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Targeting the YAP:TEAD Interface 3 domain through fluorescence-anisotropy displacement assay

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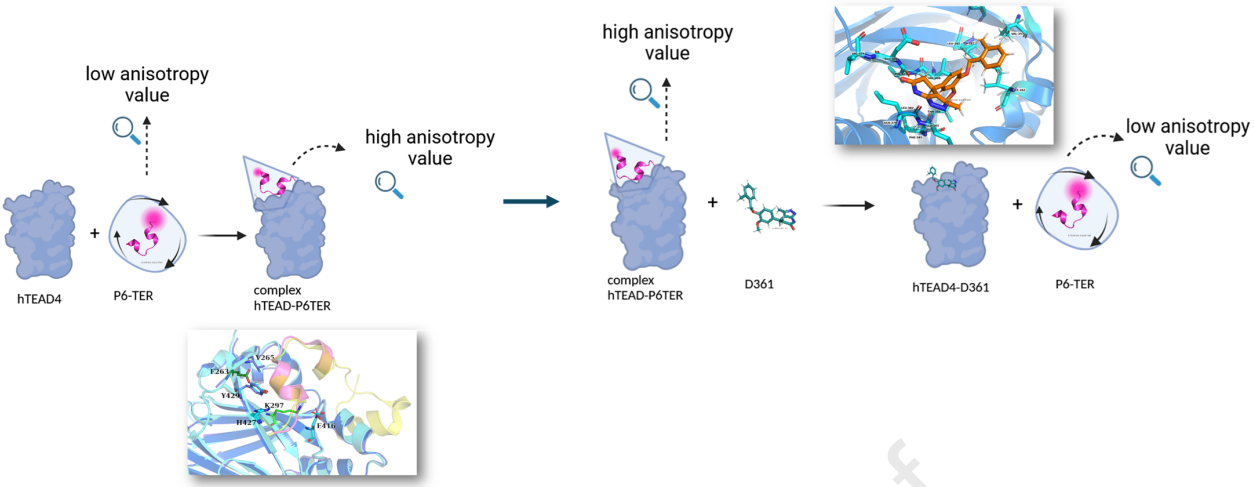
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KEYWORDS: Hippo signaling pathway, YAP, TEAD, mimetic peptide, Protein–protein interaction (PPI), Ω-loop, Fluorescence anisotropy

The Hippo pathway is a signaling cascade that regulates cell growth, proliferation, and apoptosis. Its dysregulation is linked to cancer and other hyperproliferative diseases. Transcription enhancer associated domain 1–4 (TEAD1–4) proteins are the final effectors and key transcription factors. They interact with other coactivators such as Yes-associated protein (YAP) and transcriptional coactivator with PDZ-binding (TAZ) to modulate the expression of genes involved in cell survival and proliferation, e.g., connective tissue growth factor (CTGF), cysteine-rich angiogenic inducer 61 (CYR61) and other genes coding for growth factors. The YAP:TEAD complex is the pathway's endpoint effector and a validated therapeutic target [1]. Inhibition of its formation and quantitative analysis of YAP-TEAD:ligand interaction represents a complex challenge [2,3] (Figs. S1 and S2).

Here, we describe the development and quantitative setting-up of a fluorescence-anisotropy (FA) displacement assay (DA) designed to identify compounds that bind at the TEAD4 Interface 3 region thus displacing a fluorescent peptidomimetic of the Ω-loop of YAP (Figs. S3, and 1A) [3]. First, the purified recombinant YAP binding domain (YBD) of TEAD4 was investigated by mass spectrometry (MS) to quantify its acylated and non-acylated species and to identify expression-related modifications relevant to ligand binding. The recombinant YBD of TEAD4 was obtained as an N-term-6xHisTag protein from the *E. coli* Arctic cells expression system and purified by affinity and size exclusion chromatography (S1.1). Covalent lipidation on Cys367 of TEAD with either myristic or palmitic acid, besides stabilizing the protein, putatively promotes its association to YAP. With the aim to discover YAP:TEAD protein-protein interaction inhibitors, we determined by quantitative high-resolution mass spectrometry (HRMS, Orbitrap Q-Exactive, Thermo Fisher, Waltham, Massachusetts, U.S.) the percentages of the isoforms with covalently bound thioesters in the purified protein sample (native-TEAD, S1.2). 70% of the recombinant native TEAD was found to bind either a palmitic or a myristic acid (myr). The other isoforms are shown in Fig. 1B and described in the legend. To characterize the single protein isoforms, 95% lipid-free TEAD4 and 97% myristoyl-TEAD were obtained, respectively, by alkaline hydroxylamine-mediated hydrolysis of thioesthers (S1.3) and by chemical myristoilation of native TEAD with excess (10-fold) of myristoyl-CoA (myristoyl-CoenzymeA) (S1.4, Fig. 1B).

