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DESIGNING SELECTIVE CYS-LIGANDS TO UNPAIR THE BINDING OF THE HUMAN TRANSCRIPTION ENHANCER ASSOCIATED DOMAIN 4 (hTEAD-4) WITH ITS MODULATORS TO HALT CANCER CELL GROWTH

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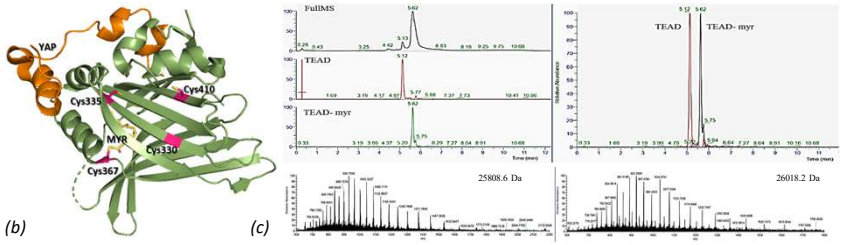
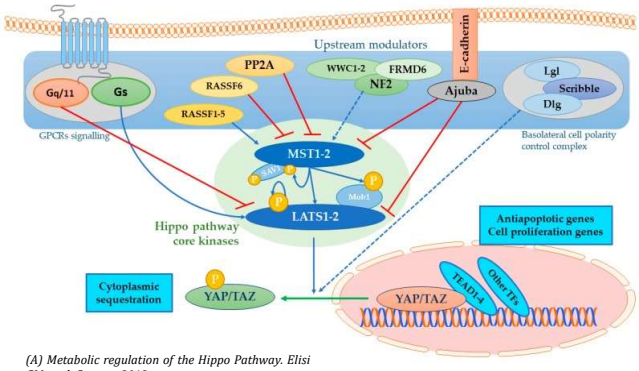
BACKGROUND & OBJECTIVES The Hippo Signalling cascade is an emerging target in tumour suppression regulation, neoplastic hypertrophy, as well as regenerative medicine. The pathway is activated by serum circulating anti-proliferative signals which lead to the LATS1/2 mediated phosphorylation of Yes Associated Protein-1 (hYAP1) on Ser127/381, thus 14-3-3σ driven cytosolic retention and proteasomal degradation. Genetic alterations and/or exogenous factors may cause YAP1 nuclear migration and association to TEAD1-4 (Transcription Enhancer Associated Domain). YAP:TEAD complex triggers the up-regulation of anti-apoptotic genes as EMT's, Bcl-xl and survinis, and cause cell hyperproliferation. Recent studies have considered **hTEAD** a promising target for **anticancer drugs development.** Its main inhibition strategies include the disruption/prevention of YAP1:TEAD4 complex formation, thus preventing the up-regulation of the above-mentioned genes. Our **objective** is to identify a new ligand/inhibitor that binds to the *Ω-loop* on YAP1:TEAD4 interface and prevents YAP1 binding, thus reducing its non-phosphorylated form and decreasing TEAD transcriptional activity. The specific aim was to develop a cysteine-directed inhibition strategy, and for this purpose we studied the effect of exposed cysteines on the protein surface and their reactivity though exposition to a small library of thiols/disulphide ligands. The four cysteines of hTEAD, indeed, are very close to the ligand binding site of our interest, and their behaviour after disulphide formation is informative of hTEAD structural modification when it binds an inhibitor.



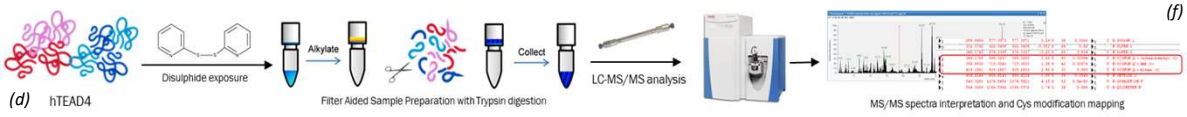
Left: (B) hTEAD4 surface Cysteines (PDB: 5OAQ) elaborated with PyMol. Right: (C) UHPLC – HRMS spectra of free and myristoylated protein in Full-MS, profile acquisition. XIC were extracted and deconvolved with FreeStyle software (ThermoFisher Scientific).
MYR mas-shift is evidenced by the RT and the predicted masses (25,808.6 Da for hTEAD4ybd and 26,018.2 Da for hTEAD4ybd-MYR)

