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Cyclic and pseudocyclic thrombin binding aptamer analogues as improved anticoagulant agents

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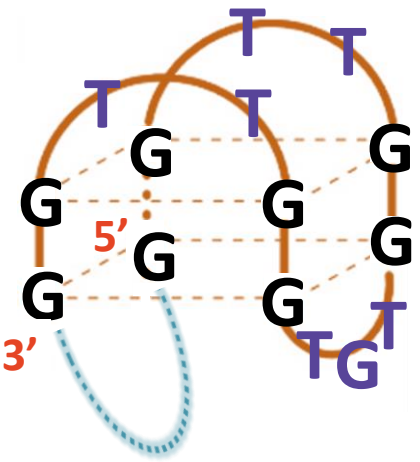
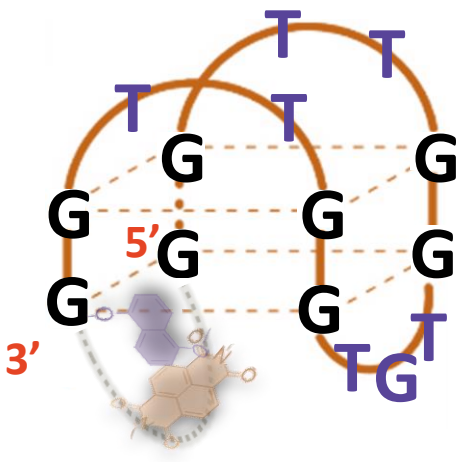
Cyclic and pseudocyclic thrombin binding aptamer analogues as improved anticoagulant agents



Cyclic TBA analogues realized via *covalent* connection of the oligonucleotide ends with flexible linkers



End-modified TBA analogues stabilized by *π/π* or *charge-transfer interactions* providing a pseudo-cyclic structure

Improved anticoagulant activity

Higher G4 thermal stability

Enhanced nuclease resistance

vs. unmodified TBA

Abstract:
The Thrombin Binding Aptamer or TBA (5'-GGTTGGTGTGGTTGG-3') is a 15-mer G-rich oligonucleotide able to **inhibit the thrombin-catalysed fibrinogen-fibrin conversion** after specific binding to its exosite I. TBA entered clinical trials but its evaluation was halted after phase I studies due to suboptimal dosing profiles.
Aiming at obtaining TBA analogues better performing in vivo, a large number of chemically modified TBA variants have been proposed.
In this frame, **we prepared a series of cyclic TBA analogues** by linking its 5' and 3'-ends with a variety of flexible linkers. The first derivative was realized introducing a 20-atom long linker. Compared to native TBA, it exhibited a G-quadruplex (**G4**) **structure with exceptionally improved stability and nuclease resistance**. However, these favourable properties were associated with reduced biological activity, suggesting that higher flexibility in the linker structure was necessary. Therefore, a mini-library of second generation cyclic TBAs (cycTBA I-IV) was prepared, carrying circularizing linkers overall spanning from 22 to 48 atoms. Among these derivatives, cycTBA II showed **improved anticoagulant activity**, associated with a dramatically stabilized G4 structure and enhanced enzymatic resistance in serum compared to the native TBA. Current studies are focused on **pseudocyclic TBA analogues**, where the cyclic structure is obtained not through covalent bonds but via π-π stacking or charge-transfer interactions of different aromatic probes inserted at the termini of the oligonucleotide. Among ten different TBA derivatives, we identified a promising candidate in this pseudocyclic series showing improved anticoagulant activity compared to native TBA, also having higher nuclease resistance and G4 thermal stability.

Keywords: anticoagulant activity; biophysical characterization; G-quadruplex; thrombin; thrombin binding aptamer.

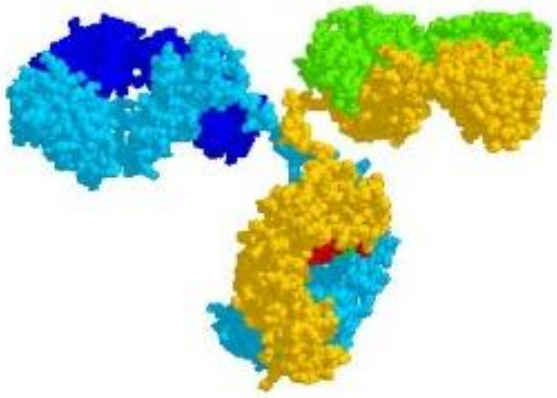
Introduction

Nucleic acid aptamers

- Short synthetic DNA or RNA sequences able to recognize with high affinity and specificity a wide range of molecular targets;
- Generally identified from combinatorial libraries by an in vitro selection procedure called SELEX (*Systematic Evolution of Ligands by EXponential enrichment*);
- Many aptamers are G-rich sequences and fold in G-quadruplex (G4) architectures.

Aptamers: promising alternatives to antibodies

VS



- ✓ Smaller size
- ✓ Lower immunogenicity
- ✓ Stability in a wide range of pH and temperature
- ✓ Easy modification to increase their stability and affinity

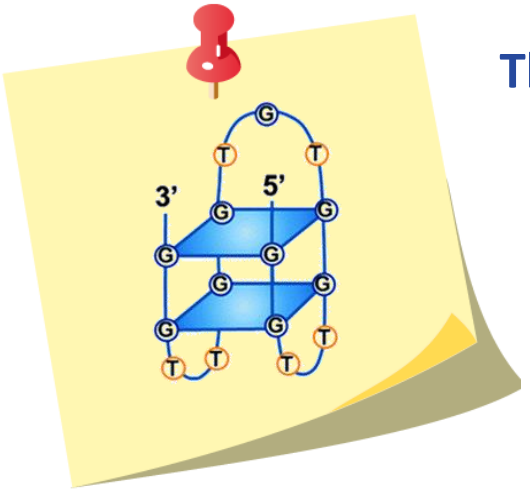
Aptamers are very attractive **therapeutic and diagnostics agents**

For recent reviews on aptamers, see for example:

Platella, C., **Riccardi, C.**, Montesarchio, D., Roviello, G.N., Musumeci, D. *Biochim. Biophys. Acta - Gen. Subj*,. **2017**, *1861*, 1429–1447;
Musumeci, D., Platella, C., **Riccardi, C.**, Moccia, F., Montesarchio, D. *Cancers*, **2017**, *9*, 174-217.

Introduction

The Thrombin Binding Aptamer



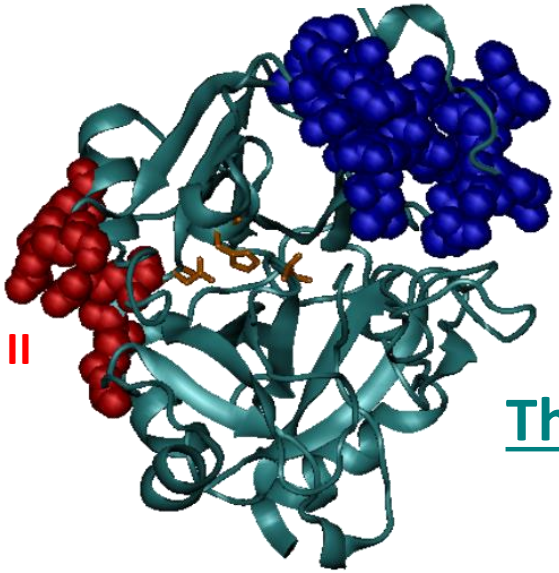
Thrombin binding aptamer or TBA_{15} d($5'$GGTTGGTGTGGTTGG$3'$)

- ✓ **Identified by SELEX against Thrombin in 1992**

  Bock L.C., *et al. Nature,* **1992**, *355*, 564–566.

- ✓ **Structure solved in 1993**

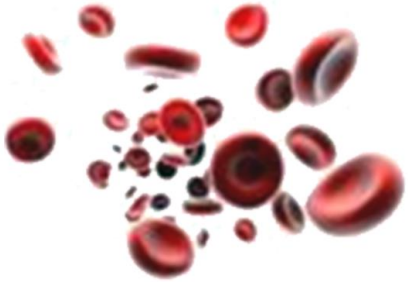
  Macaya R.F., *et al. PNAS,* **1993**, *90*, 3745-3749.