An α-N-6-phosphogluconoyl modification was detected in up to 65% of total TEAD, a common artifact of *E. coli* expression that does not affect the protein auto-acylation at Cys367. A proteomic analysis of the tryptic lysate of TEAD was performed to better identify the covalently modified Cys residue and check the above quantitative results in native conditions (Fig. S4, and Table S1). The tryptic lysates of native, myristoilated and de-lipidated TEAD were analysed in data-dependant mode. The Cys367-myr (Cys367-myristoyl) peptide ([R].SPLC(myr)EYM(ox)INFIHK.[L]) was found with a 1.23-fold higher abundance in the myristoilated than in native TEAD, while it was 5-fold less present in the de-lipidated than, again, in native TEAD (Fig. 1B). These findings confirm the above full-length HRMS results and are consistent with the affinities exhibited by the three protein forms for an Ω-loop mimicking peptide (see below Fig.1B and Fig S5).

To design the DA, we chose peptide **P6** (S1.5, Fig.S6) as the ligand to be displaced. This was previously identified as a linear Ω-loop mimetic able to bind the Interface 3 of TEAD4 with submicromolar affinity (450 nM) [4]. **P6** was synthesized and then labeled with tetramethylrhodamine (TMR) through a maleimidic linker

at either the C- or N-terminus (S1.6, Figs. S7 and S8). However, upon addition of TEAD4, the C-term variant failed to produce measurable changes from the initial free-probe value of 0.06 and, for the N-term probe, the anisotropy only increased from 0.08 to 0.11, with an estimated dissociation constant of 2.0(±0.5) μM. So, both P6 tetramethylrhodamine (**P6TMR**) variants proved unsuitable as outgoing ligands in a FA based test. These somewhat surprising findings were attributed to a large torsional freedom of the TMR probe around the maleimidic linker, due to the flexibility of the latter, even when the peptidic moiety is bound to TEAD4. A more rigid probe, **P6** tetraethylrhodamine **(P6TER**) (Fig. S9), was therefore synthesized by directly linking a TER moiety to the peptide N-terminus (Figs. 1C and Fig. S10). Indeed, the **P6TER** probe showed an FA of 0.12 ± 0.02 that increased to 0.27 ± 0.03 upon TEAD4 addition (Fig.1D). Data analysis according to eq. 4 in S1.7, gave an equilibrium constant for the dissociation of the protein:**P6TER** complex K_d = 265 ± 40 nM (Fig. S11). Structural details of the Ω-loop pocket were then refined through X-ray crystallography of the **TEAD4:P6** complex providing a high-resolution model of the interaction interface (S1.8, Figs. S12 and S13 and Tables S2 and S3). The X-ray crystal structure revealed that the peptide binds at the Ω-loop pocket and is stabilized by a network of hydrophobic and hydrogen-bond interactions (Fig.1E). The relevance of this hydrogen-bond network is reinforced by the previous observation that mutation of Tyr421 in TEAD1 (Tyr429 in TEAD4) abolishes YAP binding [4]. The same complex provided the structural model for molecular dynamics

simulations (MD) (S1.9). This finding is consistent with 1 μs MD simulations for the complexes formed by TEAD4 with **P6TER** or the two possible diastereoisomers of **P6TMR**. The hydrogen-bond network formed by the peptide recognition portion and Interface 3 is well conserved during the simulation, the fluorophore in the **TEAD4:P6TER** complex, but not in the **TEAD4:P6TMR** complex, stably takes additional van der Waals interactions with the surface of TEAD 4 (S1.9, Fig. S14). Complementary MD simulations carried out on the **TEAD4:P6TMR** and **TEAD4:P6TER** complexes showed that, because of the increase in rigidity of the **P6TER** probe, emission depolarization associated with torsional motion of the fluorophore was smaller than in the former complex (Figs. 1F, and S15–S17).

