Unique Avenues for the Inhibition of Telomerase Activity in Cancer: Selective Targeting of Higher Order G-quadruplexes

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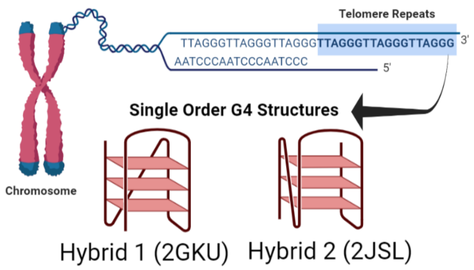
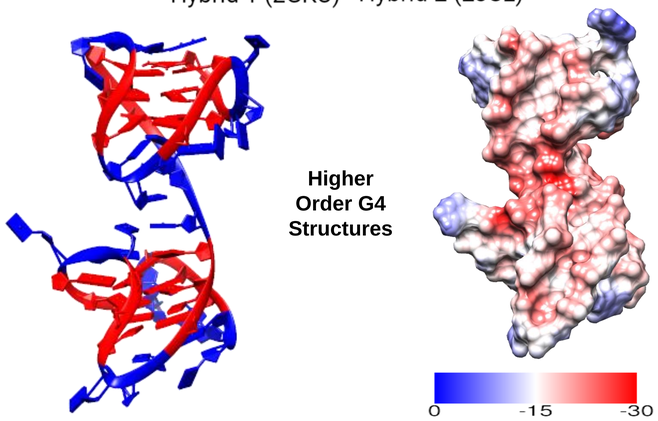
Abstract

One mechanism that is crucial for cancer cell proliferation is utilized in up to 90% of cancers, making it a promising potential target for a wide variety of cancer therapy. This mechanism is the reactivation of telomerase, an enzyme that interferes substantially with a cell's ability to self regulate by extending the 3' overhang of human telomeres, allowing cancer cells to divide repeatedly with no consequence to telomere length. In order to develop a route to block telomerase we must understand the structures where its action takes place. The telomere is rich in guanine bases and has the ability to form G-quadruplex (G4) secondary structures which consist of guanine tetrads connected by Hoogsteen hydrogen bonds and stabilized by potassium cations. The telomere G4 solution topologies are known as hybrid-1 and hybrid-2, each having 3 parallel and 1 antiparallel strand, but differing in loop position. In an extended single strand of telomere DNA with repeating bases $(TTAGGG)_n$, evidence has shown formation of higher order, or multiple, G4 systems with potentially unique junctional regions for drug targeting. This project aims to identify small molecule ligands that bind to these higher order G4s with high affinity and specificity. These G4 structures are considered promising targets for cancer treatment because the stabilization of telomeric G-quadruplexes can act as a barrier to inhibit telomerase function, blocking its ability to regulate telomere length. Our approach is unique because it emphasizes the selectivity of ligands which interact in the pocket between G4s in the higher order structure, which could help eliminate unwanted side effects due to off target interactions. We use an integrated structural biology approach to modify a lead compound in order to lower the dissociation constant and dial up affinity for the target. The goal is to create a highly selective G4 ligand which could be tested in clinical trials as an effective inhibitor of telomerase action in cancer cells.

Specific Aims

- We are applying a structure activity relationship (SAR) approach to develop a small molecule ligand to selectively bind higher order structures found in human telomeres.
- Stabilizing these structures can effectively inhibit telomerase function, the reason for cancer cell's immortality.
- We are evaluating the compound's affinity for the target by using techniques that enable us to measure K_d values such as microscale thermophoresis (MST) and isothermal titration calorimetry (ITC), as well as docking simulations *in silico*, circular dichroism (CD), and differential scanning fluorimetry (DSF) FRET analysis.
- The goal is to design a ligand with high specificity for telomere G4s that can eventually be tested in clinical trials as an inhibitor for telomerase activity in cancer.
- **There is no small molecule inhibitor of telomerase that has made it through clinical trials.**





