The 8th International Electronic Conference on Medicinal Chemistry (ECMC 2022)
01–30 NOVEMBER 2022 | ONLINE

Novel anti-HMGB1 aptamers as potential drugs in anti-inflammatory and cancer therapies

Chaired by DR. ALFREDO BERZAL-HERRANZ;
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pharmaceuticals MDPI

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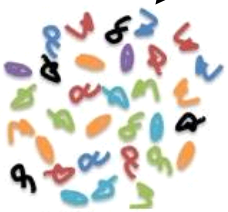
Novel anti-HMGB1 aptamers as potential drugs in anti-inflammatory and cancer therapies

tel_{26} *core*

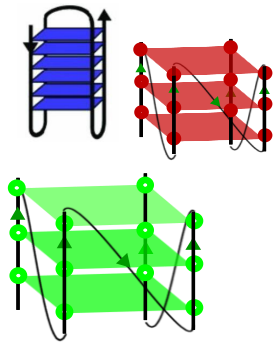
5'-TTA **GGG TTA GGG TTA GGG TTA GGG** TT-3'

5'-TTA **GGG XXX GGG XXX GGG XXX GGG** TT-3'

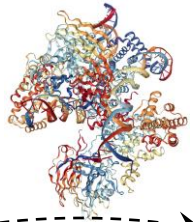


Pool of 512 different oligonucleotide aptamers

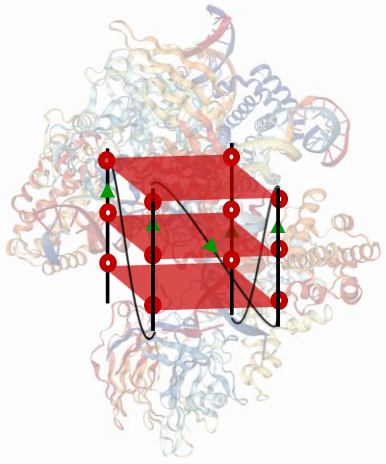
SELEX



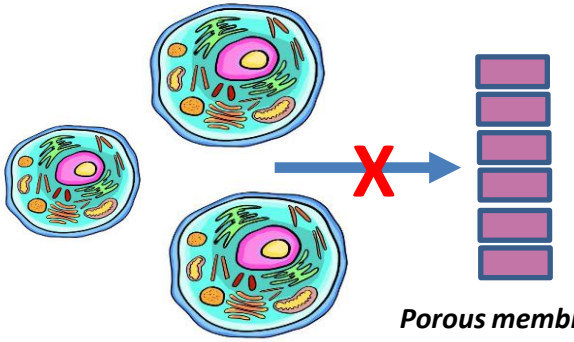
14 G-quadruplex-forming aptamers



HMGB1 protein



In vitro inhibition of HMGB1-induced cellular migration



Porous membrane

Abstract:

High-Mobility Group Box 1 (HMGB1) is an abundant, highly conserved, non-histone nuclear protein present in almost all eukaryotic cells. In inflammatory conditions, HMGB1 is actively secreted from immune cells in the extracellular matrix, where it behaves as a proinflammatory cytokine. Once released, it can bind to cell-surface receptors, such as the Receptor for Advanced Glycation End products (RAGE) and Toll-Like Receptors (TLR) 2, 4 and 9, and mediate various cellular responses, including the induction of cell migration/proliferation and the release of other proinflammatory cytokines. Moreover, HMGB1 is able to contribute to the pathogenesis of various chronic inflammatory and autoimmune diseases as well as of cancer. Given the multiple roles of HMGB1 in these pathologies, identification of inhibitors of this protein is of considerable clinical interest.
We here identified novel G-quadruplex (G4) forming aptamers as potential HMGB1 inhibitors. Using SELEX technology, we selected 14 G4-forming DNA sequences from a properly designed G-rich oligonucleotide library. These aptamers have been fully characterized in a biologically relevant buffer using several biophysical techniques to determine their preferred conformation as well as their thermal and enzymatic stability. Moreover, we evaluated the interaction between these aptamers and HMGB1, as well as their ability to inhibit HMGB1-induced migration in cancer cells so to identify the best candidates for future in vivo assays aimed at repressing the pathological functions induced by the target protein.

Keywords: aptamer; cancer; G-quadruplex; HMGB1; inflammation.

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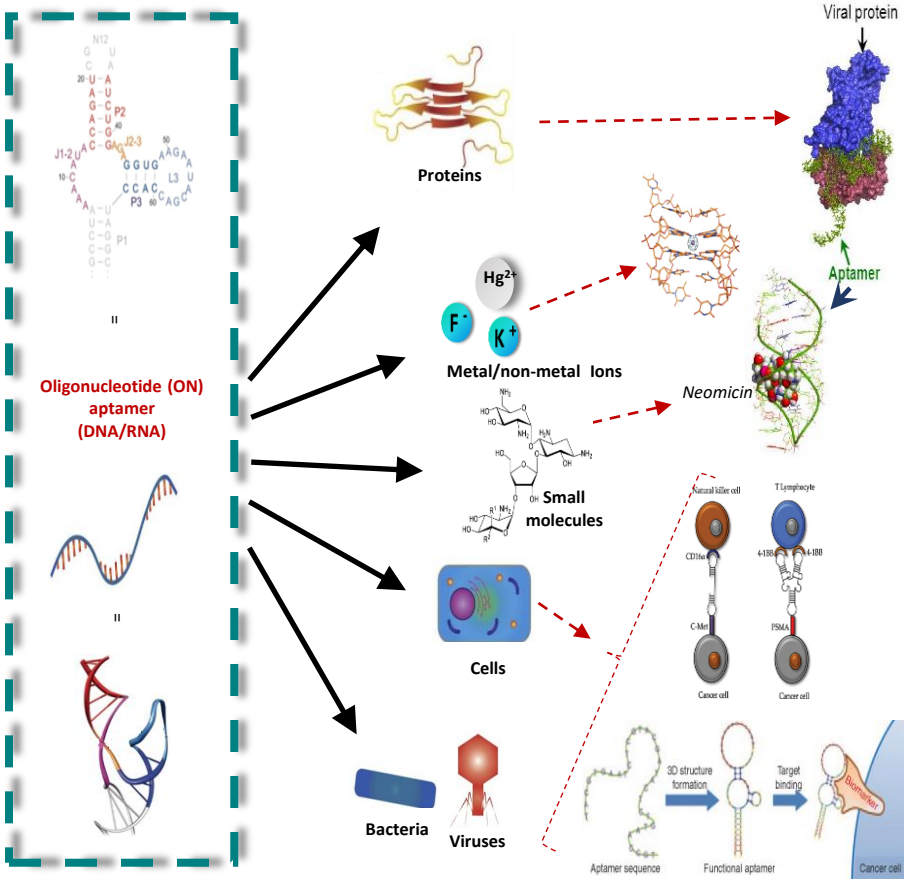
APTAMERS

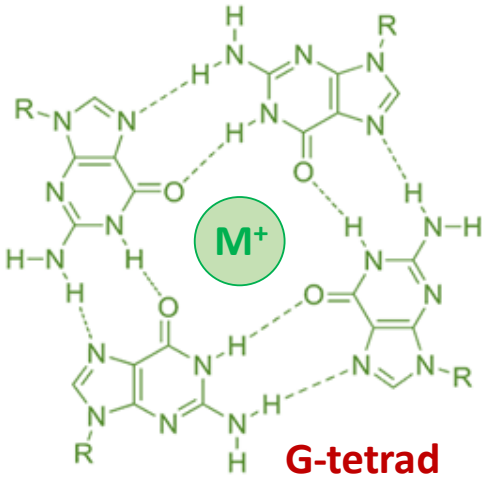
Introduction

- Short DNA or RNA sequences able to recognize with high affinity and specificity their target;
- Generally identified from large combinatorial libraries of random oligonucleotides by an *in vitro* selection procedure called SELEX.