The DA was then tested by adding compound **IAG933**, a nanomolar affinity binder of TEAD4 Interface 3 [4], to the **TEAD4:P6TER** complex. **IAG933** displaced the **P6TER** from TEAD4 in a concentration-dependent manner. Quantitative analysis of the FA data according to a competing-ligand model (S1.7) yielded a K_d = 10 ± 5 nM that compares well with the literature value of 8 nM [4]. This result confirmed both the accuracy and sensitivity of the FA-displacement assay for quantifying high-affinity ligands targeting YAP:TEAD interaction (Fig. S18). Stability of the anisotropy signal during the assay time in presence of DMSO was evaluated and confirmed (Fig. S19).

The DA was finally applied to a training set of novel pyrazolo-piperidinone compounds (Figs. 1G and H, S1.10) [2]. The compounds can effectively engage Interface 3 and displace the **P6TER** that simulates the Ω-loop of YAP. The assay is direct, quantitative, and reproducible. It provides information about the region of compound binding at the same time. The rather high ratio between maximum signal change and noise, the protein stability and the rapidity of assay execution, suggest the suitability of the here proposed FA

displacement assay for quantitative high-throughput screenings. This suitability has been substantiated by ongoing preliminary tests.

In conclusion, this fluorescence anisotropy-based assay represents a valuable translational tool for identifying and ranking YAP–TEAD inhibitors, facilitating the rational design of small molecules aimed at modulating Hippo-pathway signaling and expanding the arsenal of strategies against cancer-associated transcriptional dysregulation.

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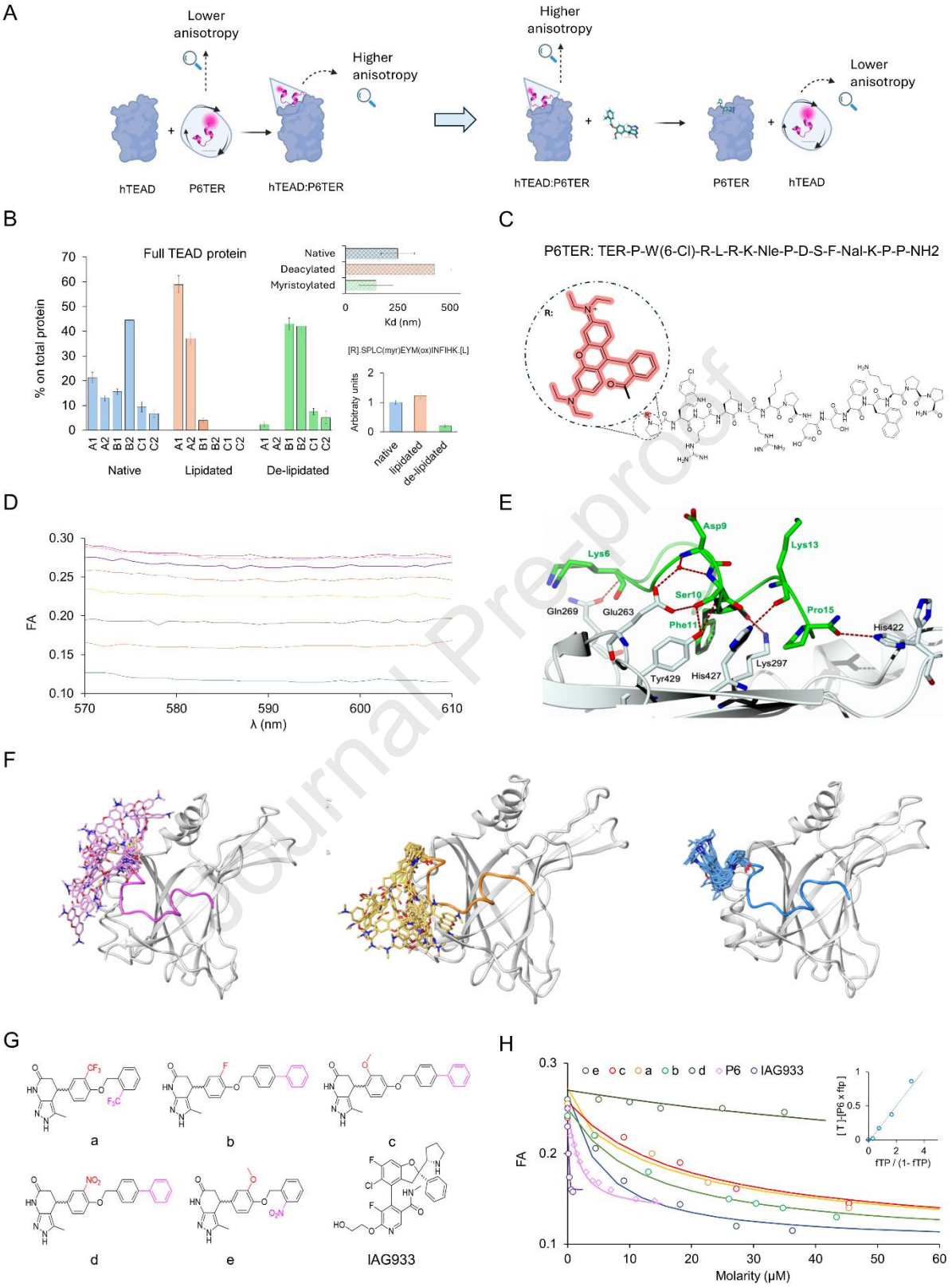