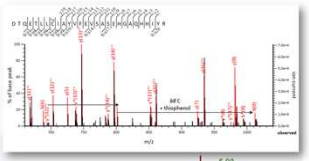


(A) Metabolic regulation of the Hippo Pathway. Elisi GM et al, Cancers 2018

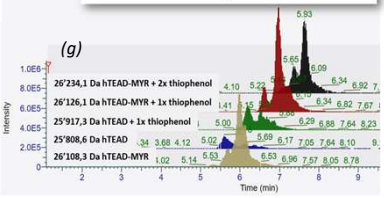
1. We expressed 6xHis-tag hTEAD4-Yap binding domain (aa. 217-434) in *E. coli* and purified it with IMAC and SEC chromatography. hTEAD was characterized with SDS page for purity evaluation, circular dichroism (CD) for it stability, top-down **LC-MS fingerprinting**, and **FASP bottom-up sequencing**. An UHPLC experiment run at high temperature on a Bioshell 1000Å IgG C4 column (Merck™) tandem HRMS (Orbitrap Q-Exactive) revealed an equilibrium of both myristoylated (Cys-thioester) and "free" Cys367, with a prevalence of the former structure.

2. A small, chemically balanced library of thiol/disulphide ligands was exposed to hTEAD at different concentrations to test their affinity towards exposed Cys. We evaluated the amount of formed disulphides on the native protein (Full-MS) and on hTEAD tryptic lysate (MS/MS experiment). HRMS spectra were deconvolved to obtain the monoisotopic exact masses of the S-S conjugated protein. Filter Aided Sample Preparation (FASP) was then employed to digest hTEAD4 after ligand exposition, followed by a C18-SPE of the peptides. A 90-min run on an Hypersil Gold C18 column (ThermoFisher) was studied to separate all peptides with a DDA Top6 acquisition mode. **MS/MS cysteines modifications** were evaluated with Mascot Matrix Suite and SkyLine, and their mass shifts validated with an *in house generated* spectral library from SwissProt database.

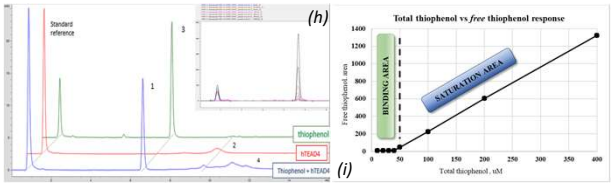




3. Combined MS and MS/MS spectra revealed full occupation of Cys330 and Cys410 when exposed to **Thiophenol / Ellman's** reagent DNTP ((5,5'-dithiobis- (2-nitrobenzoic acid)) along with 2-mercaptoethanol, but no significant ligand amount was detected for DTDP (4,4'-Dithiodipyridine), and other more lipophilic compounds.



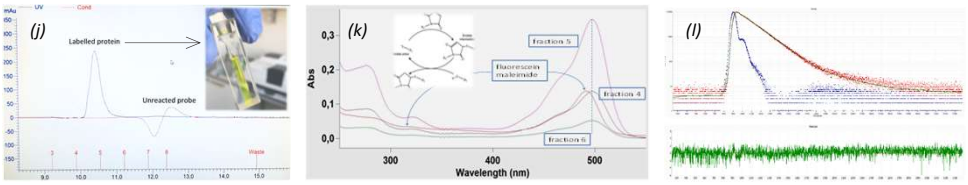
Top (f): thiophenol mass shif in MS/MS tryptic peptide spectra.
Bottom (g): 500-5000m/z ESIpos XIC spectrum of hTEAD4ybd incubated with thiophenol. hTEAD, hTEAD-thio, hTEAD-MYR, hTEAD-MYR-x1thio, hTEAD-MRY-x2thio peaks can be distinguished after [M+10H]10+ to [M+40H]40+ m/z deconvolution.



