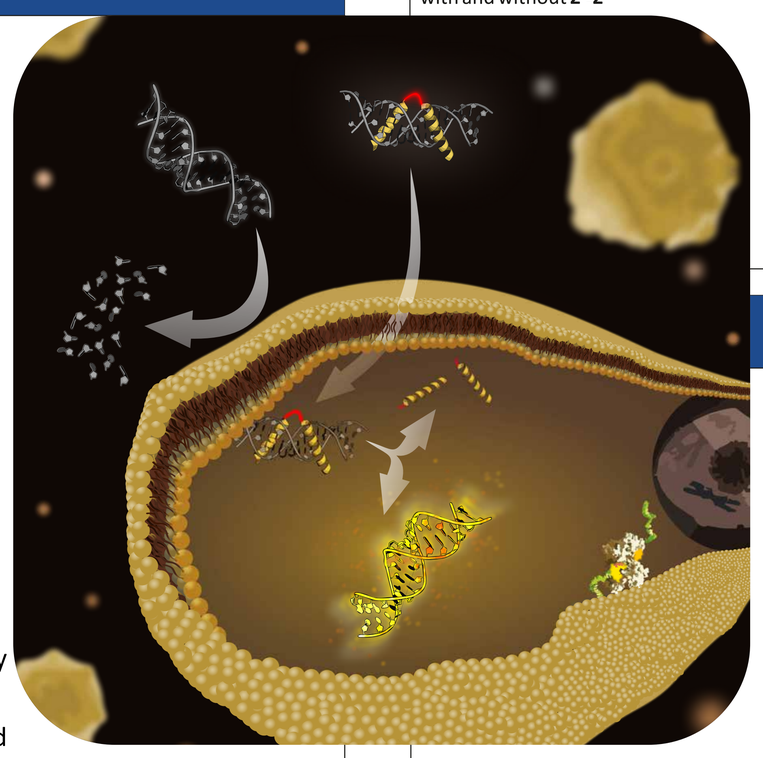
- Ribonucleic Acids are highly under-represented drug targets
- RNA interference (RNAi), activated by short RNAs, is an post-transcriptional mechanism that can degrade or block particular RNA sequences
- RNAi offers a specific and efficient therapeutic approach to silence any target gene[3]
- Despite considerable efforts the widespread application of siRNA-based therapeutics is hampered by lack of effective delivery systems

2. Design of TAV2b derived Dimers

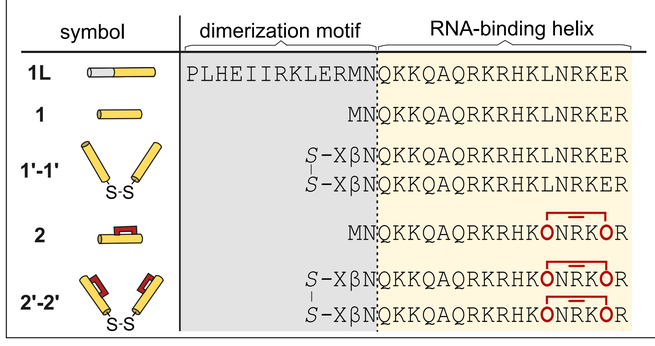
TAV2b Structure



- TAV2b is a viral gene silencing supressor that binds dsRNA[4]
- We designed TAV2b inspired disulfide linked dimers
- High affinity binding in the oxidised dimeric vs. low affinity in the reduced monomeric form



Designed Peptides



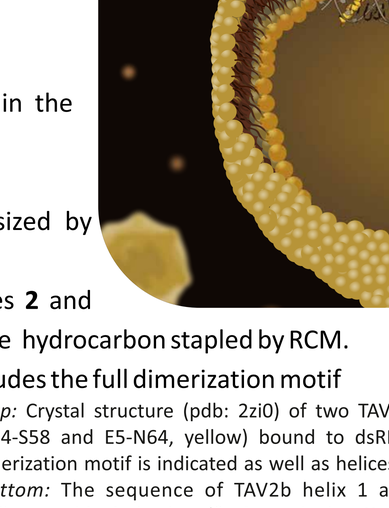
- Synthesized by SPPS
- Peptides **2** and **2'-2'** are hydrocarbon stapled by RCM.
- **1L** includes the full dimerization motif

Figures: *top:* Crystal structure (pdb: 2zi0) of two TAV2b subunits (I4-S58 and E5-N64, yellow) bound to dsRNA (grey). Dimerization motif is indicated as well as helices 1 and 2. *bottom:* The sequence of TAV2b helix 1 and corresponding peptide derivatives (β = beta-alanine, X = 3-mercaptopropionic acid, O = (S)-2-(4-pentenyl)alanine).