SELEX: Systematic Evolution of Ligands by EXponential enrichment

G-QUADRUPLEX-FORMING APTAMERS



G-tetrad

Many biologically active aptamers are G-rich oligonucleotides, able to fold into peculiar G4 structures consisting of guanine-based tetrads, further stabilized by positive ions, *e.g.* Na^+ or K^+.

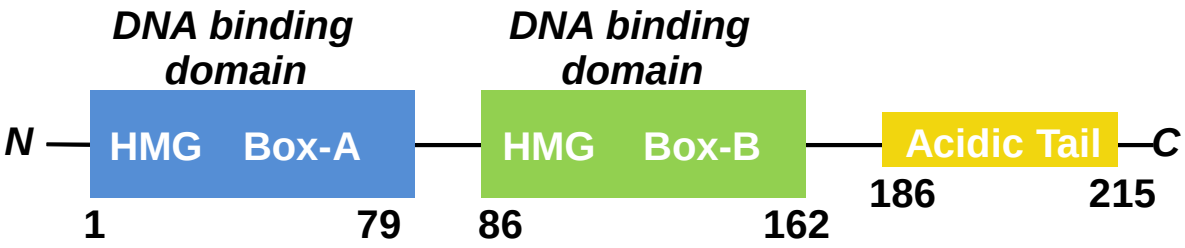
- ✓ **Polymorphism**
- ✓ **High density of negative charges**

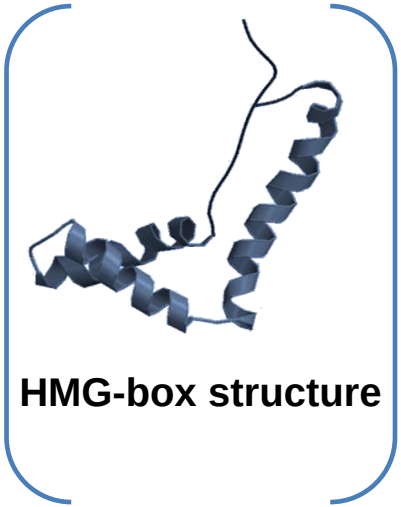
For a review, see: C. Platella, C. Riccardi, D. Montesarchio, G. N. Roviello, D. Musumeci, Biochim. Biophys. Acta - Gen. Subj. 2017, 1861, 1429–1447.

Development of novel G4-forming aptamers targeting HMGB1 protein

The target: HMGB1 protein

- Small non-histonic protein (27 kDa, 215 amino acids) associated with chromatin present in almost all eukaryotic cells;
- Characterized by three independent domains.





HMGB1 is involved in the pathogenesis of various chronic inflammatory and autoimmune diseases, as well as cancer

- **Sepsis**
- **Rheumadoid arthritis**
- **Atherosclerosis**
- **Obesity**
- **Type II Diabetes**
- **Systemic lupus erythematosus (SLE)**
- **Cancer**

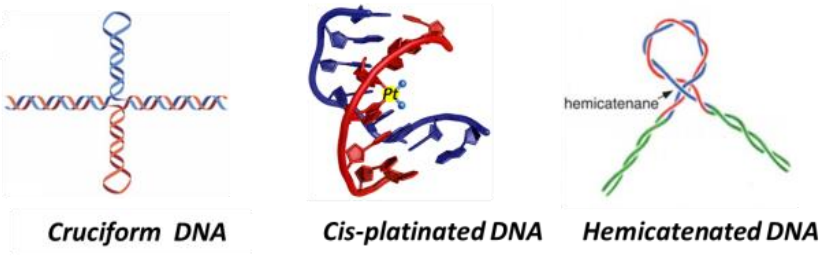
Identification of HMGB1 inhibitors: HMGB1-RAGE interaction inhibition

RAGE: Receptor for Advanced Glycation End products

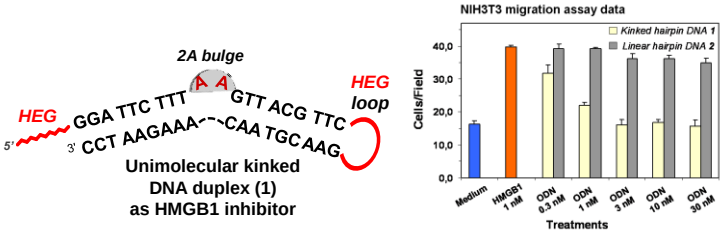
For a review, see: D. Musumeci, G.N. Roviello, D. Montesarchio, *Pharmacology & Therapeutics* **2014**, 141, 347–357

On the basis of previous findings:

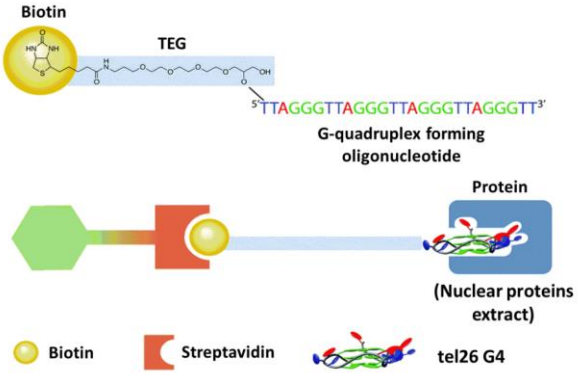
- HMGB1 is considered a ***DNA binding protein***, *with high affinity against* ***distorted DNA structures****;*



- kinked DNA duplexes are able to **inhibit HMGB1** *in vitro*;



- the 26-mer truncation of human telomeric DNA sequence d(TTAGGGTTAGGGTTAGGGTTAGGGTT) (**tel$_{26}$**), able to fold into G-quadruplexes (G4), can interact with HMGB1 from nuclear extract;



Pagano B, *et al.*, Identification of novel interactors of human telomeric G-quadruplex DNA, *Chem Commun* **2015**, 51, 2964-67

Design of a novel putatively G4-forming DNA library

tel_{26} *core*

5'-TTA **GGG TTA GGG TTA GGG TTA GGG** TT-3'

5'-TTA **GGG XXX GGG XXX GGG XXX GGG** TT-3'

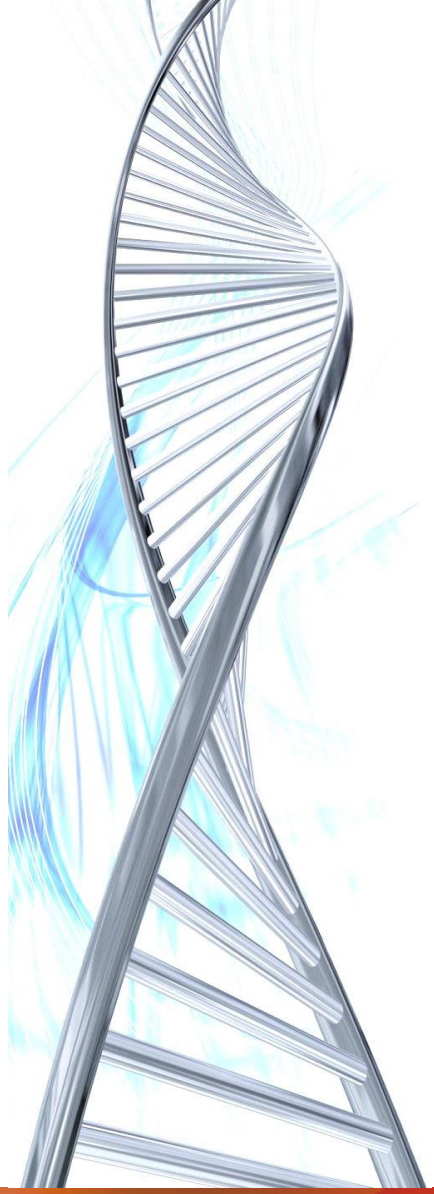
VARIABLE POSITIONS

X= Adenine or Thymine

The DNA library was designed to:

- ✓ **avoid the formation of *hairpin* or duplex structures;**
- ✓ **form only G4s.**