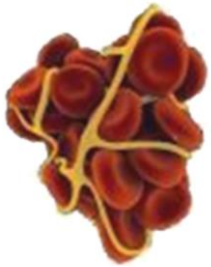
Exosite I

Exosite II

Thrombin



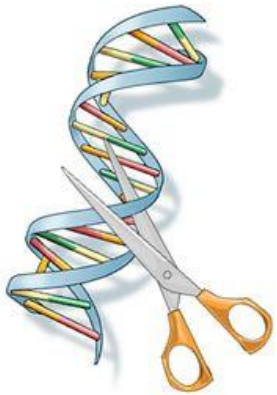
Fibrinogen



Fibrin

Improving the in vivo properties of TBA

The general approaches so far exploited to overcome the disadvantages of aptamers, in general, and of TBA, in particular, were based on:

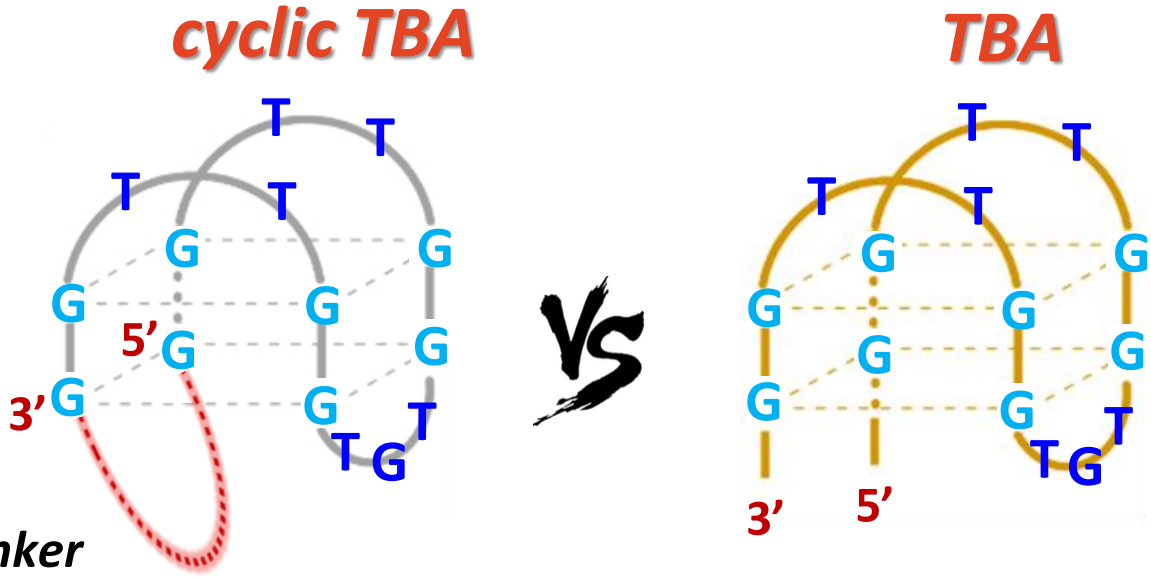


enzymatic degradation

- **modifications**
  - **on the nucleobase**
  - **on the sugar**
  - **on the phosphodiester linkages**
- **conjugations**
  - **with PEG**
  - **with lipids**
  - **with nanoparticles**

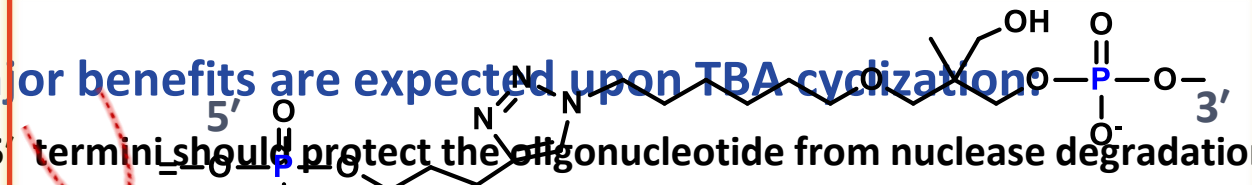
For recent reviews on chemically modified TBA analogues, see for example:
Riccardi C., *et al. Pharmacol Ther.*, **2021**, *217*, 107649;
Musumeci D., Montesarchio D. *Pharmacol Ther.*, **2012**, *136,* 202-215.
For a recent article on nanoparticle decoration, see for example:
Riccardi C., *et al. ACS Appl. Mater. Interfaces,* **2017**, *9*, 35574–35587.

A cyclization approach



cycTBA

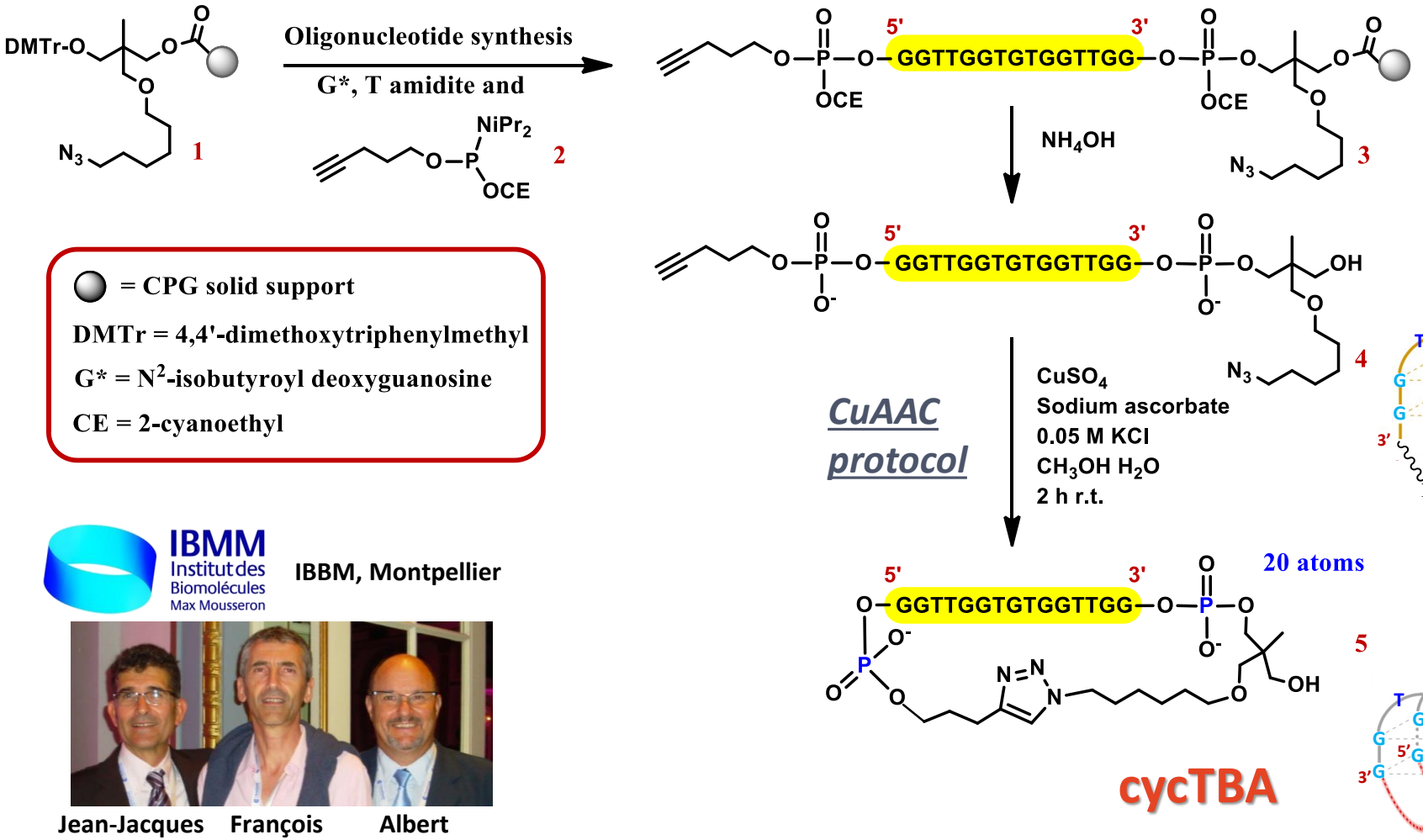
- ✓ Two major benefits are expected upon TBA cyclization:
- ✓ the absence of the 3’ and 5’ termini should protect the oligonucleotide from nuclease degradation
- ✓ the cyclization should impose a structural pre-organization favouring the G4 formation



Riccardi C., et al. *ChemBioChem.* **2019**, *20(14)*, 1789-1794. doi: 10.1002/cbic.201900045.

Results and discussion

Synthetic scheme



= CPG solid support

DMTr = 4,4'-dimethoxytriphenylmethyl

G* = N^2-isobutyroyl deoxyguanosine

CE = 2-cyanoethyl

IBMM Institut des Biomolécules Max Mousseron

IBBM, Montpellier

Jean-Jacques Vasseur | François Morvan | Albert Meyer

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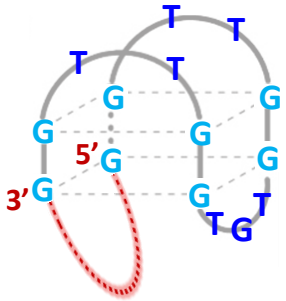
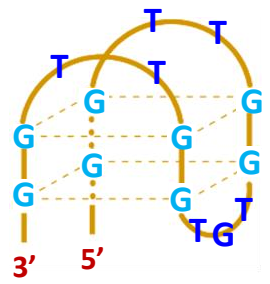
Biophysical characterizazion

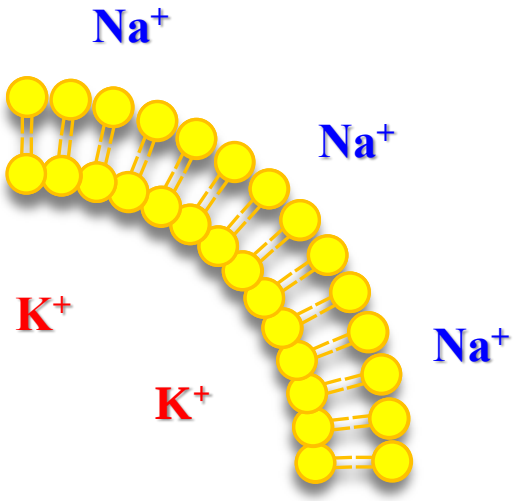


cycTBA



unmodified TBA

Anticoagulant activity *vs.* thrombin determined by Dynamic Light Scattering (DLS) analysis