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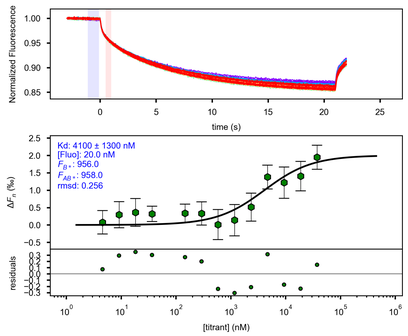
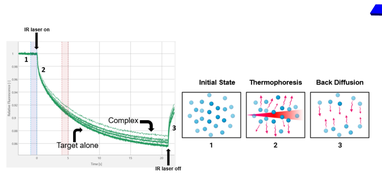
Methods and Results

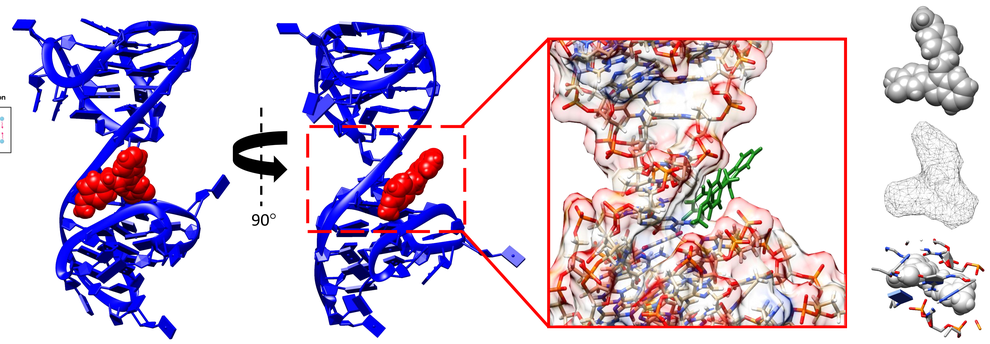
Lead compound (C37) confirmed with microscale thermophoresis screening





MST technology detects the migration of fluorescently labeled molecules along a temperature gradient created with an infrared laser. Thermophoresis shifts are dependent upon changes in the size or charge of molecules, as well as the solvation shell surrounding the complex.

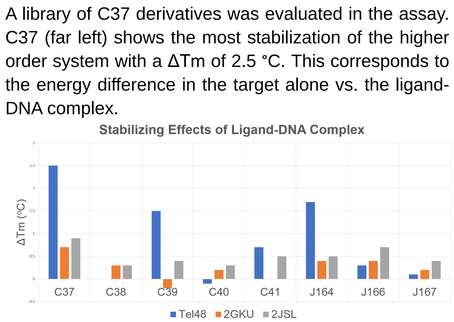
Docking simulations identify ligand conformations for binding in the pocket between G4s

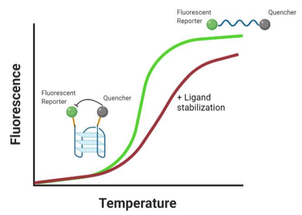


(Left) The ligand-DNA complex formed after docking simulations generated the lowest possible energy conformation. (Right) Spherical and mesh representations of the surface area surrounding C37 and target DNA within 5 Å. By examining the pocket of binding relative to the proximate nucleotides we hope to demonstrate C37's ability to interact across the quadruplexes, establishing a binding event that is selective to this specific chemical environment.

Fluorescence thermal shift assay shows selective stabilization of the higher order structure (Tel48) over hybrids 1 & 2

A library of C37 derivatives was evaluated in the assay. C37 (far left) shows the most stabilization of the higher order system with a ΔTm of 2.5 °C. This corresponds to the energy difference in the target alone vs. the ligand-DNA complex.

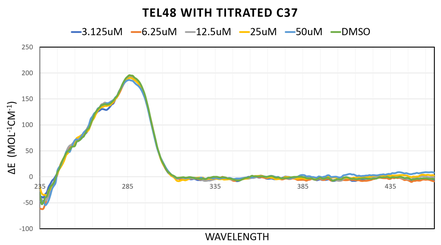


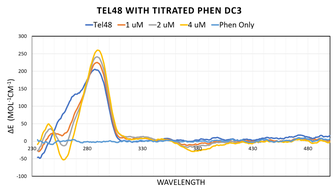


FRET labeled DNA emits fluorescent signal as a function of the distance between reporter and quencher. This helps us visualize G4 unfolding with temperature increase.