Pool of 512 different oligonucleotide sequences

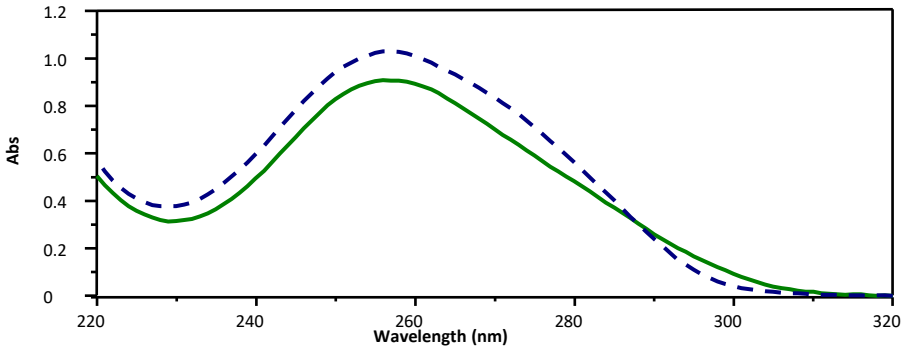


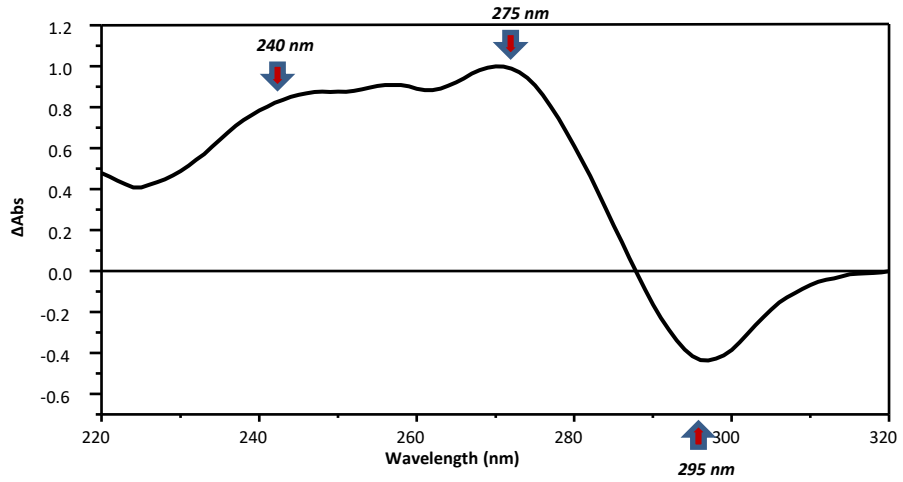
A32	TTA	GGG	XXX	GGG	XXX	GGG	XXX	GGG	GGG	TT
B6	TTA	GGG		GGG		GGG		GGG	GGG	TT
D40	TTA	GGG		GGG		GGG		GGG	GGG	TT
L12	TTA	GGG		GGG		GGG		GGG	GGG	TT
L13	TTA	GGG		GGG		GGG		GGG	GGG	TT
L16	TTA	GGG		GGG		GGG		GGG	GGG	TT
L17	TTA	GGG		GGG		GGG		GGG	GGG	TT
L21	TTA	GGG		GGG		GGG		GGG	GGG	TT
L23	TTA	GGG		GGG		GGG		GGG	GGG	TT
L27	TTA	GGG		GGG		GGG		GGG	GGG	TT
L30	TTA	GGG		GGG		GGG		GGG	GGG	TT
L33	TTA	GGG		GGG		GGG		GGG	GGG	TT
L37	TTA	GGG		GGG		GGG		GGG	GGG	TT
L41	TTA	GGG		GGG		GGG		GGG	GGG	TT

PBS (*Phosphate-buffered saline*): 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4,1.86 mM KH_2PO_3

UV Thermal Difference Spectra (TDS)

Results and discussion





Fully unfolded aptamers

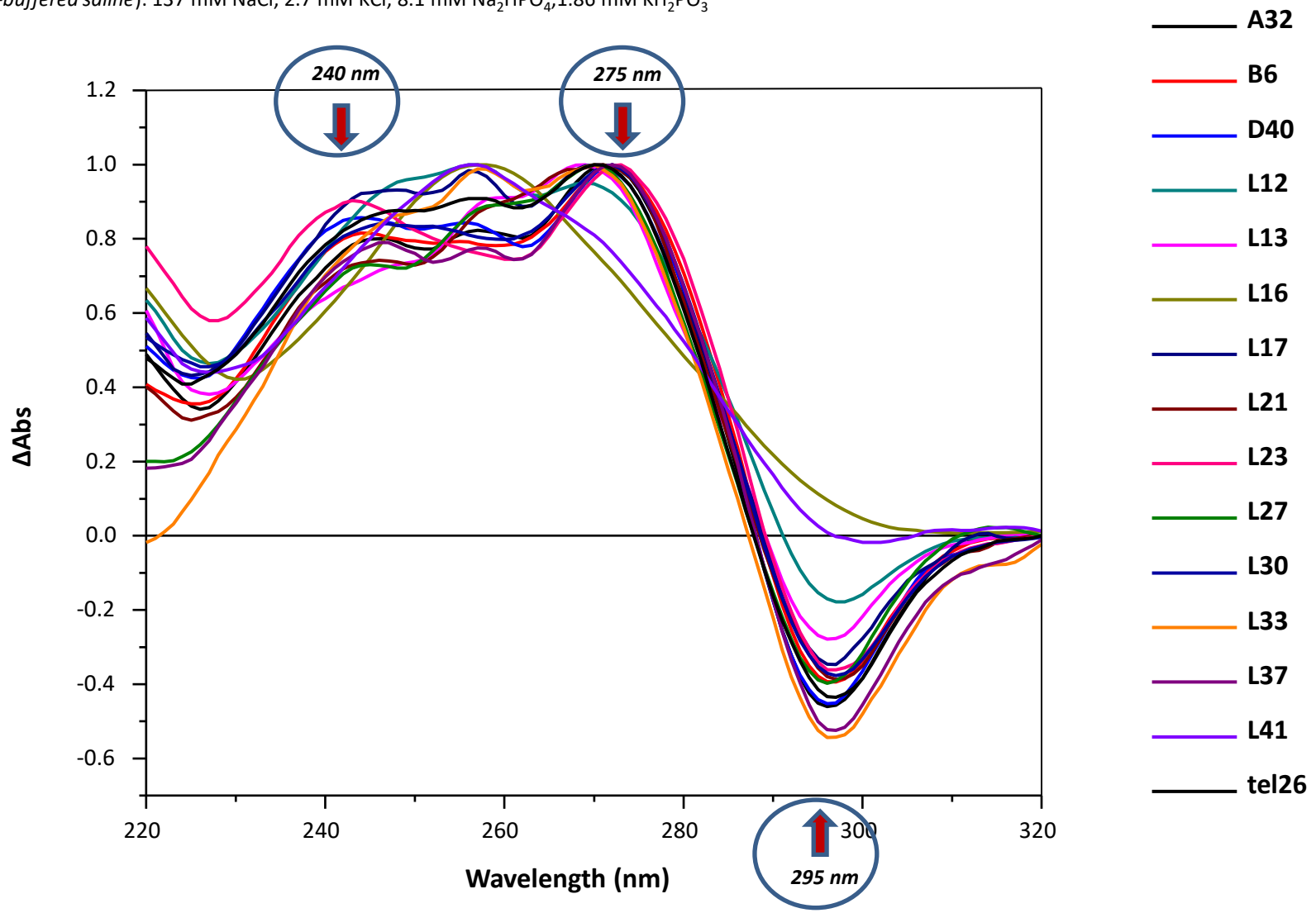
tel_{26} Abs spectrum at 95°C

tel_{26} Abs spectrum at 10°C

Fully folded aptamers

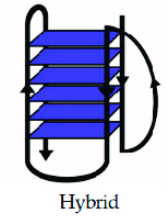
J.L. Mergny et al., **Thermal difference spectra: A specific signature for nucleic acid structures.** *Nucleic Acids Res.*, **2005**, 33, *e138.*