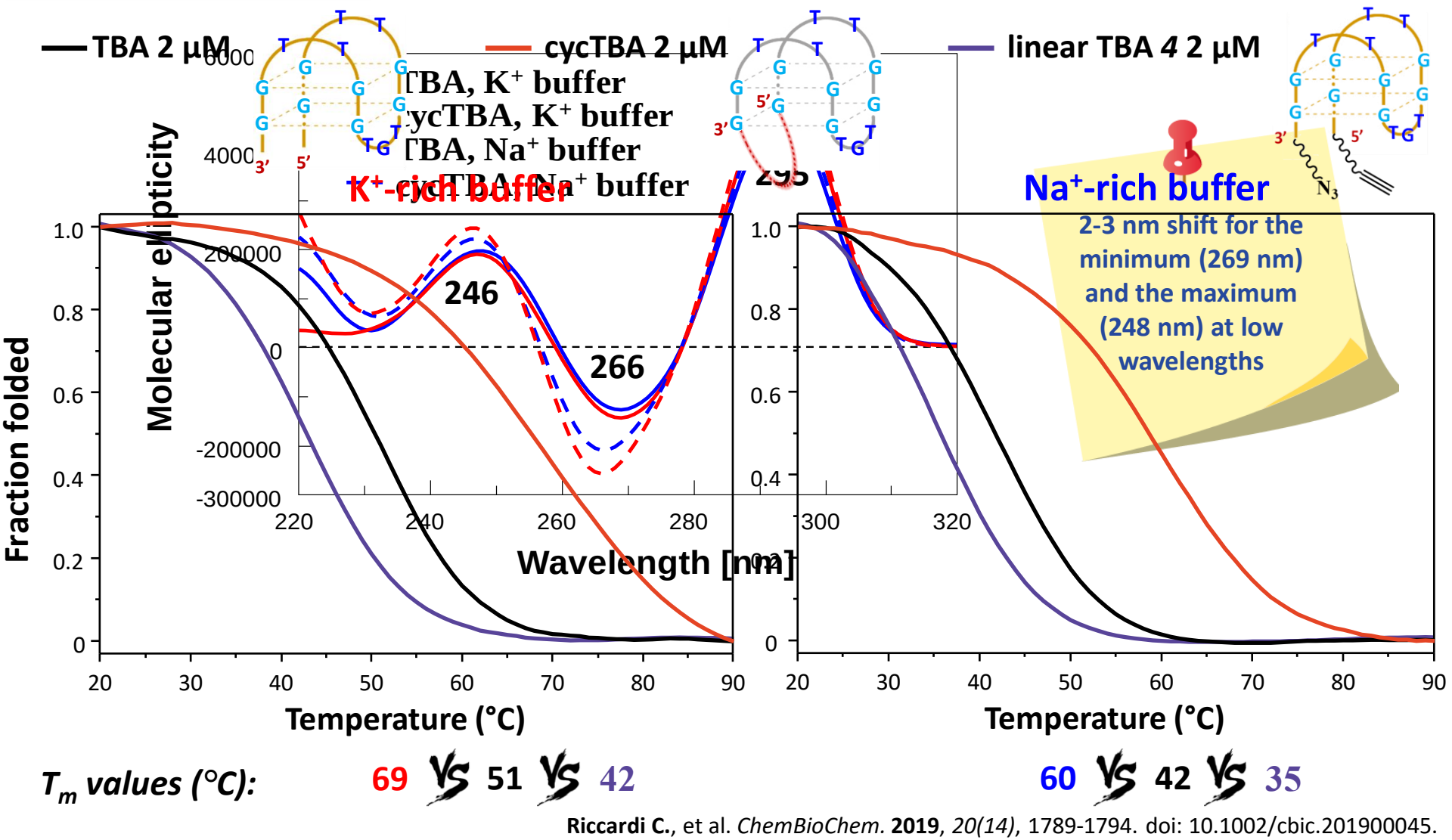


K⁺ buffer: 10 mM KH_2PO_4/K_2HPO_4, 70 mM KCl, 0.2 mM EDTA, pH = 7.0

Na⁺ buffer *i.e.* **PBS**: 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH = 7.2

Riccardi C., et al. *ChemBioChem.* **2019**, *20(14)*, 1789-1794. doi: 10.1002/cbic.201900045.

CD and CD-melting experiments



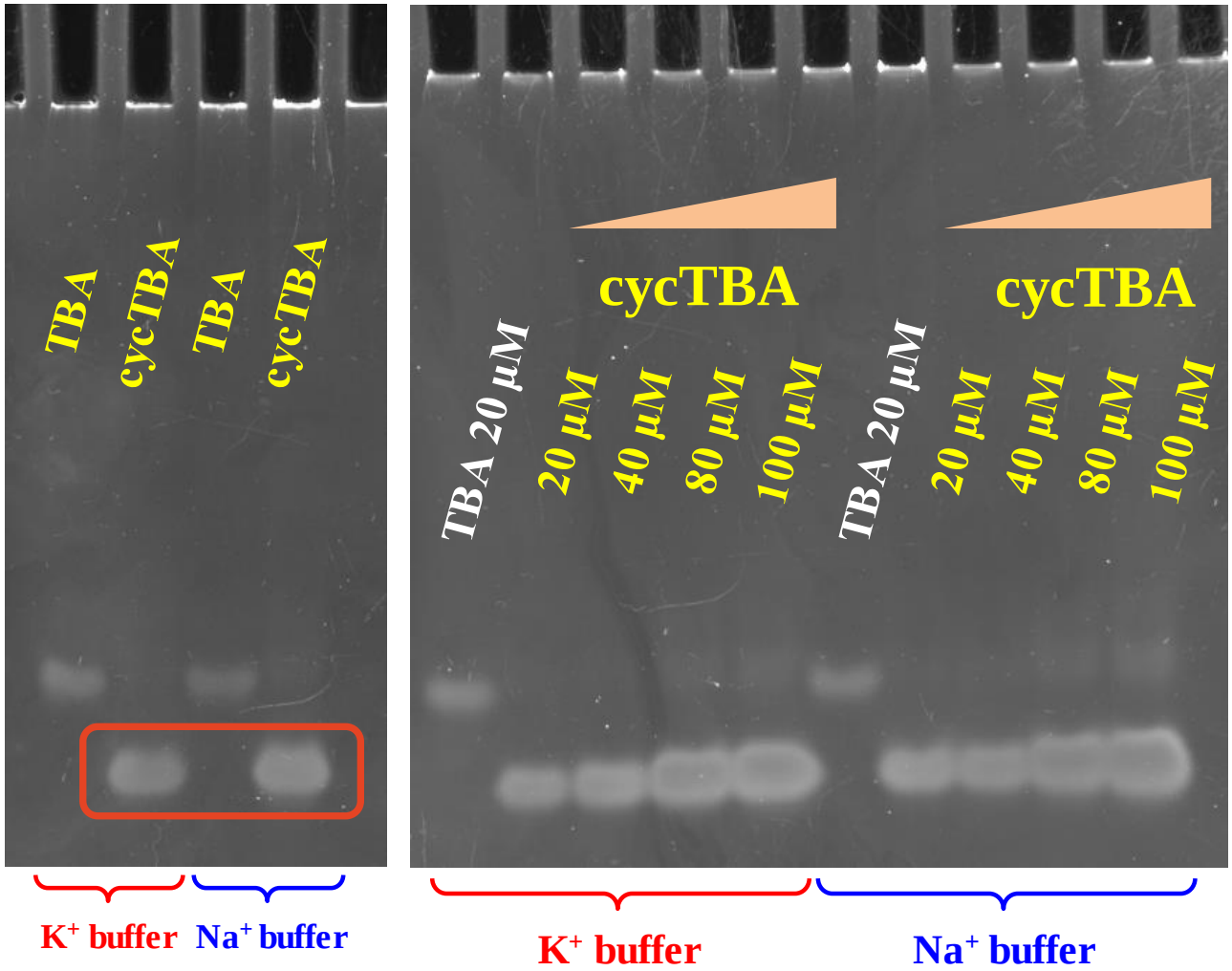
T_m values (°C): 69 vs 51 vs 42

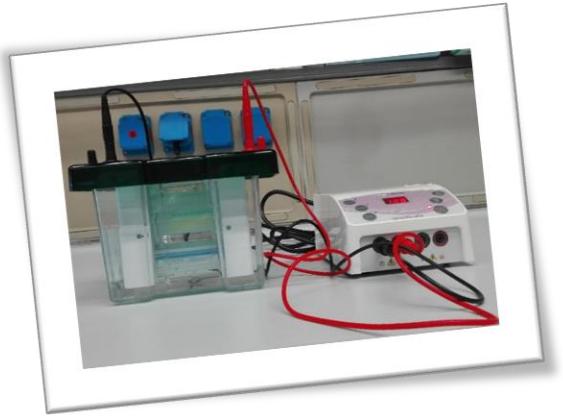
60 vs 42 vs 35

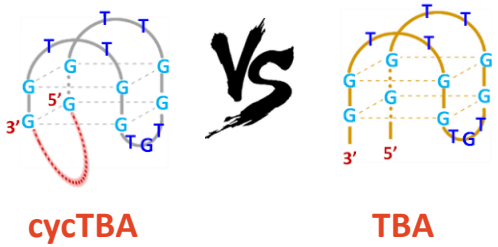
Riccardi C., et al. *ChemBioChem.* **2019**, *20(14)*, 1789-1794. doi: 10.1002/cbic.201900045.

