A toolbox of biophysical and analytical assays helps to confirm the activity of novel *h*TS dimer disrupters (Ddis) with anticancer properties

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Background and Aim

Thymidylate synthase (hTS) is an homodimeric protein existing in a dimer-monomer equilibrium. Drugs that target the hTS are widely used in anticancer therapy. However, treatment with classical substrate site-directed TS inhibitors such as 5-FU binds to dimer and induce *h*TS overexpression.

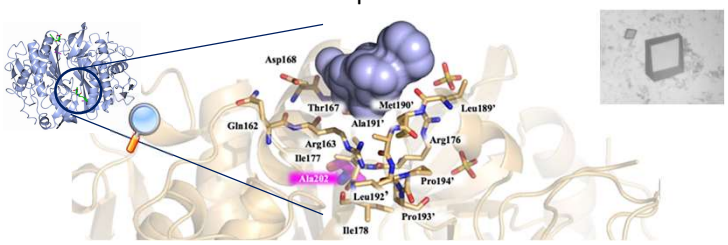

Figure 1. *particular of a hTS monomer interface binding a Ddis*

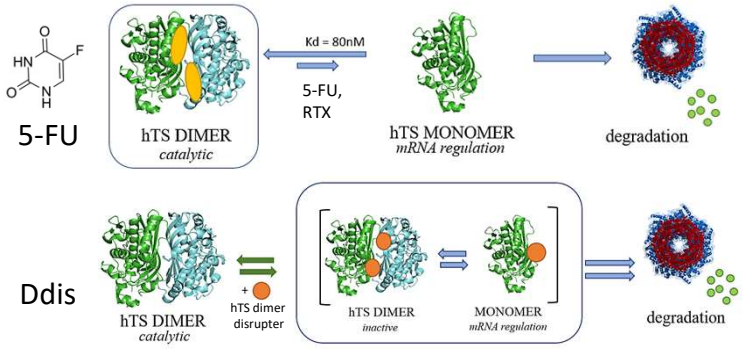

Figure 2. (a) *hTS Monomer dimer-equilibrium when a 'traditional' inhibitor (i.e. 5-FU, RXT) is administered.* ***(b)*** *Novel* ***dimer disrupters*** *move hTS equilibrium towards the monomer form, causing catalytic inhibition without enzyme overexpression.*

We have then synthesized and tested a large library of dissociative inhibitors (Ddis) of *h*TS that bind to the monomer interface, resulting in enzyme inhibition without overexpression. A toolbox of biophysical assays was developed to screen their activity.

Results and discussion

1. The direct kinetic studies on the target revealed a mixed mode inhibition model, with Ddis binding preferably to the monomers. The equilibrium is described by a Michaelis Menten mathematical model in Figure 3.

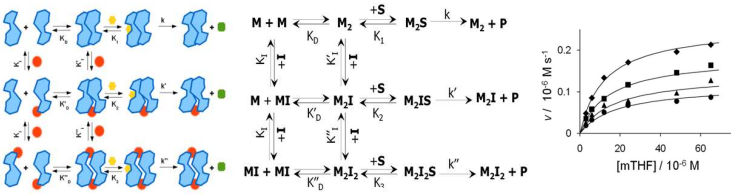

Figure 3. *(left) kinetic panel describing hTS equilibrium when perturbed by Ddis (Mixed Mode). (right) MM graph at increasing cofactor concentration*

2. We labelled each *h*TS monomer with fluorescent probes to test the actual percentage of dimers getting disrupted by the Ddis with a FRET assay.

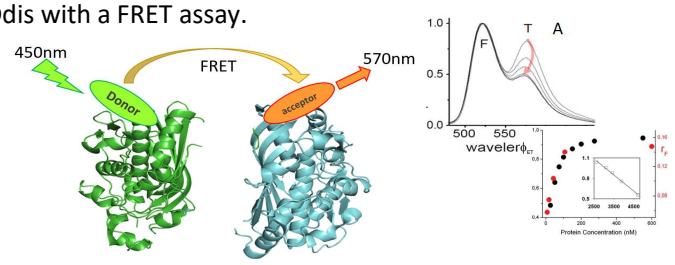

Figure 4*. Representation of a FRET assay on hTS. Each monomer is biolabelled with fluorescein as donor, and rhodamine-5-maleimide as FRET acceptor.*

3a. The co-elution of *h*TS with Ddis was checked by Anionic Exchange Chromatography (AEX) on FPLC. Increasing Ddis concentration in *h*TS solution causes monomer peak to rise.

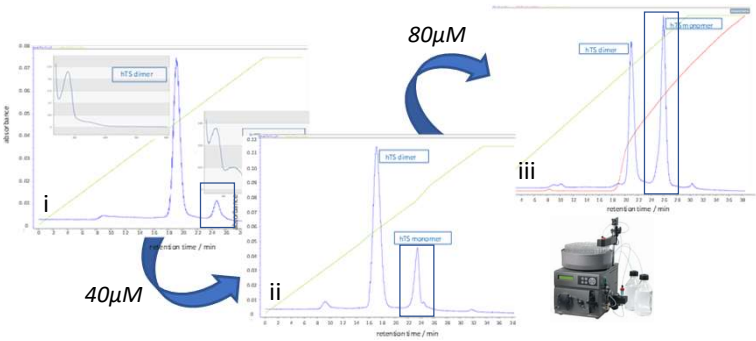

Figure 5*. AEX chromatograms of hTS with 4 (i), 40 (ii) and 80 (iii) μM Ddis.*

3b. We have employed LC-MS to analyse the actual amount of Ddis that binds *h*TS monomer and dimer fractions.

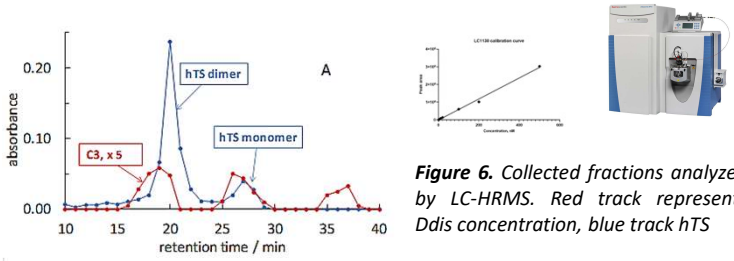

Figure 6. *Collected fractions analyzed by LC-HRMS. Red track represents Ddis concentration, blue track hTS*

Conclusions

Biophysics + bioanalytical techniques → target engagement and *Kd*'s
Elution profile with AEX → *Ddis : hTS co-elution confirmation*
Future investigations: Photoaffinity labelling (PAL) to validate binding

References:
- Genovese F, Ferrari S et al. *Dimer-monomer equilibrium of human thymidylate synthase monitored by fluorescence resonance energy transfer*. Protein Sci. 2010 May;19(5):1023-30.
- Costantino L, Ferrari S et al. Disrupters of the thymidylate synthase homodimer accelerate its proteasomal degradation and inhibit cancer growth, 2021 ChemRxiv

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