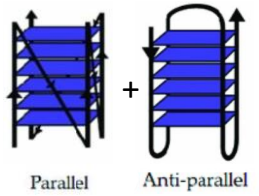
PBS (*Phosphate-buffered saline*): 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4,1.86 mM KH_2PO_3

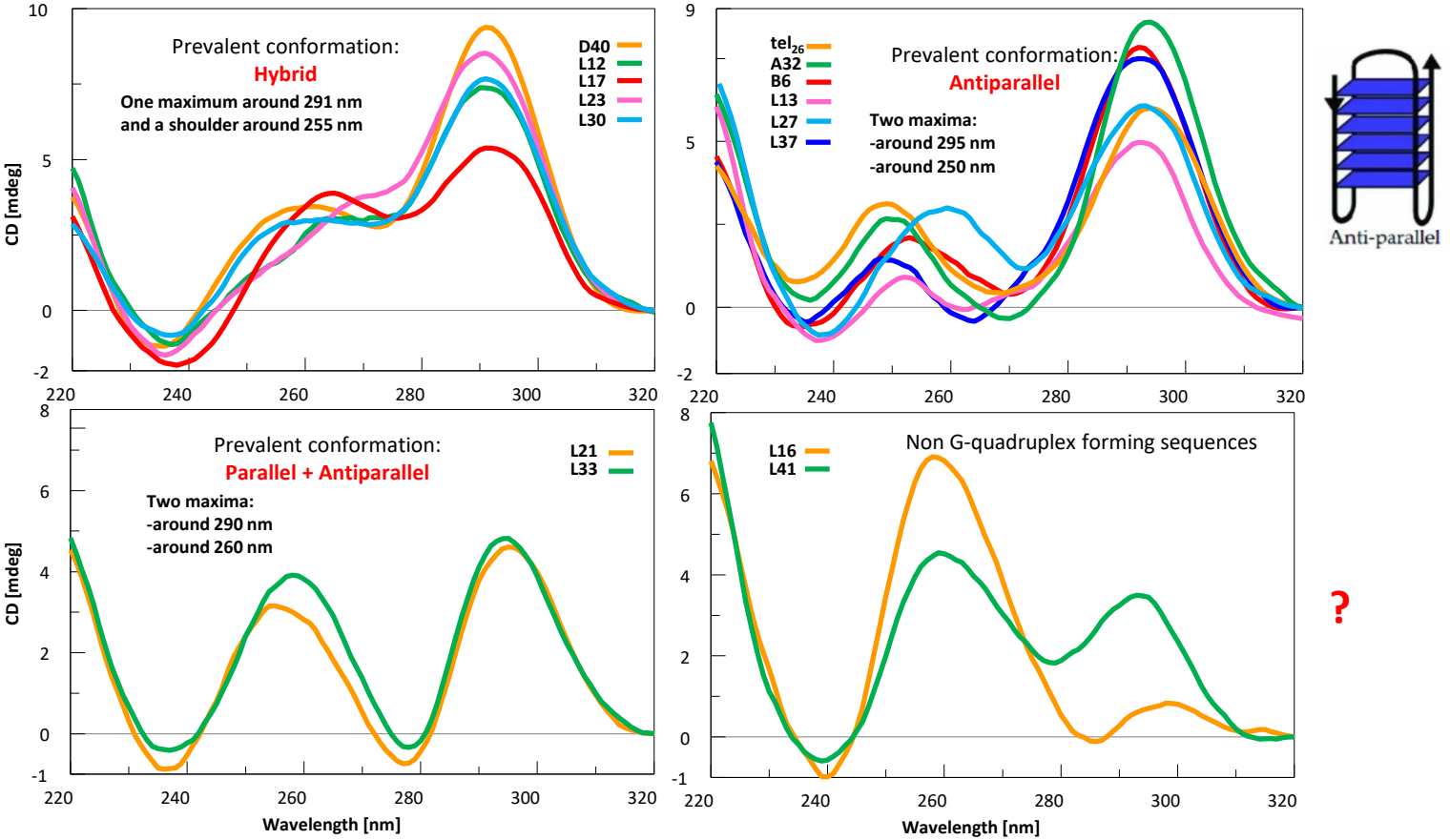


J.L. Mergny et al., **Thermal difference spectra: A specific signature for nucleic acid structures.** *Nucleic Acids Res.*, **2005**, 33, *e138.*

CD spectra of the selected anti-HMGB1 aptamers





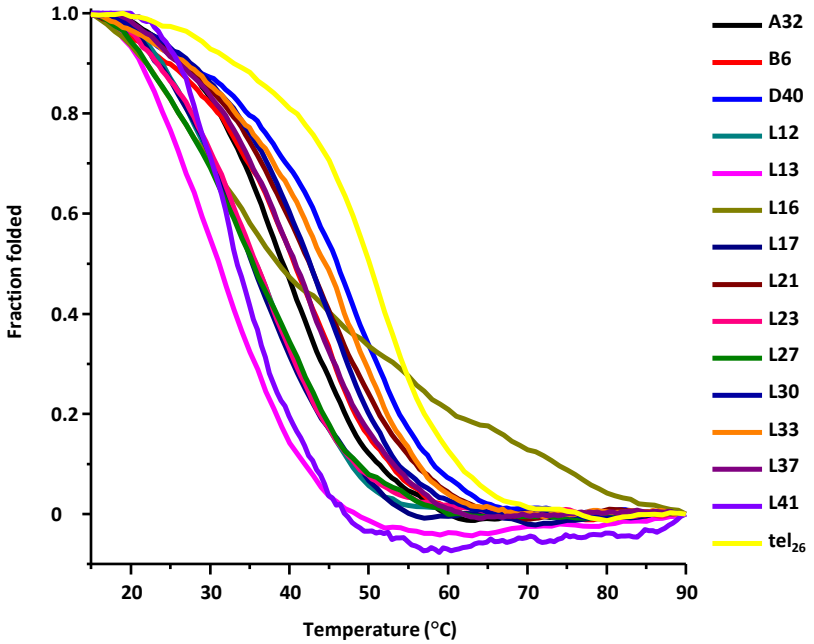


?

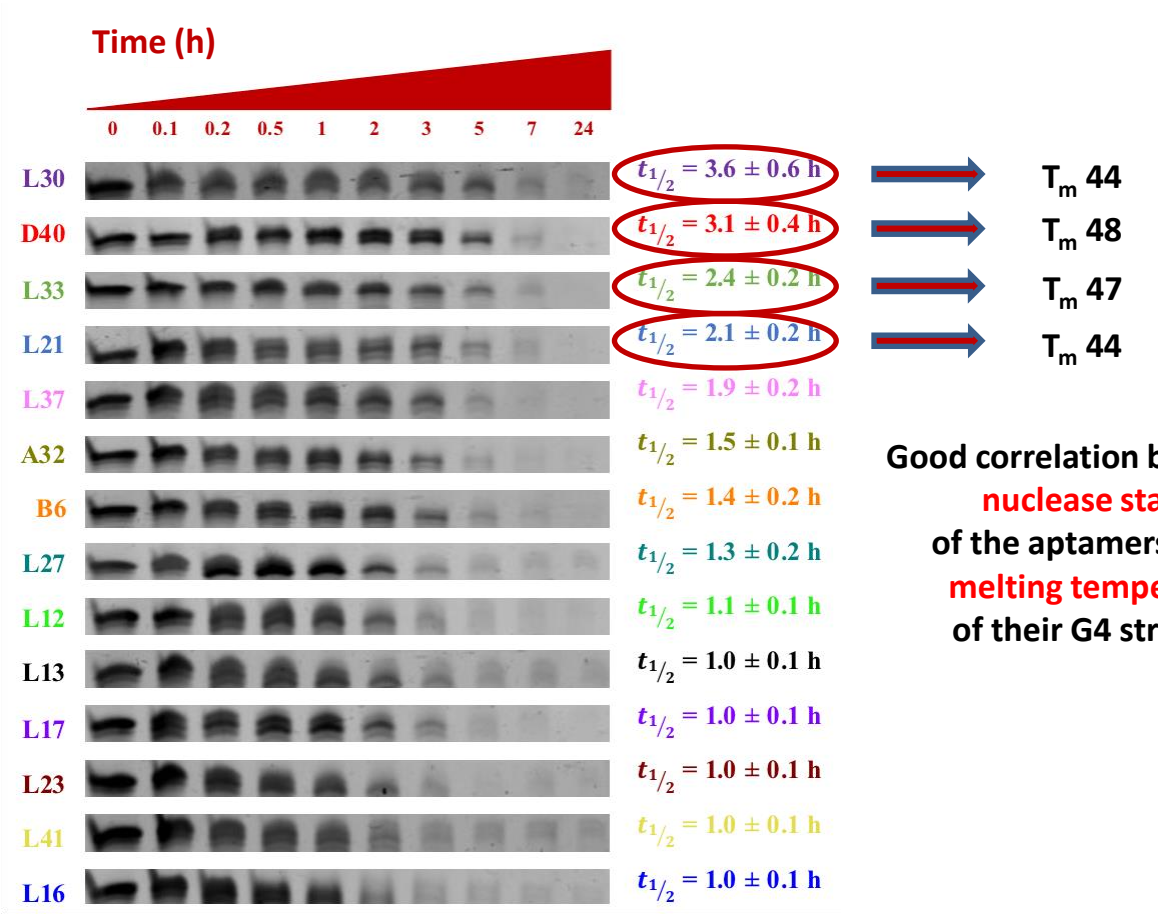
PBS (*Phosphate-buffered saline*): 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4,1.86 mM KH_2PO_3