Results and discussion

Native gel electrophoresis analysis





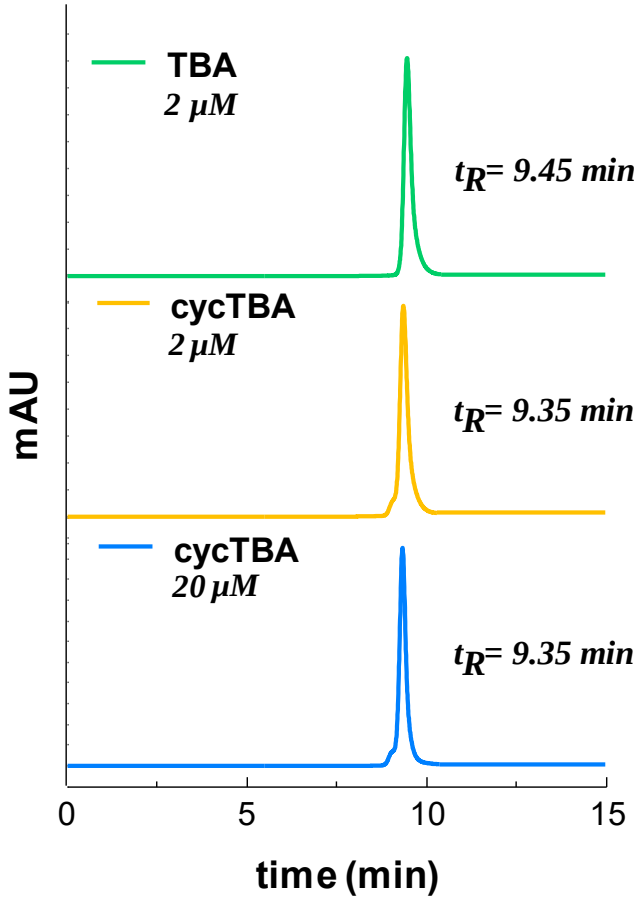


Riccardi C., et al. *ChemBioChem.* **2019**, *20(14)*, 1789-1794. doi: 10.1002/cbic.201900045.

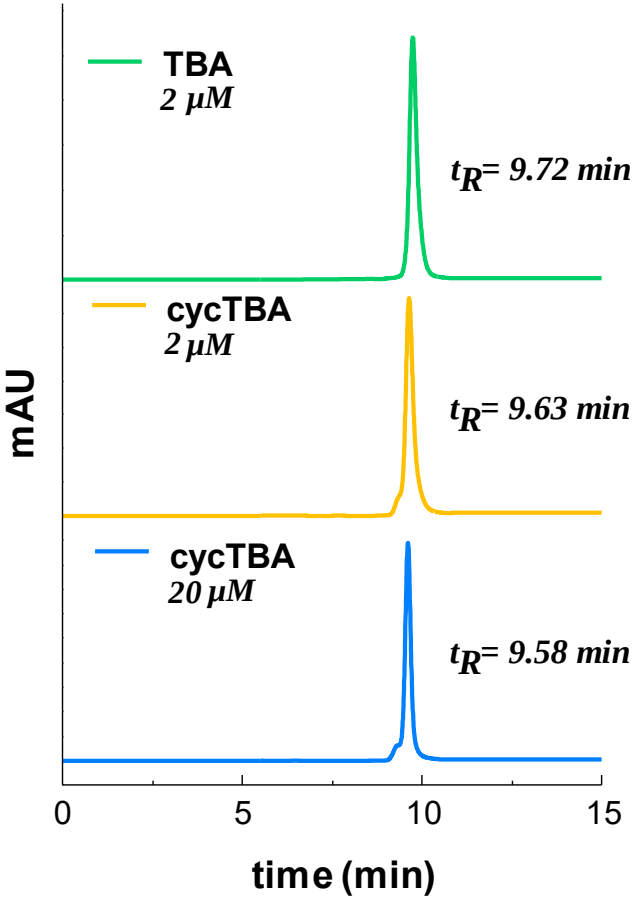
Results and discussion

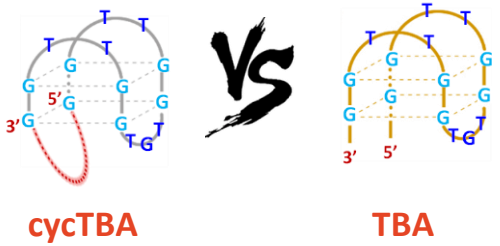
SE-HPLC analysis

K^+ buffer



Na^+ buffer

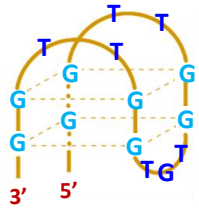


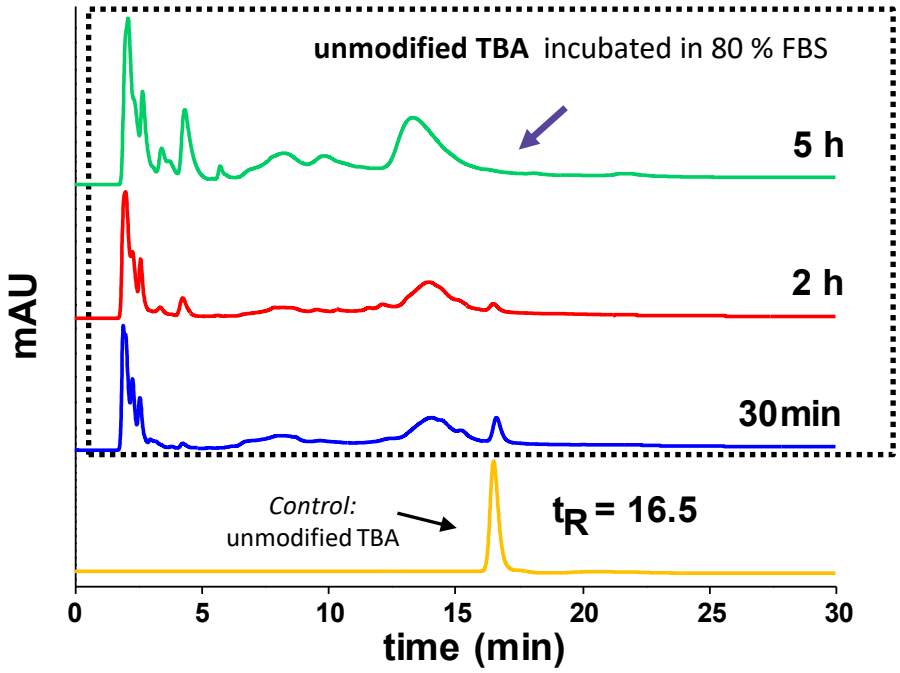


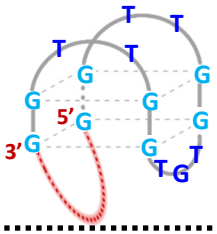
Riccardi C., et al. *ChemBioChem.* **2019**, *20(14)*, 1789-1794. doi: 10.1002/cbic.201900045.

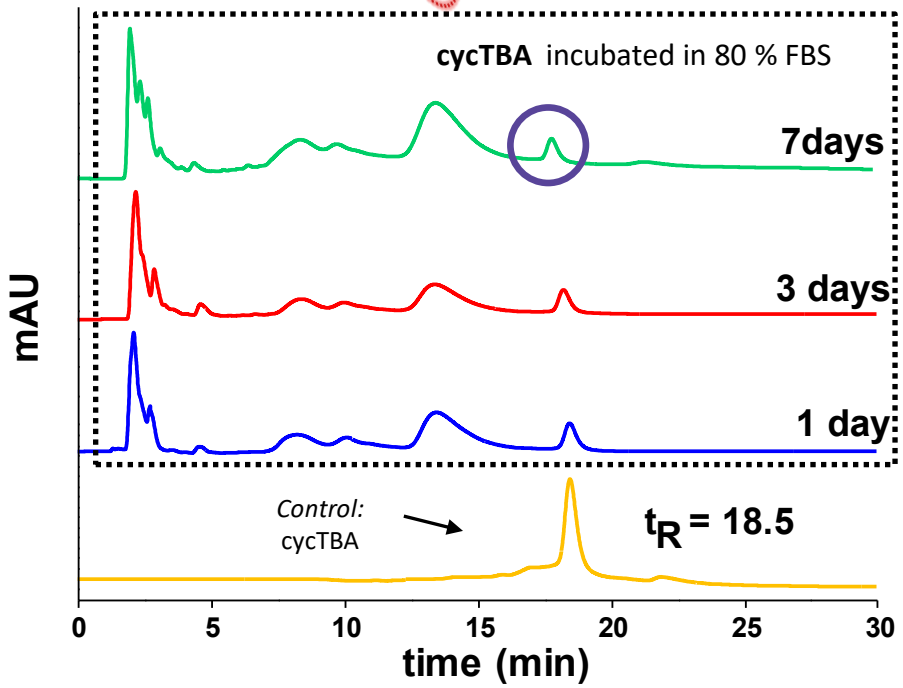
Results and discussion

Enzymatic stability in serum









Nucleogen DEAE 60-7 column 7 µm, 125 x 4 mm, λ= 254 nm; Linear gradient of B (0 to 100 %) in A in 30 min
Solution A: 20 mM KH_2PO_4/K_2HPO_4 aq. solution, pH 7.0, containing 20 % (v/v) CH_3CN;
Solution B: 1 M KCl, 20 mM KH_2PO_4/K_2HPO_4 aq. solution, pH 7.0, containing 20 % (v/v) CH_3CN.