4. To validate previous MS experiment, we reversed-titrated hTEAD4ydb with increasing thiophenol concentration, and the ligand excess was quantified by HPLC-UV-ELDS. From the peak areas (λ=250nm) and the initial concentration added, we calculated thiophenol-bound fraction, which reveals to be **3:1 ligand:protein**. When further ligand is added above to initial 15uM hTEAD4ybd, excess thiophenol peak appears, and increases linearly (saturation zone) to indicate that no more ligand can bind to our protein domain. Furthermore, the protein pattern peaks at rt 10-12 modify their moiety, confirming the occurring binding.

Left figures (h): 15uM h*TEAD4 titrated with increasing thiophenol concentration (1-400 equivalent nmoles). (i) After 3 Cys are saturated, "free" thiophenol peak appears.*

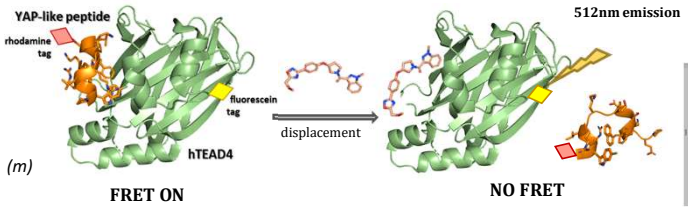
5. To study hTEAD spectroscopic features, we labelled one of its cysteine with a fluorescent probe. Indeed, hTEAD4 was incubated with excess fluorescein-5-maleimide, then purified from the unreacted probe through a Sephadex column with an Akta™ FPLC system. Protein-to-probe ratio was established 1.2:1 thought their respective molar extinction coefficients in UV-Vis, and a bottom-up LC-MS/MS confirmed that only Cys335 is actually susceptible to fluorescein conjugation.
Also, from a **time-decay fluoresce** measurement, we concluded that fluorescein-5-maleimide half-life (2.3 and 4.2ns, two exp. decay, with 88% and 8% relative abundances) results significantly more rapid than the emission decay of the conjugated probe (0.9 and 4.3ns, 10% and 90%). A **NED analysis** proved that the saturation of the maleimide double bond inhibits the quenching activity of the ring, and confirmed the occurred reaction.



Left (j): SEC chromatogram of hTEAD4ybd and probe separation. Middle (k): absorption spectra of hTEAD4ybd labelled with fluorescein-5-maleimide. 280 typical Trp protein peak is visible along with 492nm fluorescein peak, red-shifted when its ring is open/saturated due to conjugation. Right (l): Time decay fluorescence spectra of labelled protein excited at 450nm (blue) and free probe decay (red).

CONCLUSIONS & NEXT STEPS

We used spectrometric and spectroscopic techniques to characterize the Cys surface reactivity of recombinant *hTEAD4 YAP1 binding domain.* Cys367-MYR emerged to be an important feature for hTEAD stabilization, despite its functional role remains unclear. The remaining Cys residues, located near the druggable binding site (so called "interface 3"), display a medium-to-low level of polar solvent exposure. Cys330 and Cys410 - sited on a different β-sheet but pointing towards the same outer surface - are more likely to react to thiols and disulphides, as long as the examined compounds are not hindered, either too lipophilic. The inner Cys335 –SH easily reacts as nucleophile towards maleimide Michael's acceptors.
The ongoing work is currently engaging the screening of a larger compound library to study YAP:TEAD interaction with a ligand displacement assay of labelled TEAD to a tetramethylrhodamine-tagged peptidomimetic probe to help achieve more structural information about the heterodimer interface, and to start a hit-optimization programme. The Fluorescence Resonance Energy Transfer (FRET) signal measured in our assay will be quenched by molecules with higher affinity after peptide displacement. This will allow to confirm the target engagement of the synthesized molecules.



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[3] Malpezzi G MSc Degree Thesis, Solvent exposure, and reactivity of the cysteines of Transcription Enhancer Associate Domain (TEAD), a potential anticancer target, 2021. University of Pavia – University of Modena and Reggio Emilia.