CD-melting experiments

	Aptamers	$T_m \pm 1$ (°C) in PBS
Mainly Hybrid	D40	48
	L12	36
	L17	35
	L23	36
	L30	44
Mainly Antiparallel	tel_{26}	51
	A32	39
	B6	43
	L13	30
	L27	36
	L37	42
Parallel + Antiparallel	L21	44
	L33	47
Non G4-forming	L16	30
	L41	32



Evaluation of the nuclease stability of oligonucleotide aptamers in FBS (Fetal Bovine Serum)



Good correlation between the **nuclease stability** of the aptamers and the **melting temperatures** of their G4 structures

APTAMERS ANALYSIS IN DIFFERENT CONDITIONS
Annealed vs Not-annealed

Annealed (A.) aptamer solutions in PBS:
the solutions were treated at 95 °C for 5 minutes and slowly cooled to r.t. overnight
(aptamers in their thermodynamically-favoured conformations)

Low T_m (32-48 °C), close to physiological body temperature

Half-lives in serum (FBS) ranging between 1 and 3.6 h

Not-Annealed (N.A.) aptamer solutions in PBS:
no thermal treatment
(aptamers in their kinetically-favoured conformations)

Same analysis as for the annealed aptamers:

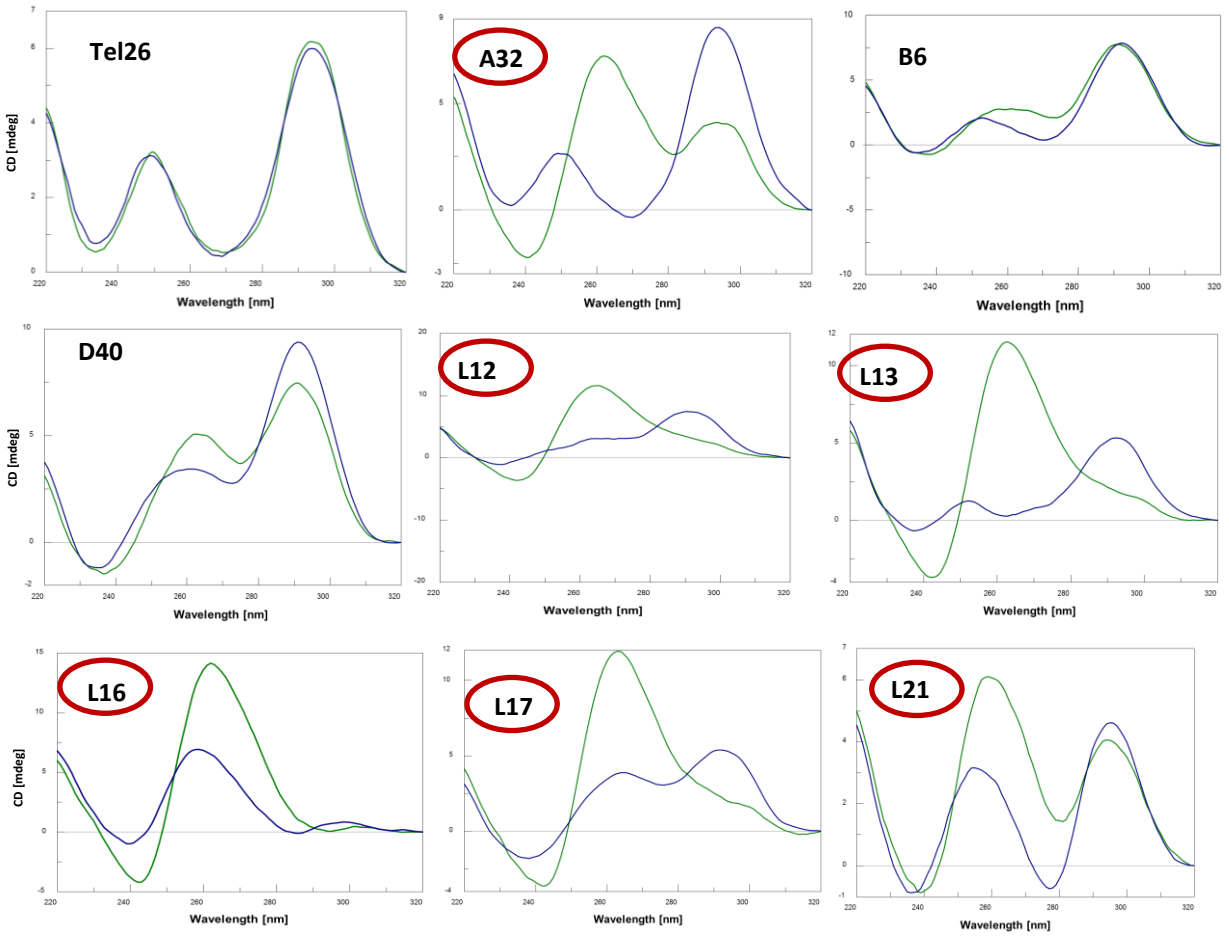
- **TDS,**
- **CD,**
- **CD-MELTING,**
- **CD-DECONVOLUTION,**
- **UV-MELTING,**
- **NATIVE PAGE,**
- **ENZYMATIC RESISTANCE**

PBS (*Phosphate-buffered saline*): 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4,1.86 mM KH_2PO_3