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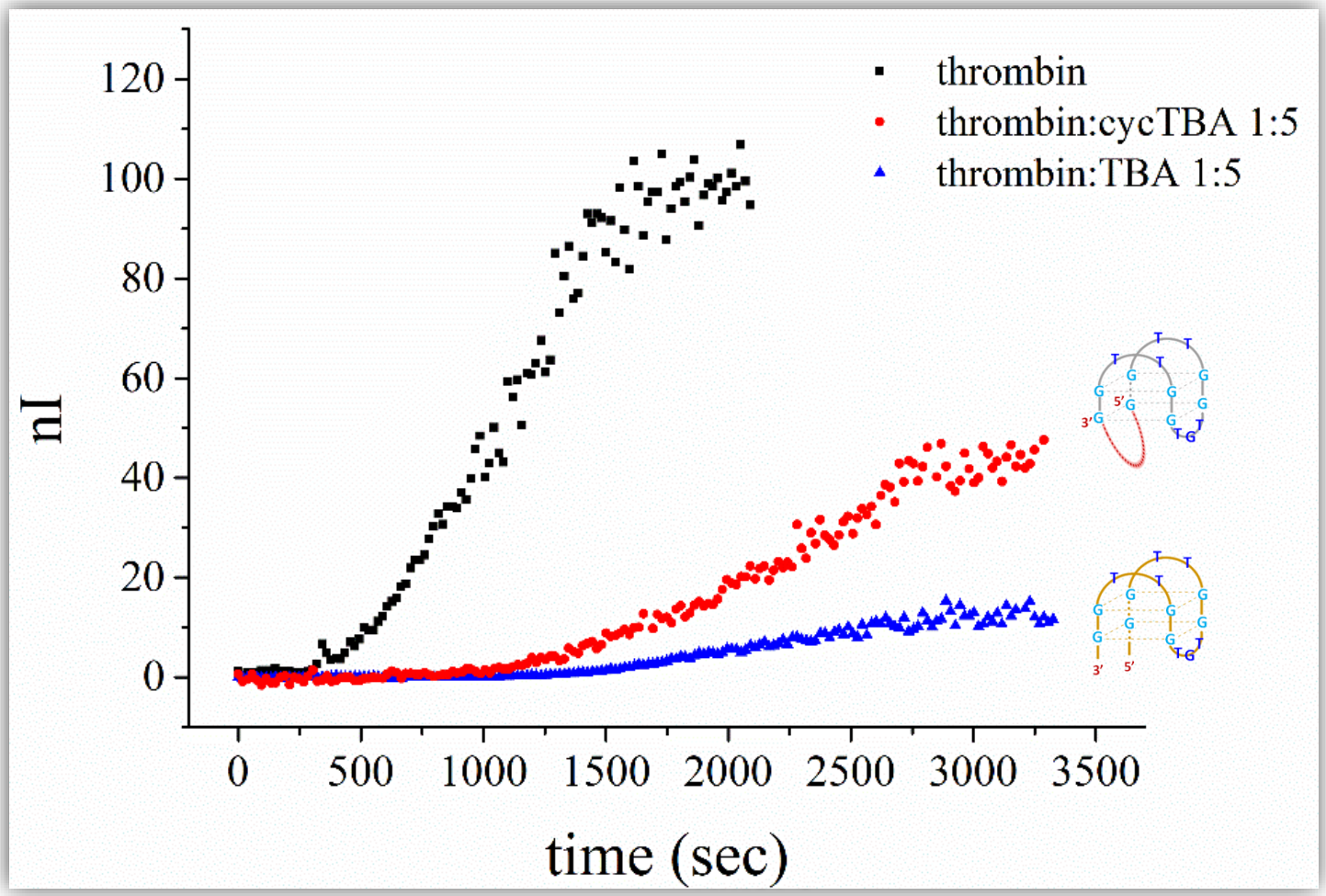
Results and discussion

Thrombin inhibition



prof. Luigi Paduano

Dr. Irene Russo Krauss

Dept. of Chemical Sciences, Naples

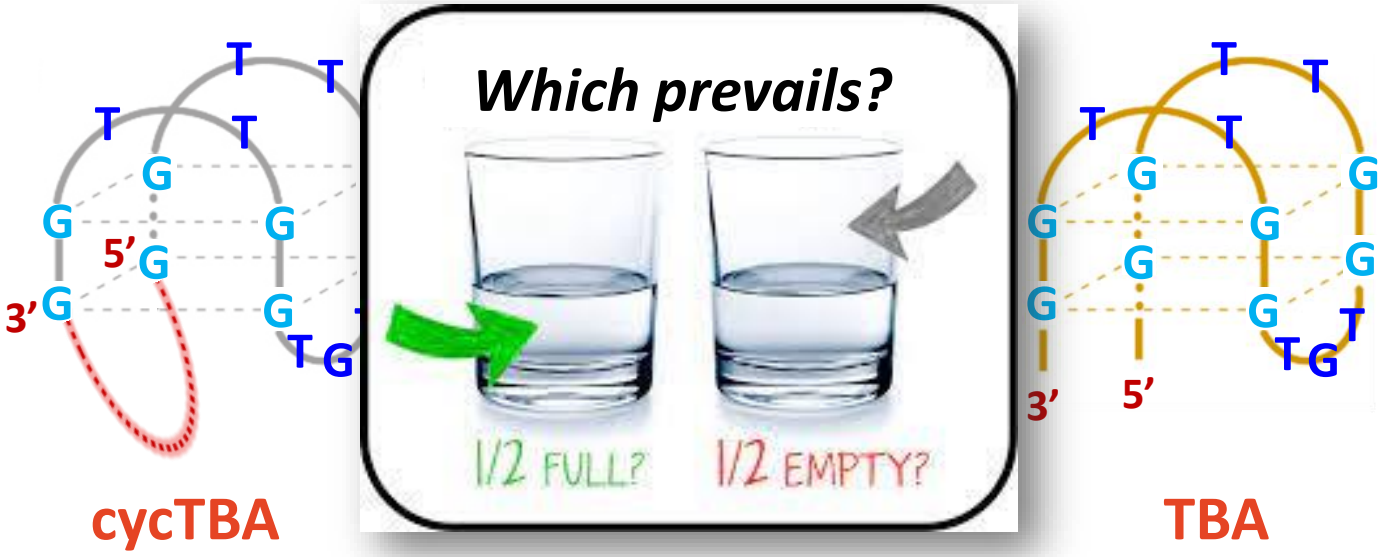
Fibrinogen: 1.2 µM
Thrombin: 5 nM

Riccardi C., et al. *ChemBioChem.* **2019**, *20(14)*, 1789-1794. doi: 10.1002/cbic.201900045.

Summarizing

- Remarkable increase in the thermal stability of the G-quadruplex structure;
- Noteworthy higher resistance to nuclease degradation;

- Anticoagulant activity reduced by about 50 %;
- Binding affinity reduced by about 50 %.

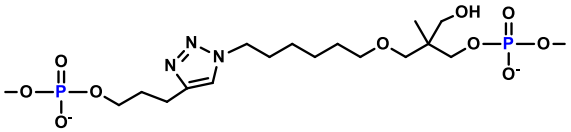
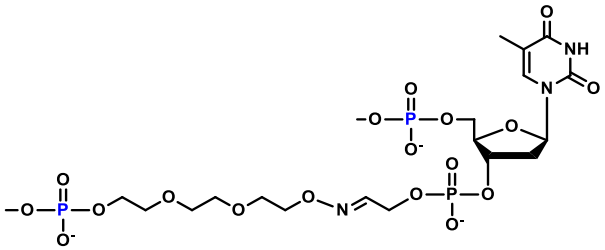
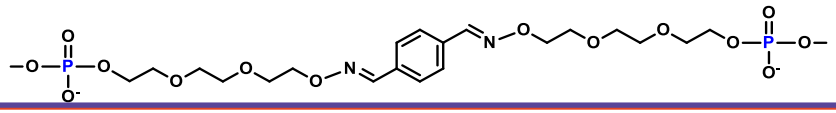
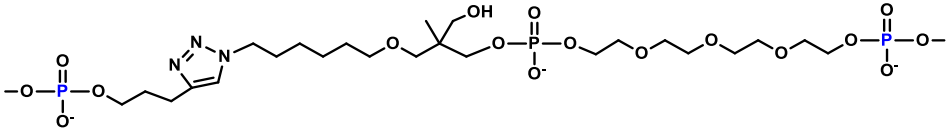
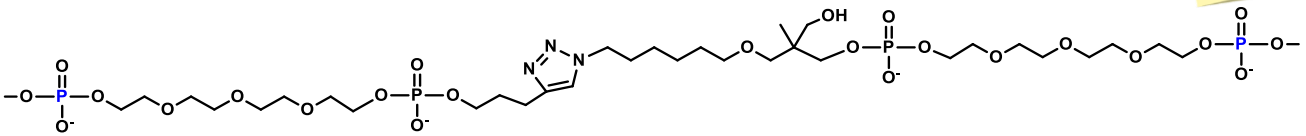


Riccardi C., et al. *ChemBioChem.* **2019**, *20(14)*, 1789-1794. doi: 10.1002/cbic.201900045.