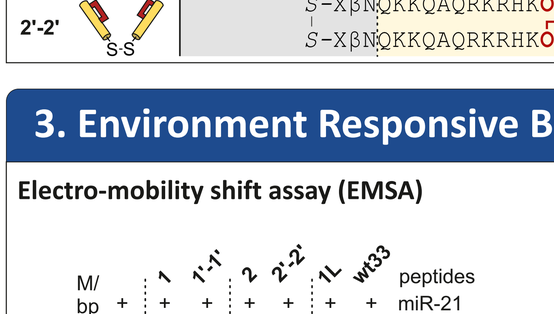
3. Environment Responsive Binding

Electro-mobility shift assay (EMSA)

Isothermal titration calorimetry



Reversible binding



- **2'-2'** binds dsRNA (Kd = 31.7 nM)
- RNA binding is sequence independent (data not shown)
- Reducing conditions result in loss of binding
- Disulfide cleavage also at physiological glutathione concentrations (5mM) within 5 min(data not shown)

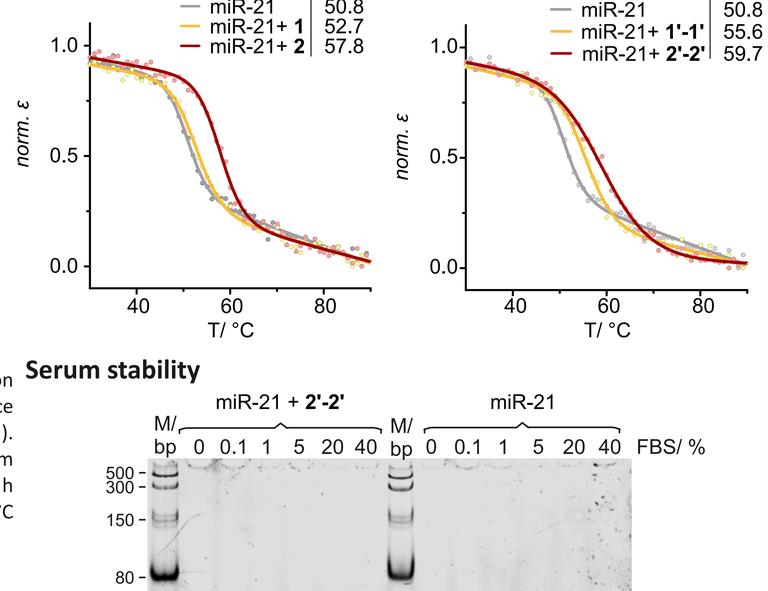
Figures: *top left:* EMSA of miR-21 (c = 3 µM) incubated with **1**, **1'-1'**, **2**, **2'-2'**, **1L** and **wt33** (c = 6 µM), native PAGE, TAE-buffer, SYBR gold stain. **top** *right:* ITC of **2'-2'** (c = 305 µM) with miR-21 (c = 15 µM) at 30 °C in 1xPBS. *bottom:* EMSA of miR-21 co-incubated with peptide dimers **1'-1'** or **2'-2'** and increasing concentrations of reducing agent TCEP (c = 6, 60 and 600 µM)

4. RNA Stabilization

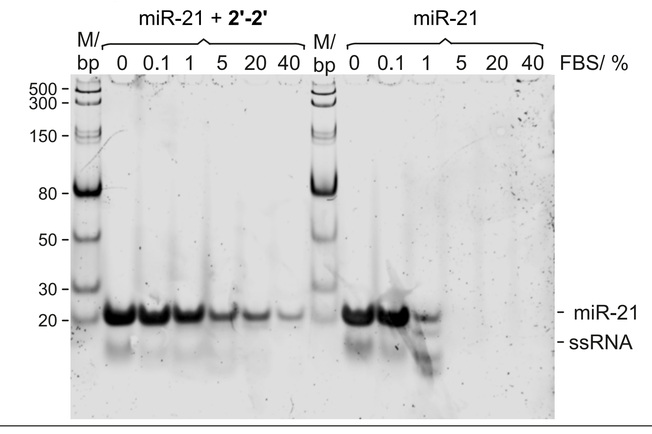
- Increased miR-21 melting temperatures upon binding
- T_m increases in complex with **1'-1'** and **2'-2'**
- ΔT_m with **2'-2'** ~ 9 °C
- **2'-2'** extends miR-21 life-time in fetal-bovine serum (FBS) preventing its degradation by nucleases
- Up to 6h in 27.5 % FBS at rt