Comparison of the CD spectra of A. and N.A. anti-HMGB1 aptamers

Not-annealed

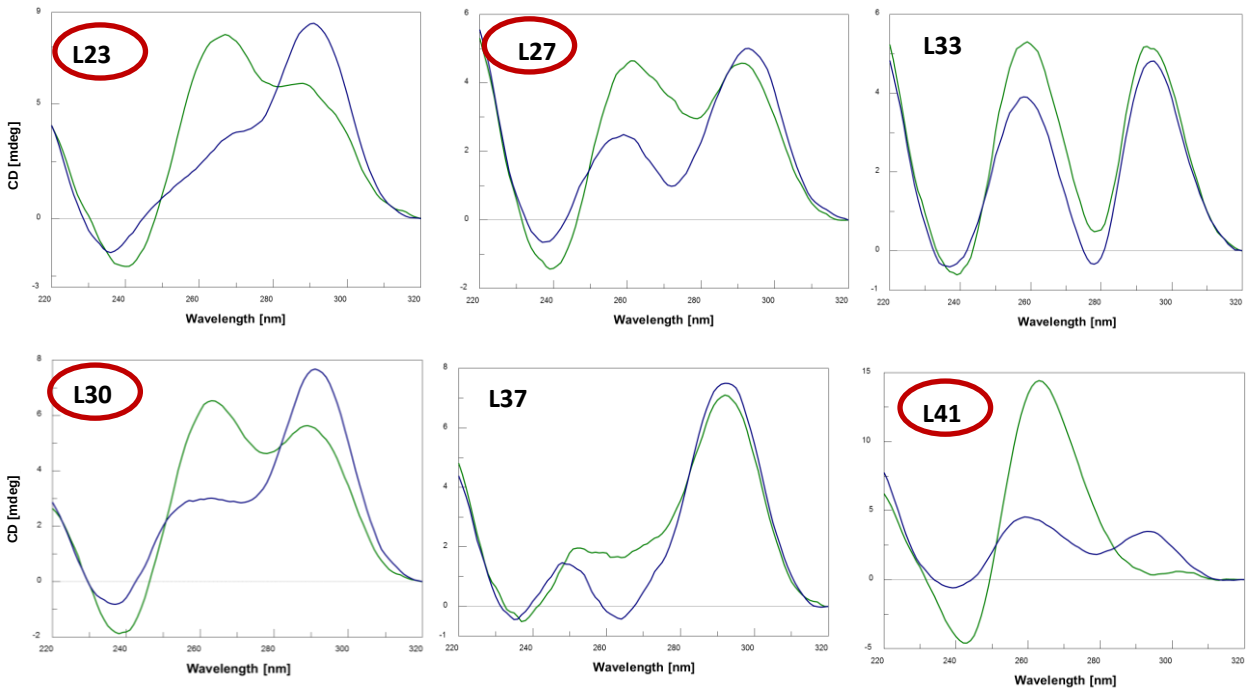
Annealed



Comparison of the CD spectra of A. and N.A. anti-HMGB1 aptamers

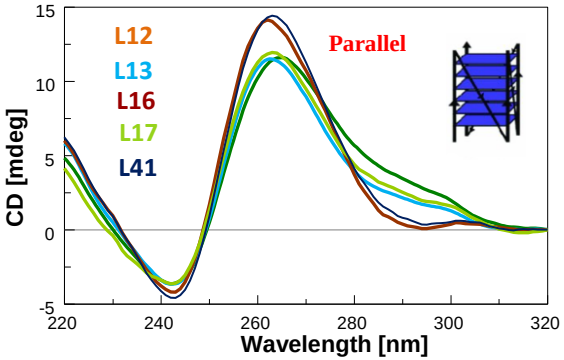
Not-annealed

Annealed

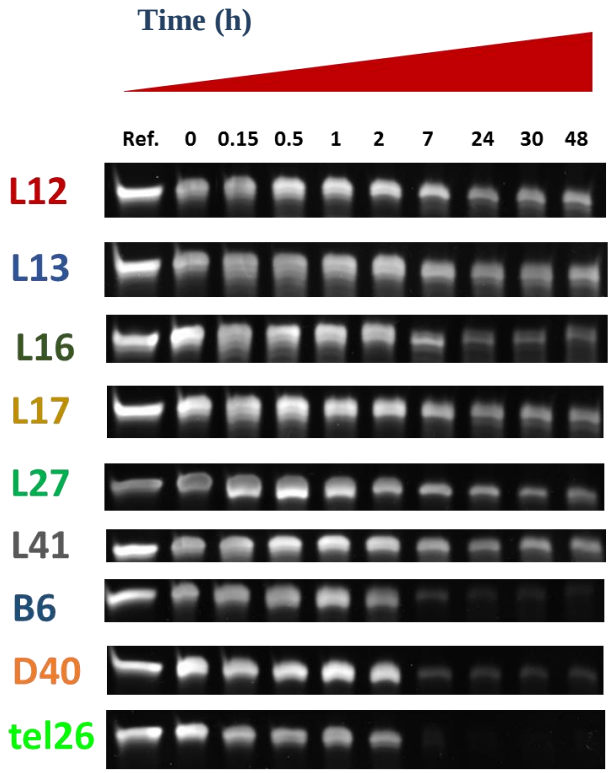


Comparison of the T_m of A. and N.A. anti-HMGB1 aptamers

	T_m±1 (°C) annealed	T_m ±1 (°C) not-annealed
tel26	51	52
A32	39	63
B6	43	44
D40	48	48
L12	36	62
L13	30	61
L16	n.d.	57
L17	35	61
L21	44	57
L23	36	61
L27	36	63
L30	44	54
L33	47	48
L37	42	42
L41	32	61

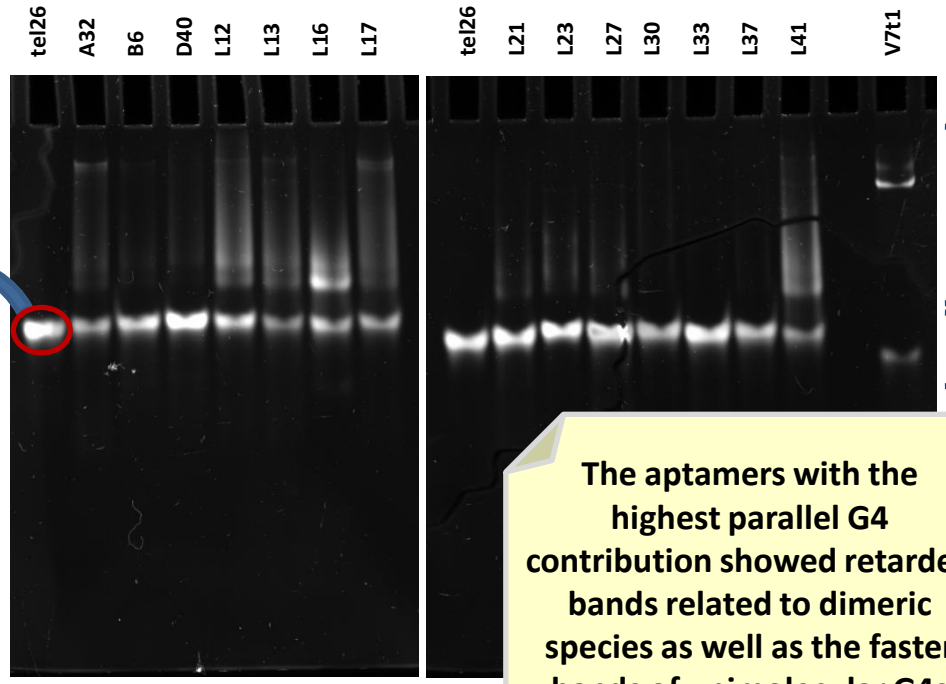


Nuclease stability evaluation of the N.A. G4 aptamers in FBS (Fetal Bovine Serum) at 37 °C



	$t_{1/2}$ (h) N.A.	T_m (±1 °C) N.A.		$t_{1/2}$ (h) A.	T_m (±1 °C) A.
L12	**10.1**	62		1.1	36
L13	**17.2**	61		1.0	30
L16	8.5	57		1.0	n.d.
L17	**12.3**	61		1.0	35
L27	6.5	63		1.3	36
L41	8.7	61		1.0	32
B6	2.0	44		1.4	43
D40	3.5	48		3.1	48
tel26	1.2	52		1.0	51

Native polyacrylamide gel electrophoresis (20 %) of the N.A. G4 aptamers

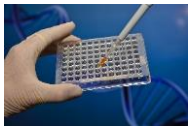


Unimolecular structure for tel_{26} in PBS

The aptamers with the highest parallel G4 contribution showed retarded bands related to dimeric species as well as the faster bands of unimolecular G4s

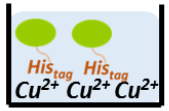
***Running buffer**: TBE 1x*
***[G4]**: 3 µM; not-annealed in PBS (30 pmol loaded)*
***Staining**: Gel Green*

HMGB1-aptamers interaction: ELISA-like assay

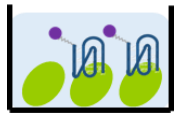


The assay was based on the following steps:

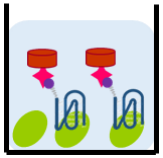
1) **Immobilization of HMGB1** protein, labelled with an His-tag (**HMGB1-His**), on a Cu-coated 96-well ELISA-type micro-plate;



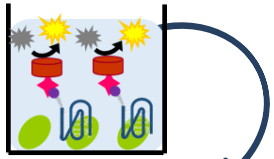
2) **Interaction of the biotinylated aptamers** (**ON-Biot**) with the HMGB1-functionalized plate, exploiting their potential affinity for the target protein;



3) **Attachment of horseradish peroxidase-streptavidin** (**HRP-Strep**) to the bound **ON-Biot**, trough streptavidin-biotin interaction;



4) **Addition of an HRP-substrate** (ECL) to produce a chemiluminescence signal proportional to the amount of bound aptamer.