Results and discussion

Optimized cyclic TBA analogues

good balance between the compactness of the G-quadruplex core and its structural flexibility

Name	Linker connecting sequence (5'-3')	Linker connecting length
cycTBA		20 atoms
cycTBA I		22 atoms
cycTBA II		30 atoms
cycTBA III		34 atoms
cycTBA IV		48 atoms

oxime ligation method

CuAAC protocol

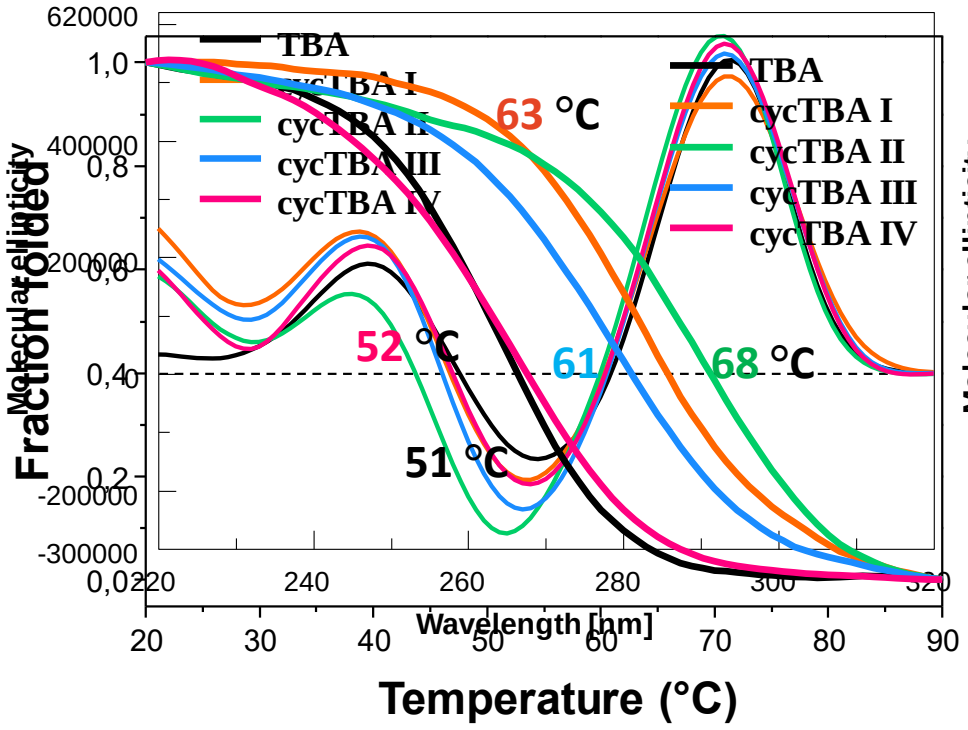
Riccardi C., et al. *Bioorg. Chem.* **2020**, *94*, 103379. doi: 10.1016/j.bioorg.2019.103379.

Biophysical characterizazion

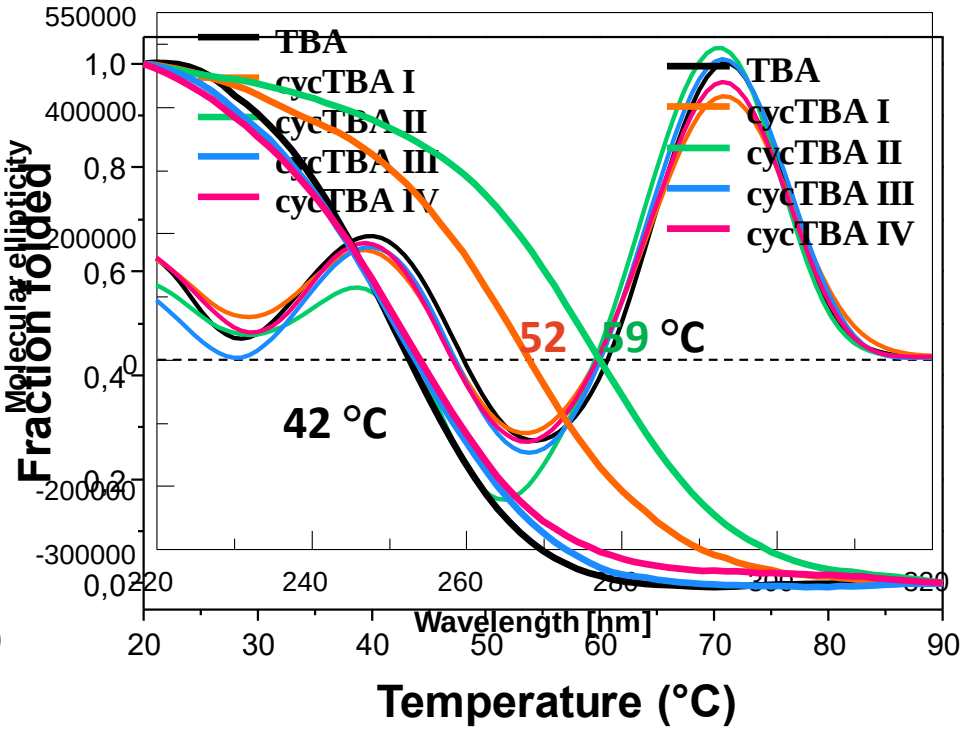
- **UV and TDS analysis: in both saline solutions, TDS profiles confirmed the formation of G-quadruplex structures, providing the typical "fingerprint" of these architectures;**

- **CD analysis:**

K^+-rich buffer



Na^+-rich buffer



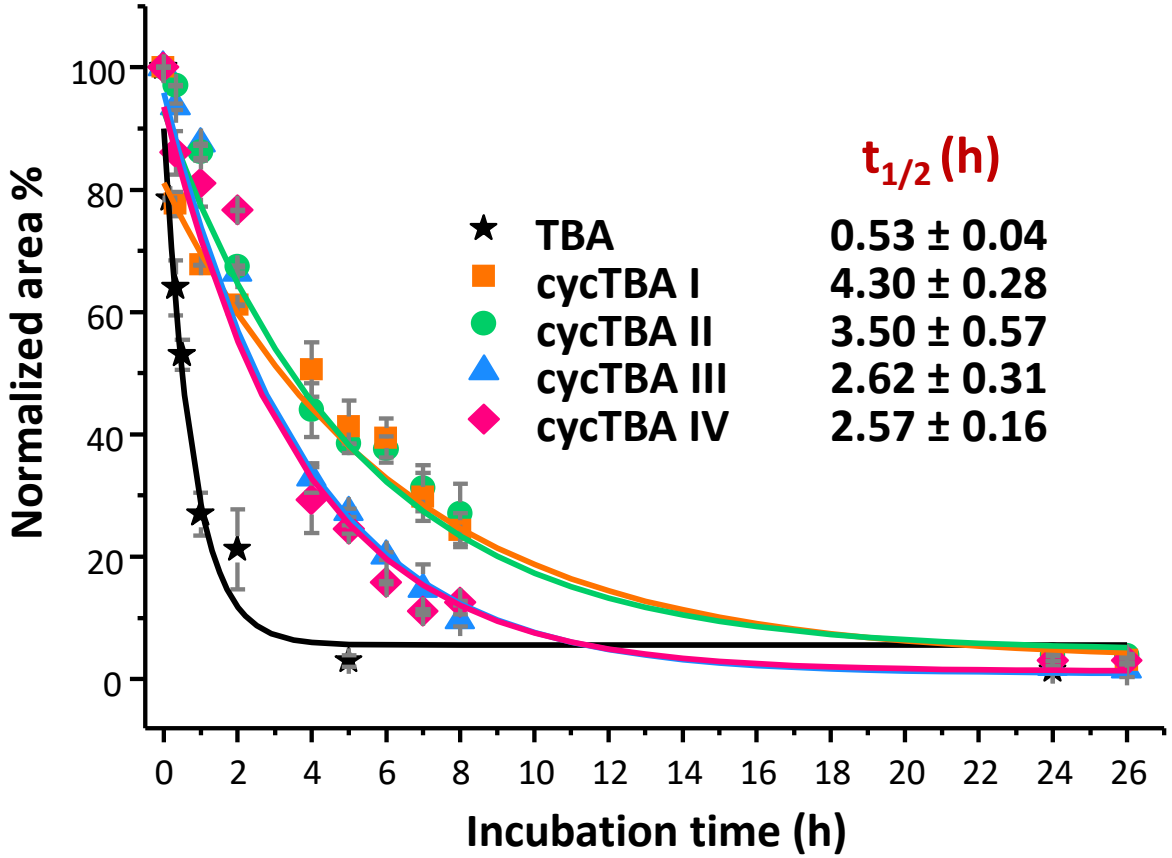
- ✓ In both saline conditions, **cycTBA I** and **cycTBA II** are the most stable derivatives

Riccardi C., et al. *Bioorg. Chem.* **2020**, *94*, 103379. doi: 10.1016/j.bioorg.2019.103379.