Fig. 1. Overview and main results of the fluorescence anisotropy (FA) displacement assay developed to identify ligands that bind at the Interface 3 of the Transcriptional enhancer associated domain (TEAD) thus inhibiting its complexation with Yes-associated protein (YAP). (A) Principle of the FA assay: the emission

anisotropy of the fluorescent probe, tetraethylrhodamine (TER), increases upon formation of the TEAD4–P6TER complex and decreases when the tagged peptide is displaced by a competing ligand. (B) Left: Relative abundance (% on total TEAD protein) of acylated, deacylated, and native TEAD4 isoforms expressed in *E. coli*, as determined by high resolution mass spectrometry (HRMS). Protein species: A1, native TEAD4 (TEAD protein as from purification, i.e., not submitted to chemical modifications), without α-N-6-phosphogluconoylation; A2, TEAD4, with α-N-6-phosphogluconoylation; B1, acylated TEAD4 (TEAD protein submitted to chemical myristoylation with myristoyl-CoA), without α-N-6-phosphogluconoylation; B2, acylated TEAD4, with α-N-6-phosphogluconoylation; C1, deacylated TEAD4 (TEAD protein submitted to thioester hydrolysis with hydroxylamine), without α-N-6-phosphogluconoylation; C2, deacylated TEAD4 - with α-N-6-phosphogluconoylation. (B) Lower right: MS determination of the Cys367-myr (Cys367-myristoyl) peptide ([R].SPLC(myr)EYM(ox)INFIHK.[L]) after tryptic digestion of native TEAD4, and TEAD4 obtained by the described myristoylation and de-lipidation process. (B) Upper right: Corresponding binding affinities of P6TER toward the three TEAD4 samples: native (mixture of acylated and deacylated forms, light blue), K_d = 265(±30) nM; 95% deacylated (yellow), K_d = 426(±50) nM; and 97% myristoylated (green), K_d = 144(±20) nM. (C) Chemical structure of the tetraethylrhodamine-labeled probe, P6TER, with the fluorophore linked to the peptide N-terminus. (D) Emission FA spectra of P6TER (1 μM) measured at, bottom to top, [TEAD4] = 0, 300, 600, 900, 1200, 1500, 1800 and 2100 nM. The FA increases from 0.12, for the free peptide, to 0.27 for the protein-complexed peptide. The typical signal noise, 0.03, is about 50 times lower than the maximum signal change, 0.15. At each addition step, the protein concentration was determined spectrophotometrically with an estimated error within 3%. (E) Detail of the X-ray crystal structure of the TEAD4-YAP binding domain (YBD) and P6 complex showing the Ω-loop binding region of TEAD4 (in grey) and the main interacting residues that contribute to the recognition of peptide P6 (in green). (F). Representative molecular dynamic (MD) conformations of P6TMR (P6 tetramethylrhodamine) (*R*-diastereomer, pink carbon atoms for the fluorophore and cartoon for the peptide portion, left, and *S*-diastereomer, orange carbon atoms and cartoon, center) and of P6TER (cyan carbon atoms and cartoon, right) all bound to TEAD4. Geometries of the conformers are taken from one of the forty 20 ns-long MD simulations performed on each of the tagged peptide-TEAD4 complexes (TEAD4 represented with grey cartoon). The chemical structure of the tagged peptide is represented in each MD conformation of the complexes. (G) Chemical structures of pyrazolo-piperidinone derivatives (**a**, **b**, **c**, **d**, **e**) used to validate the displacement assay for identifying compounds able to inhibit YAP–TEAD4 binding. (H) Changes in TER emission anisotropy (FA, λ_{em} = 590 nm) measured after subsequent additions of **P6**, **IAG933** and the test compounds (**a–e**) to the **TEAD4:P6TER** complex. The observation that FA decreases with the first additions of the displacers suggests that these have no other high-affinity binding sites in TEAD4. In the inset, an example of the linear fitting used for K_d determination (S1.7) [5]. The binding affinities measured by fluorescence anisotropy (FA) assay showed that compound **a** (cLogP = 5.70) displayed a K_d of 2.4 ± 0.5 μM, while compound **b** (cLogP = 5.66) exhibited a K_d of 2.1 ± 0.5 μM. Compound **c** (cLogP = 5.26) showed a slightly weaker affinity, with a K_d of 4.0 ± 0.8 μM. Compound **d** (cLogP = 5.34) demonstrated improved binding, with a K_d of 0.6 ± 1.6 μM. In contrast, compound **e** (cLogP = 2.86)