Induced circular dichroism spectra suggests no conformational changes in bound state: evidence for junctional interaction

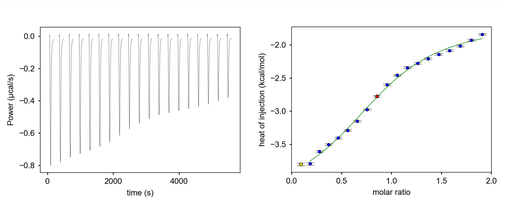
ICD aims to visualize changes in how a system rotates polarized light. If a molecule causes a conformational shift, it will show up as a change in CD signature.

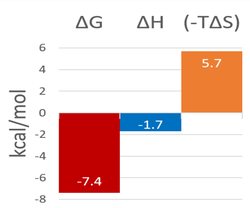




Phen DC3 is a known quadruplex binder with high affinity for hybrid 1 and 2. This served as a positive control for drug induced G4 conformational changes, which were not apparent in the Tel48-C37 CD.

ITC curve gives full thermodynamic characterization of C37-DNA binding

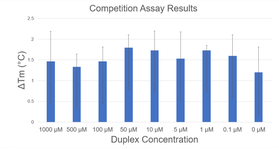




Thermodynamic data suggests that C37 is a groove binder and its interaction with the target is driven largely by the displacement of solvent molecules leading to a favorable change in the entropy of the system. This method measured a dissociation constant between 2-4 μM.

C37 shows selective stabilization of the target even in the presence of duplex DNA at a similar concentration ratio of duplex to G4 as is found in human cells

The competition assay provides evidence that the stabilization of the higher order structure is unaffected even in the presence of large concentrations of duplex DNA. This is critical in the development of a potential drug because it shows the compound is highly selective to the telomere G4 and has the potential to limit off target interactions: a major problem with previous small molecule telomerase inhibitors.



Modifying the lead compound to dial up affinity

Alteration of the Lead Compound	Glide Docking Energy Score	Molecular Dynamics Energy Score
Original Compound	-5.4	-10.7
Removed hydroxyl	-4.0	N/A
Moved hydroxyl from para to meta position (neutral/charged)	-5.1/-1.5	**-14.7**/-8.7
Moved hydroxyl from meta to ortho position (neutral/charged)	-5.2/-1.2	**-12.1**/-8.7
Reduced heterocyclic ring and removed hydroxyl (neutral/charged)	-2.6/**-6.6**	**-13.8**/**-14.6**
Removed nitrogen atom (neutral/charged)	-5.2/**-6.3**	-8.1/**-19.5**
Removed methoxy	-5.7	-2.0

Conclusion and Future Directions

- From the library of compounds identified in a virtual screening, experimental data determined C37 to have the highest level of affinity for the target G4 system. The interaction provided a small signal change in thermophoresis traces which corresponded to a K_d value of 4.1 μM.
- In order to inhibit telomerase function, we must observe stabilization of the higher order telomere structure. This was visualized in the form of melting temperature shifts in the FRET assay. When the C37-DNA complex was formed the melting temperature was increased by 2.5 °C.
- Induced circular dichroism revealed little to no signal change as the complex was formed, which gives evidence that C37 is not pasting directly to the tetrads or altering the conformation of the system.
- Isothermal titration calorimetry was performed in order to understand the thermodynamic changes driving the reaction. This method also allowed for an accurate measurement of the dissociation constant which agreed with the value measured in MST.
- We hypothesize that the unique chemical environment of the target DNA allowed for the observed selective ligand stabilization even in the presence of highly concentrated duplex DNA, an important finding for translating the compound to use in clinical studies.
- The above conclusions make C37 an attractive lead compound, and to increase target affinity a library of derivatives with small alterations to the compound's structure were modeled and tested *in silico*. This shows the possibility that small changes can help to increase the strength of the drug's binding affinity. The derivate without the methoxy group was synthesized and evaluated with MST and ITC, and it was determined to have a decreased affinity for the target. This leads to the conclusion that the methoxy group specifically is involved in the interaction. After examining ligand interaction maps, we hypothesized that the methoxy was acting as a hydrogen bond acceptor or possibly attenuating electronics to nearby aromatic ring systems. The next steps of this project include a synthesis of the full library of derivates in order to identify all relevant functional groups. With this potential increase in affinity coupled with the compounds high level of selectivity, the hope is that it will move to studies *in vivo* and eventually to clinical trials as a small molecule inhibitor of telomerase function in cancer cells.