Chemiluminescence
(Luminescence reader)

HMGB1-His

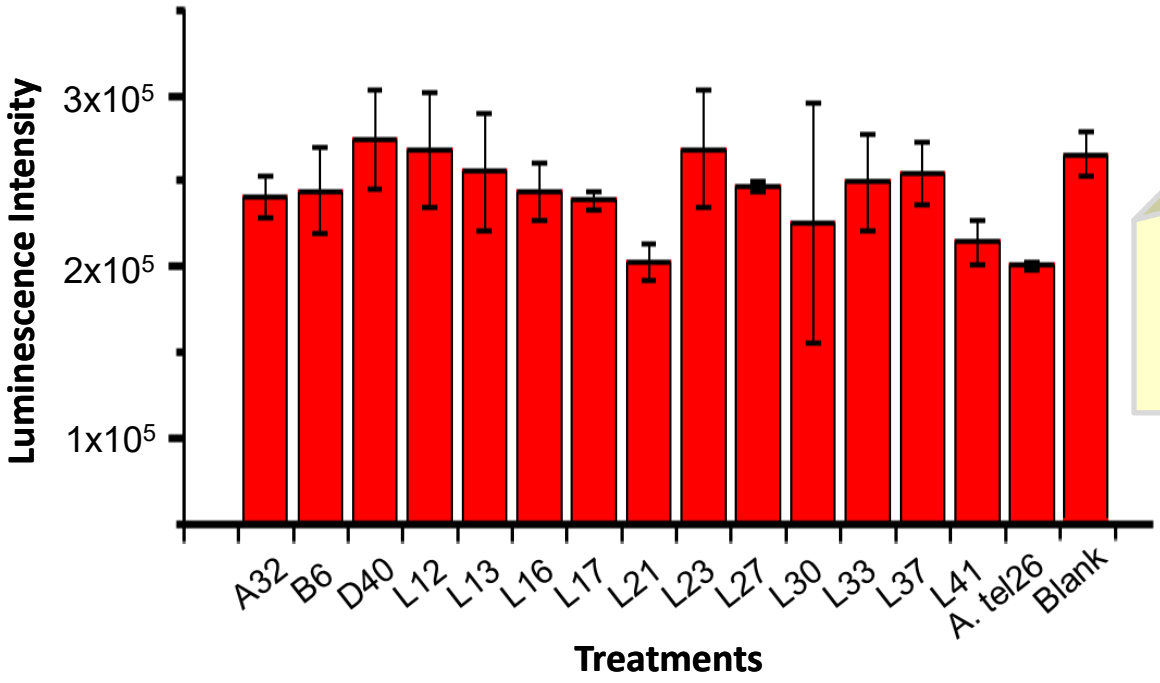
ON-Biot

HRP-Strep

ECL-substrate

ECL-substrate (oxidized form)

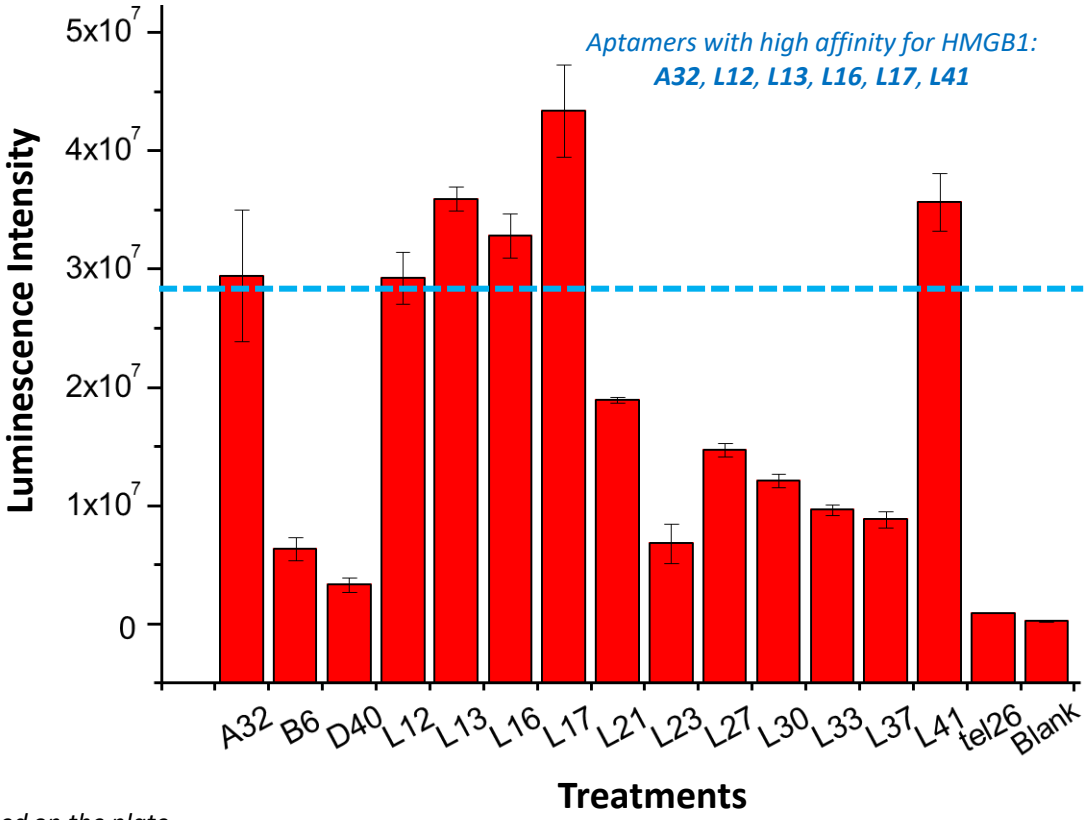
HMGB1-binding assay with A. aptamers



The luminescence signals were very low and close to the Blank;

HMGB1-His immobilized on the plate
Assays with aptamers in their annealed form (PBS)