displayed very weak binding, with a K_d greater than 100 μM. Finally, the reference compound **IAG993** (cLogP = 4.05) showed markedly higher affinity, with a K_d of 0.01 ± 0.005 μM.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Key input and output structures, and trajectories used to estimate the FAnisotropy decay are available as a public repository in GitHub at https://github.com/medchemunipr/TEAD_fluorescence-anisotropy-

Author contribution

Author	Affiliation(s)	CRediT roles
Giulia Malpezzi	1,2	Conceptualization; Methodology; Investigation; Data curation; Validation; Visualization; Writing – original draft; Writing – review & editing
Laura Scalvini	3	Methodology; Software; Formal analysis; Visualization; Writing – review & editing
Lorenzo Tagliazucchi	1,3	Formal analysis; Investigation; Methodology; Data curation; Writing – review & editing
Anna Lisa Chiaravalle	1	Methodology; Formal analysis
Daniele Aiello	1	Methodology; Investigation; Writing – review & editing
Giulia Saporito	1	Investigation; methodology
Davide Illuminati	4	Investigation; methodology;
Salvatore Pacifico	4	Investigation; methodology
Remo Guerrini	4	Supervision; Investigation; methodology, Writing – review & editing
Ludovica Lopresti	5	Investigation; Visualization
Luisa Raucci	5	Investigation; Visualization
Cecilia Pozzi	5,6	Methodology; Validation; Supervision; Visualization; Writing – review & editing
Alberto Venturelli	1	Supervision; methodology
Glauco Ponterini	1	Conceptualization; Methodology; Investigation; Supervision; Writing – review & editing
Marco Mor	3	Conceptualization; Methodology; Supervision; Investigation;Writing – review & editing

Maria Paola Costi	1	Funding acquisition; Supervision; Investigation; Conceptualization; Writing – review & editing

Supplementary Material

Additional experimental details, figures, tables, and datasets supporting the findings of this study are available in the Supplementary Material (SM) section.

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Highlights

- Interface 3 of the YAP:TEAD complex a key Hippo pathway effector driving oncogenesis.
- A robust fluorescence anisotropy displacement assay was developed using an Ω-loop peptide probe to identify TEAD4 Interface 3 inhibitors.
- Successful probe displacement by pyrazolopiperidone inhibitors confirms the assay's suitability for hit and lead screening.

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Declaration of competing interest

The authors declare no competing financial interests.

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