Biophysical characterizazion

- **Native gel electrophoresis and SE-HPLC investigations confirmed the presence of a single unimolecular G4 structure;**

- **Nuclease resistance experiments:**



	$t_{1/2}$ (h)
TBA	0.53 ± 0.04
cycTBA I	4.30 ± 0.28
cycTBA II	3.50 ± 0.57
cycTBA III	2.62 ± 0.31
cycTBA IV	2.57 ± 0.16

✓ **cycTBA I** and **cycTBA II** are the most resistant analogues in serum

Nucleogen DEAE 60-7 column 7 µm, 125 x 4 mm, λ= 254 nm;
Linear gradient of B (0 to 100 %) in A in 30 min
Solution A: 20 mM KH_2PO_4/K_2HPO_4 aq. solution, pH 7.0, containing 20 % (v/v) CH_3CN;
Solution B: 1 M KCl, 20 mM KH_2PO_4/K_2HPO_4 aq. solution, pH 7.0, containing 20 % (v/v) CH_3CN.

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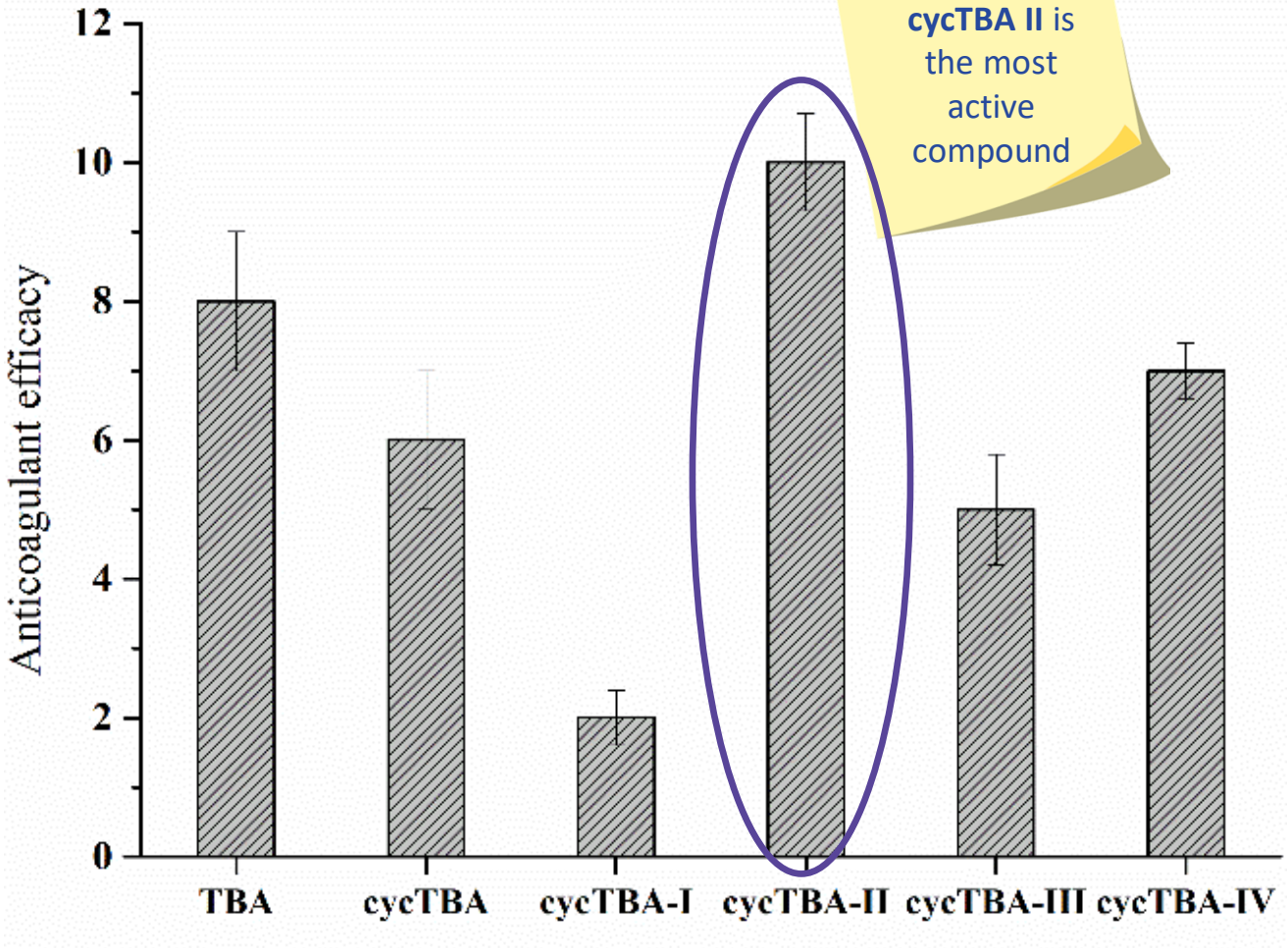
Results and discussion

Thrombin inhibition



cycTBA II is the most active compound

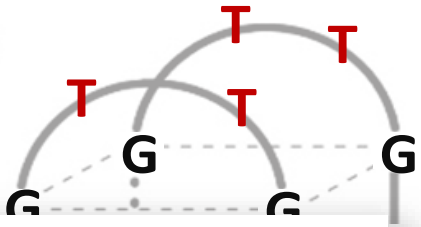
prof. Luigi Paduano **Dr. Irene Russo Krauss**

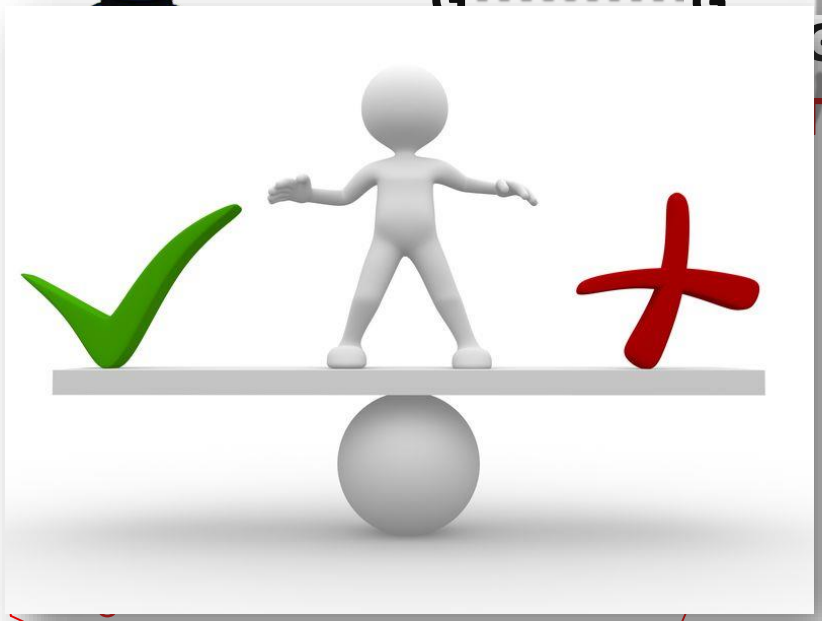
Dept. of Chemical Sciences, Naples

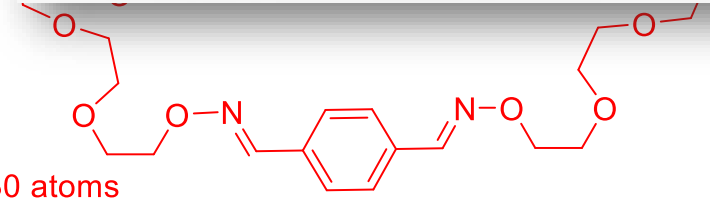
Coagulation efficacy (defined as the ratio between the coagulation rate in the presence of thrombin and in the presence of both thrombin and each aptamer) of cycTBAs **I-IV**

Riccardi C., et al. *Bioorg. Chem.* **2020**, *94*, 103379. doi: 10.1016/j.bioorg.2019.103379.

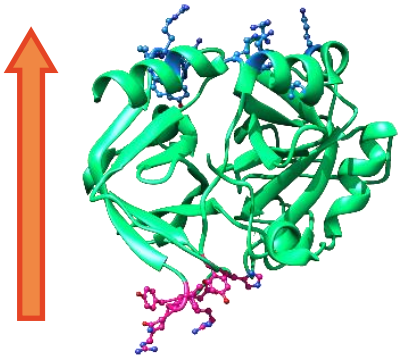
A first improved TBA analogue



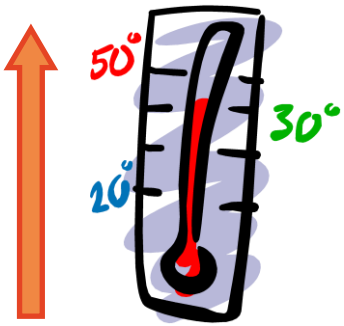




30 atoms



Improved inhibition of thrombin activity



Increased G4 stability (ΔT_m= +17 °C)

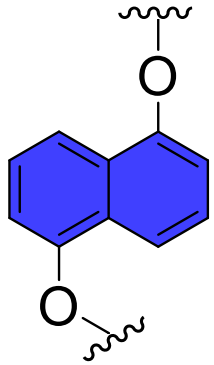
6.6-fold higher nuclease resistance

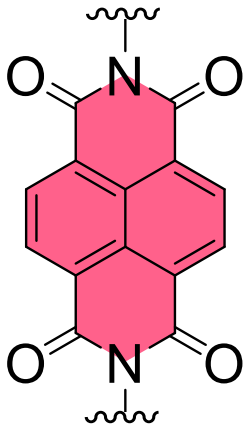
Riccardi C., et al. *Bioorg. Chem.* **2020**, *94*, 103379. doi: 10.1016/j.bioorg.2019.103379.