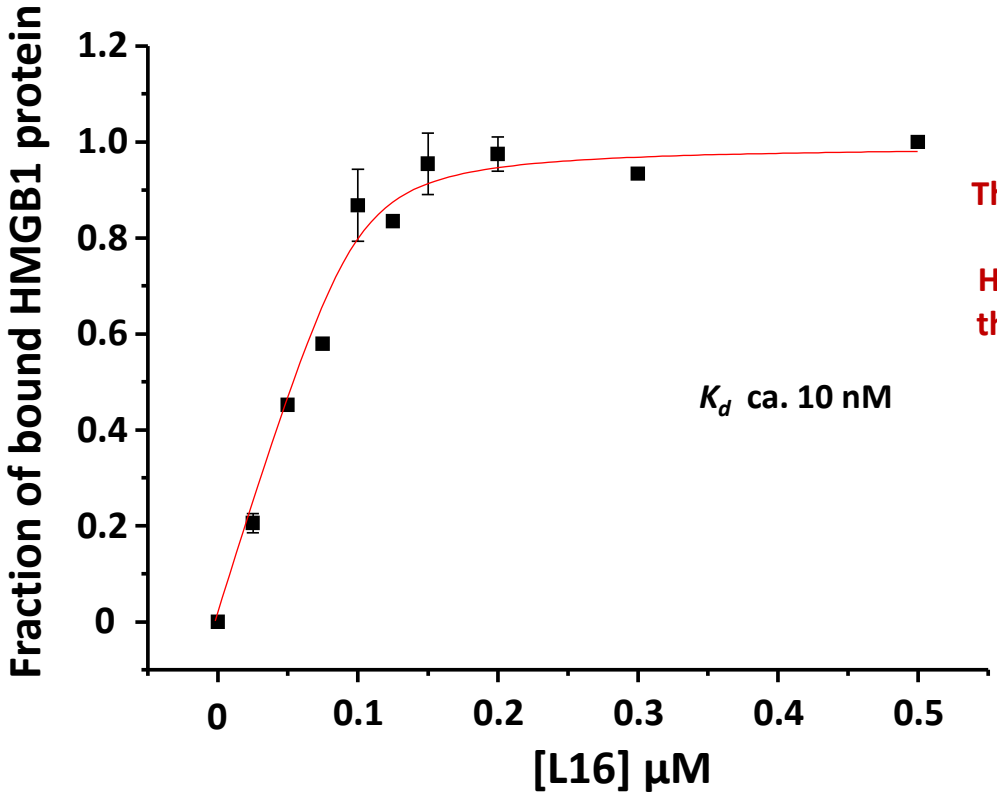
HMGB1-binding assay with N.A. aptamers



Higher affinity for parallel G4s

HMGB1-His immobilized on the plate
Assays with aptamers in their not-annealed form (PBS)

HMGB1-aptamers (N.A.) interaction assay



The G4 aptamers with the highest affinity for HMGB1 have K_d values in the low nanomolar range

Binding curve obtained by plotting the fraction of bound HMGB1 as a function of N.A. L16 aptamer concentration. The black squares represent the experimental data, the red line is the best fit obtained with an **independent and equivalent-sites model.**

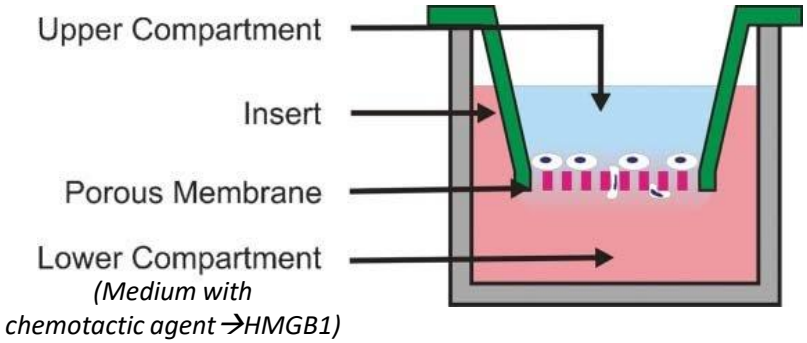
In vitro biological assays

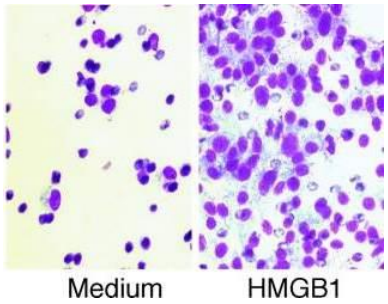
Consiglio Nazionale delle Ricerche

ISTITUTO PER L'ENDOCRINOLOGIA E L'ONCOLOGIA SPERIMENTALE "G.SALVATORE"

Dr Carla Esposito

➢ Inhibition of HMGB1-induced cellular migration





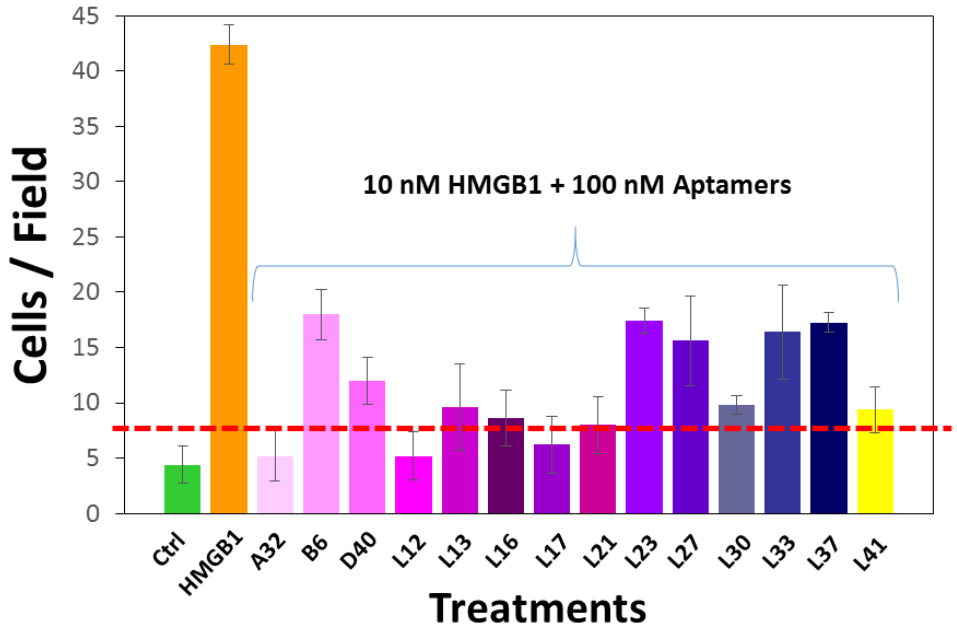
Selected cells: NIH3T3 cells (Murine Embryonic Fibroblast Cell Line).

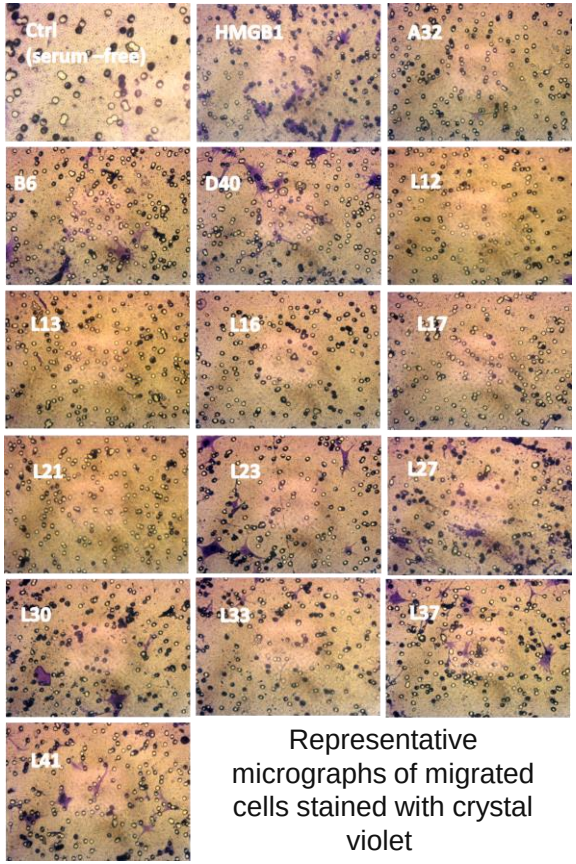
Medium = Negative control: in the absence of HMGB1, cell migration was only basal

Positive control: At 10 nM HMGB1, cells migrated significantly

Incubation time: 4 h

Migration assay data on NIH3T3 cells (N.A. aptamers)





Representative micrographs of migrated cells stained with crystal violet

Aptamers with the highest activity against HMGB1:

A32, L12, L13, L16, L17, L41

→ **GOOD CORRELATION BETWEEN**
→ **AFFINITY AND ACTIVITY**

Parallel G4 aptamers are the most active ones

Conclusions

We were able to select novel G4-forming aptamers targeting HMGB1. In detail, the not-annealed aptamers in PBS can interact with the target protein with higher affinity (in the low nanomolar range) than the annealed ones and they are characterized by very high melting temperatures and nuclease resistance, probably due to their ability to form high order species. Moreover, these aptamers showed a great ability to inhibit HMGB1-induced migration in cells, so these findings provide fundamental information to develop valuable and highly selective agents for anti-inflammatory and cancer therapies.

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Thank you for your attention!!!