Figures: *top:* CD-derived thermal denaturation profiles (λ = 267 nm) of miR-21 in the presence and absence of **1, 1'-1'** (yellow) and **2, 2'-2'** (red). *bottom:* native PAGE of phenol/chloroform extracted miR-21 after incubation with increasing amounts of serum for 10 min at 37 °C with and without **2'-2'**

Thermal stability

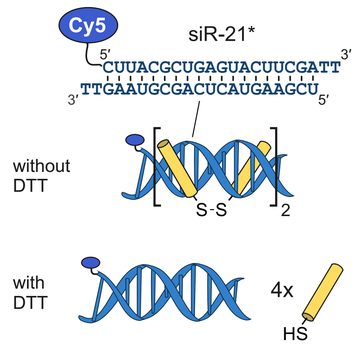


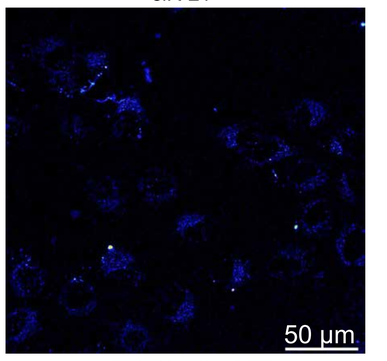
Serum stability

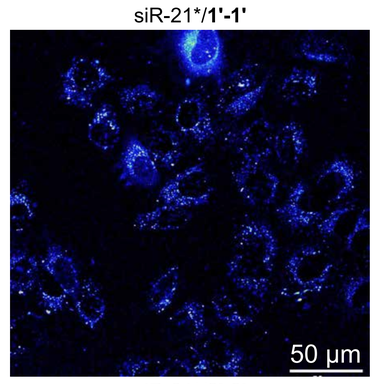


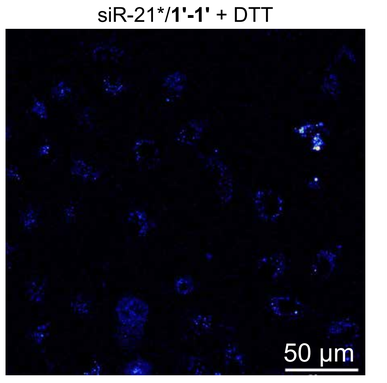
5. Promotion of Cellular Uptake

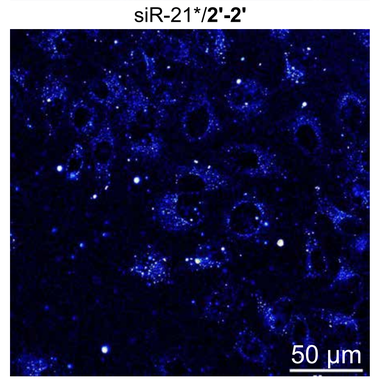
Confocal Microscopy

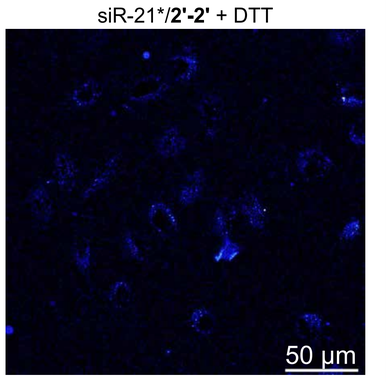












- HEK293 cells were incubated with Cy5-siRNA* with and without peptides **1'-1'** and **2'-2'**
- siR-21* alone showed low intracellular signal
- Increased cellular uptake with **1'-1'** and **2'-2'** (presumably endosomes).
- Again, low uptake with prior DTT treatment

Figures: *top left:* Cy5-labeled siRNA (siR-21*) and schematic representation of pre-treatment of **1'-1'** and **2'-2'** (c = 2 µM) with and without DTT before incubation with HEK293 cells. *Panels:* Confocal micrographs of HEK293 cells after incubation with siR-21* (c = 1 µM) for 1h with and without peptides **1'-1'** and **2'-2'**.

6. Conclusion

In this study, we designed and synthesized **novel dimeric peptides** derived from TAV2b, that **reversibly bind dsRNA**. Interestingly, dimer **2'-2'** increased the capability of RNA to **withstand degradation in serum** and **promoted the cellular uptake** of labeled siRNA. This study highlights how high affinity RNA ligands, can **stabilize dsRNA** and be equipped with **environment-responsive** binding properties, creating the basis for a significant advancement of dsRNA delivery strategies using peptides.

References

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