Further developments: pseudo-cyclic TBAs

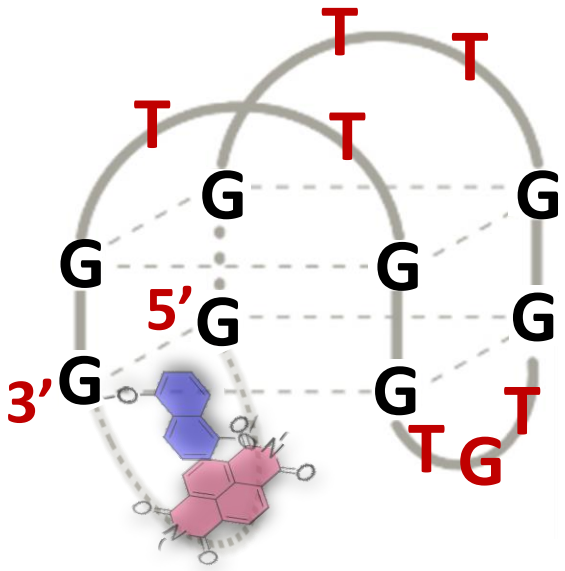
End-modified thrombin binding aptamers stabilized by π/π or charge-transfer interactions

DAN





NDI



DAN: 1,5-dialkoxy naphthalene

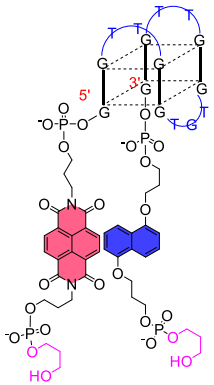
NDI: 1,8,4,5-naphthalenetetra-carboxylic diimide

G-quadruplex structural flexibility necessary for thrombin recognition combined with the intriguing advantages found for the previously investigated cyclic TBA analogues

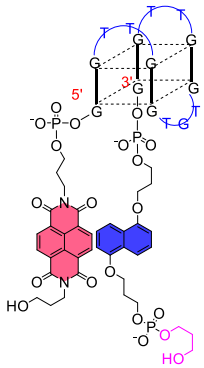
Pérez de Carvasal K., **Riccardi C.**, et al. *Int. J. Mol. Sci.* **2021**, *22(17)*, 9510. doi: 10.3390/ijms22179510.

Further developments: pseudo-cyclic TBAs

TBA-Np/Dp



TBA-N/Dp



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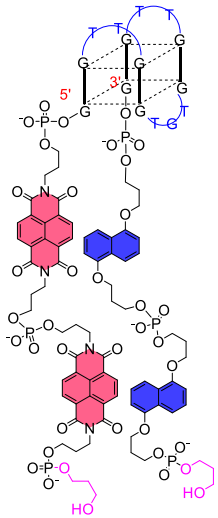
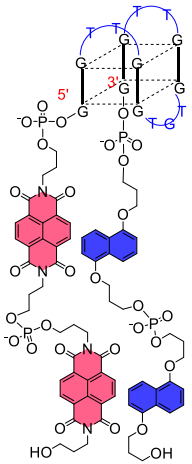


TBA-NN/DD



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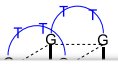
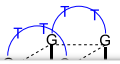
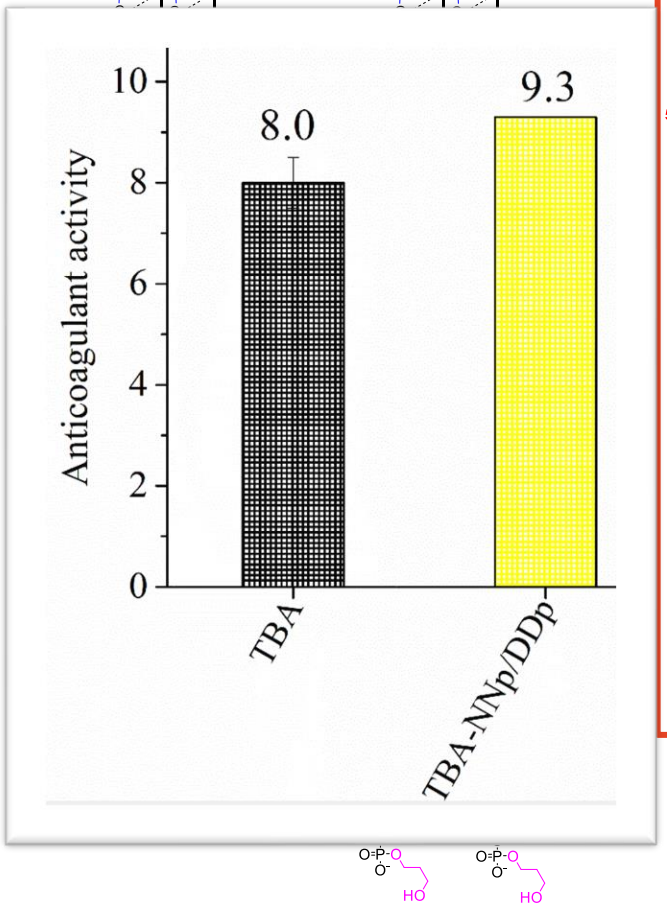
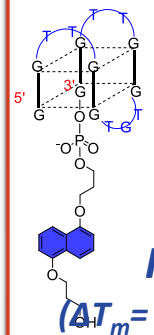


TBA-N/D

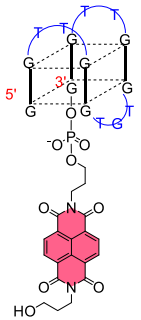


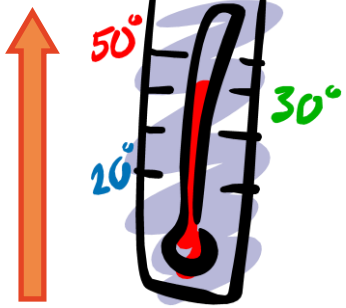


TBA-D



TBA-N





Increased G4 stability

(ΔT_m = +11 and + 8°C, respectively in K^+- and Na^+-rich buffers)

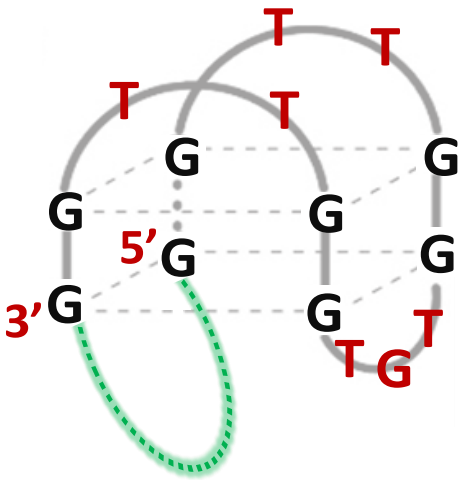
4.5-fold higher nuclease resistance

Pérez de Carvasal K., **Riccardi C.**, et al. *Int. J. Mol. Sci.* **2021**, *22(17)*, 9510. doi: 10.3390/ijms22179510.

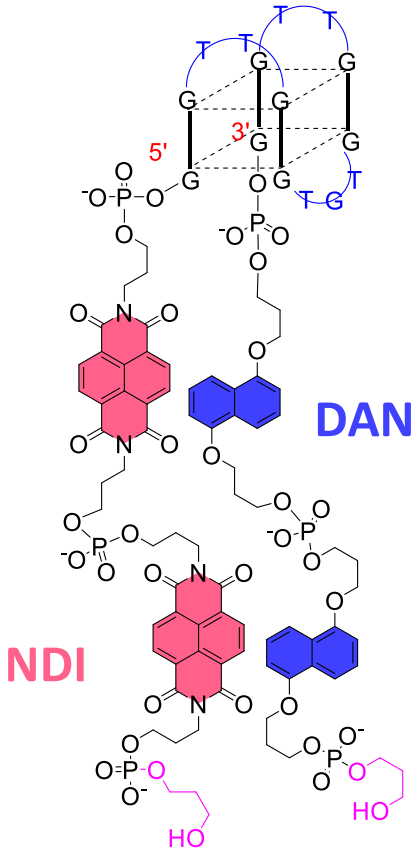
Conclusions

What's next???

Analysis of the crystal structures obtained for thrombin with the most promising TBA derivatives

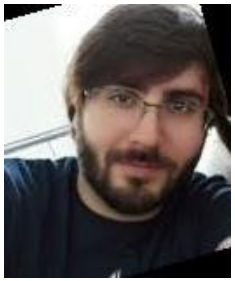


cycTBA II



TBA-NNp/DDp

prof. Filomena Sica



Dr. Romualdo Troisi

Dept. of Chemical Sciences, Naples

...to be continued!!!

Pérez de Carvasal K., **Riccardi C.**, et al. *Int. J. Mol. Sci.* **2021**, *22(17)*, 9510. doi: 10.3390/ijms22179510.

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